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Crystallographic Study of a Novel Sub-Nanomolar Inhibitor Provides Insight on the Binding Interactions of Alkenyldiarylmethanes with Human Immunodeficiency Virus-1 (HIV-1) Reverse Transcriptase[†]

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Abstract

Two crystal structures have been solved for separate complexes of alkenyldiarylmethane (ADAM) non-nucleoside reverse transcriptase inhibitors (NNRTI) **3** and **4** with HIV-1 reverse transcriptase (RT). The structures reveal inhibitor binding is exclusively hydrophobic in nature and the shape of the inhibitor-bound NNRTI binding pocket is unique among other reported inhibitor-RT crystal structures. Primarily, ADAMs **3** and **4** protrude from a large gap in the backside of the binding pocket, placing portions of the inhibitors unusually close to the polymerase active site and allowing **3** to form a weak hydrogen bond with Lys223. The lack of additional stabilizing interactions, beyond the observed hydrophobic surface contacts, between **4** and RT is quite perplexing given the extreme potency of the compound (IC₅₀ ≤ nM). ADAM **4** was designed to be hydrolytically stable in blood plasma, and an investigation of its hydrolysis in rat plasma demonstrated it has a significantly prolonged half-life in comparison to ADAM lead compounds **1** and **2**.

Introduction

The human immunodeficiency virus (HIV), the causative agent of the acquired immune deficiency syndrome (AIDS), is a retrovirus that relies on a myriad of viral proteins to help infect CD4⁺ cells and enable its replication. One of these proteins, reverse transcriptase (RT), facilitates viral replication by transcribing HIV's single-stranded, RNA-based genome to a double-stranded DNA equivalent that is compatible with the host cell's replication machinery. The genomic transcription abilities of RT are made possible by both the DNA polymerase and RNase H activities, which are absolutely required for the virulence and replication of HIV.¹

[†]PDB code for **3** with HIV-1 RT: 3IS9; PDB code for **4** with HIV-1 RT: 3IRX.

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As such, RT is an attractive target for antiviral therapy, with an array of drugs currently in use for the treatment of HIV infection through inhibition of RT enzymatic function.

Structurally, RT is a heterodimeric protein composed of 66 kDa and 51 kDa subunits (known as p66 and p51, respectively), each of which consists of connection, thumb, palm, and finger domains. The four domains of the p66 and p51 subunits are identical in amino acid sequence from their N-termini for 440 residues.² However, the heavier p66 subunit bears an additional 120 residues at its C-terminus, which form the RNase H domain. Despite their near identical sequences, the p66 and p51 subunits have surprisingly different ternary structures, which results in RT assuming the shape of a hand that is capable of grasping genomic material for enzymatic processing.³

As previously mentioned, several antiretroviral drugs have been developed to inhibit RT, and they are classified by their mechanism of action.⁴⁻⁷ The nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs) halt RT's polymerase activity by acting as dNTP mimics that, when incorporated into viral DNA, terminate synthesis of the viral DNA chain.^{8,9} The other category of HIV-1 RT inhibitors, non-nucleoside reverse transcriptase inhibitors (NNRTIs), bind to a hydrophobic, allosteric site located ~10 Å from the polymerase active site. When NNRTIs, such as nevirapine¹⁰ and efavirenz,¹¹ bind to this hydrophobic site, the native arrangement of amino acid side chains is forced to change such that several of the residues point toward or intrude upon the polymerase active site.^{3,12} In fact, crystallographic studies on various HIV-1 RT-NNRTI complexes have shown that binding of the inhibitors causes significant conformational changes within RT, primarily through the displacement of sheets β12-14 in the DNA primer grip, resulting in the enzyme binding DNA in a non-productive manner.^{3,12} Because of their usually tolerable toxicities, NNRTIs have enjoyed increased use in highly active antiretroviral therapy (HAART) in recent years. However, the clinical efficacy of these inhibitors has been attenuated by the emergence of drug resistant HIV-1 strains that bear mutant forms of RT.^{6,13} There is great need for the development of NNRTIs that are efficacious against both wild-type and drug-resistant forms of HIV-1 RT.

For several years, our group has been developing a series of alkenyldiarylmethane-based NNRTIs that exhibit antiviral activity against a number of the common drug-resistant strains of HIV-1 that bear RT mutations.¹⁴⁻²⁰ Previous hypothetical binding models developed for potent inhibitors **1** and **2** showed the alkenyldiarylmethanes (ADAMs) oriented in accordance with the “butterfly” model, as described by Schäfer, which reflects the binding orientation of NNRTIs like nevirapine, TIBO, and alpha-APA.^{16,19,21} However, these models are not sufficiently general for the ADAM class of inhibitors, nor do they correlate with the RT SAR. It is well known that the NNRTI binding pocket is plastic in nature and will conform to the structure of the specific inhibitor that is bound, thus making molecular modeling studies problematic. Recent crystallographic studies involving new structural classes of NNRTIs have revealed compounds that are highly flexible (large number of rotatable bonds) and similar in structure can each bind to RT in different orientations.^{22,23} Furthermore, potent inhibitors can even have more than one binding orientation if the structure is flexible enough to allow for “wiggling” and “jiggling” inside the binding pocket.^{22,23} Yet, the malleable nature of the NNRTI binding pocket obviates attempts to generate an accurate binding model via de novo methods.

Given that current ADAM-RT interaction models lack correlation with SAR data, our desire to identify key protein-ligand interactions for this class of NNRTIs necessitated the acquisition of ADAM-RT crystal structures. Herein we reveal the first X-ray crystal structures of HIV-1 RT in complex with an ADAM (compounds **3** and **4**). The present report also discloses the synthesis and antiviral evaluation of the incredibly potent NNRTI **4** (IC₅₀ ≤ 1 nM). ADAM **4** was designed as part of an investigative effort to replace the enzymatically labile methyl

esters in lead compound **1** with bioisosteres that would confer hydrolytic stability in blood plasma while maintaining the desired pharmacological activity.

Results and Discussion

Chemistry

Several routes for the synthesis of ADAM analogues have been reported.^{17,24,25} The synthesis of ADAM **3** has been previously described.²⁶ The synthesis of compound **4** centers on a series of palladium-catalyzed cross-coupling reactions recently utilized to prepare a set of hydrolytically stable ADAM analogues.²⁰ Briefly, the synthesis of ADAM **4** began with the coupling of known compounds **5** and **6** under standard Sonogashira conditions (Scheme 1). Cross-coupling product **7** was treated with tributyltin hydride and Pd(PPh₃)₄ to obtain stannane **8**,²⁰ which was elaborated with iodide **9**²⁰ through the Stille reaction to afford ADAM **4**.

Evaluation of RT Inhibition, Antiviral Activity, and Hydrolytic Stability

As is standard in our investigations, ADAM **4** was evaluated in a functional enzyme assay for inhibition of HIV-1 RT. Additionally, the cytotoxicity and ability of ADAM **4** to protect cells from the cytopathic effects of HIV-1_{RF}, HIV-1_{IIIB}, or HIV-2_{ROD} were evaluated. The metabolic half-life of **4** in rat plasma was also determined in a metabolic stability assay. The results of the RT inhibitory, antiviral, metabolic, and cytotoxicity analyses for ADAM **4** are presented in Table 1. Associated data for compounds **1-3** are included in Table 1 for comparison.¹⁷⁻¹⁹

Concerning inhibition of RT enzymatic activity, ADAM **4** is the most potent analogue among the four compounds under discussion, exhibiting an impressive IC₅₀ of less than or equal to 1 nanomolar. The detection limits of our functional enzyme assay precluded a more exact IC₅₀ determination for **4**. Despite ADAM **4**'s remarkable potency in the in vitro assay, the manifestation of this analogue's cytoprotective efficacy in whole cells is surprisingly lower than expected and comparable to that of ADAM **1** (IC₅₀ = 0.3 μM). The discrepancy between ADAM **4**'s enzyme inhibitory potency and its cytoprotective activity could be the result of many factors, including the fact that the enzymatic assay, in contrast to the cellular system, uses the synthetic template/primer poly(rC)-oligo(dG). Significant cytotoxicity is observed for each of the analogues with ADAM **4** unfortunately presenting the narrowest therapeutic index (TI ≈ 32). Because the current goal of our research is to improve on the metabolic stability of the ADAMs through isosteric replacement of enzymatically labile methyl esters, ADAM **4** was evaluated in our rat blood plasma stability assay and the results were compared to previous leads (ADAM **1**). ADAM **4** was found to be at least 40-fold more stable (t_{1/2} = 238 min) than **1**, indicating the fused-oxazolidinone and oxadiazole isosteres rendered **4** less prone to enzymatic degradation.

As our initial interest in studying the ADAM-scaffold was spurred by the ability of initial leads to retain antiviral activity against several common NNRTI-resistant HIV-1 strains, the antiviral activity of **4** was evaluated against NNRTI-resistant HIV strains. Specifically, CEM-SS cells infected with HIV-1 strain NL4-3 which bore either the K103N or Y181C RT mutant were treated with ADAM **4** to determine the residual cytoprotective activity of the inhibitor. The results of the experiment are presented in Table 2, with data for ADAM **1** and nevirapine included for comparison. Unlike that of ADAM **1**, the cytoprotective activity of **4** was completely abolished (EC₅₀ >100 μM) by two of the most common NNRTI resistance mutations, K103N and Y181C. A priori one would have expected ADAM **4** to exhibit moderate antiviral activity, even in the presence of the two mutations, on the basis of the point mutation data for ADAM **1**. Such a drastic change in activity suggests that ADAM **4** is incapable of adapting conformationally to the structural changes induced in the NNRTI binding pocket by

the K103N and Y181C mutations, whereas the slightly more flexible ADAM **1** can adopt an alternative binding conformation or orientation.

ADAM-RT Complex Structure and Analysis

The ADAM-RT structures were determined at final resolutions of 2.6 and 2.8 Å for compounds **3** and **4**, respectively, with associated diffraction and refinement data presented in Table 3. The overall conformation of RT in the crystal structures is typical of most NNRTI-RT complexes (Figure 1) in which the p66 thumb region is hyper-extended. The side chains of Tyr181, Tyr188, and Trp229 are rotated or shifted toward the polymerase active site and the β 12-14 sheet is displaced by approximately 2 Å relative to the structure of apo-RT.

In general, both **3** and **4** occupy nearly the entire volume of the NNRTI-binding pocket, making very few, if any, polar contacts with the protein (Figure 2). The latter observation is surprising, but not unprecedented given the recent crystal structure of the highly potent NNRTI, TMC278 (**10**), which showed that drastic improvements in potency were achieved by the addition of an increased number of hydrophobic interactions.²⁷ Although the NNRTI-binding pocket is known to be predominantly hydrophobic in nature and known non-nucleoside inhibitors have significant hydrophobic character, most NNRTI-RT complexes, including **10**, exhibit one or more hydrogen bonds between the inhibitor and protein. Our past SAR studies indicated the ester functionalities are a crucial component of the ADAM pharmacophore and were suspected of participating in one or more hydrogen bonds with RT.

In comparing the NNRTI-binding pockets containing **3** and **4** to those of other published NNRTI-RT crystal structures, it was observed that the shape of the ADAM-binding pocket resembles that of the bis(heteroaryl)piperazine (BHAP) series, to which delavirdine belongs. For NNRTI binding, it is commonly accepted that inhibitors bind to one of two NNRTI-binding pocket 'shapes', one of which is represented by nevirapine (PDB code: 1VRT) and the other, delavirdine (PDB code: 1KLM). The nevirapine-binding pocket is characterized as small and encapsulating, where no surface gaps are observed in the pocket. In the case of delavirdine, the inhibitor is quite large and the NNRTI-binding pocket expands to accommodate its volume. The binding pocket acquires additional space by breaking the hydrogen bond between the carbonyl of Pro236 and main chain amide of Lys101, which creates a large gap in the pocket near the surface of the protein. Additionally, the β 12-14 sheets are displaced further than in the nevirapine pocket, which provides supplementary volume and results in the opening of another gap toward the back of the pocket. The aforementioned hole in the back of the binding pocket is significantly larger in the two ADAM structures, relative to the delavirdine structure, and allows both inhibitors to protrude from the pocket, placing them unusually close to the RT polymerase site (Figure 3). In fact, the 5' nitrile functionality on ADAM **3** is held only 6 Å, with an unobstructed 'line-of-site,' from the catalytically active residue Asp186 in the only polymerase active site! The previous observation can be made for the crystal structure of diphenylether R165481 (**11**) (pdb 2B5J), which shows the nitrile of the ligand lays only 4.9 Å from aspartate 186.²⁸ In comparison to the nevirapine and delavirdine structures, the shortest path from each inhibitor to Asp186 are 8.6 and 8.9 Å in length, respectively, where the paths are obstructed by Y181 and Y188.

A more detailed analysis of the structures revealed that ADAM **4** creates a smaller pocket and binds slightly deeper relative to **3**. Yet in both crystals the alkenyl side chains of the ADAMs display significant edge-to-face interactions with the aromatic rings of Tyr181, Tyr188, and Trp229, in addition to general hydrophobic interactions with the side chains of Leu100 and Leu234. Most notably, the vinyl hydrogen on each ligand is nearly buried in the pi cloud of Trp229's indole ring. The Trp229 residue is highly conserved among HIV strains and is part of the DNA/RNA primer grip, thus disrupting the alignment of this residue can significantly impair RT polymerase activity.²⁹⁻³¹ The aryl rings situated trans to the side chain in both

ligands are buried deep in the pocket and occupy a region normally blocked by residues Phe227, Trp229, and Tyr188. With **3** and **4** bound so deeply in the pocket, the side chain of Phe227 becomes pinned against the peptide backbone, forcing a chain rotation of the local residues (219-228). This chain displacement opens a large hole at the back of the binding pocket and, in the case of ADAM **3**, brings the side chain of Lys223 in close proximity to the 'trans' aryl ring's ester. The proximity of Lys223 hints at a potential hydrogen bond, but the inter-atomic distance of 3.6 Å from the side chain amine to the nearest ether-type oxygen of **3** is at the outer limits of hydrogen bonding distance. In comparison, electron density for residues 218-223 in the RT-ADAM **4** crystal structure is completely absent, suggesting a large degree of disorder in this region of the protein. Such an observation precludes the assignment of a hydrogen bond between **4** and Lys223 in the crystal structure; however, such an association may theoretically arise under physiological conditions.

Further analysis of the crystal structures shows stacking between the aromatic ring of Tyr181 and the terminal side chain functionalities of the ligands in both structures. Additionally, the methyl ester (ADAM **3**) and oxazolone (ADAM **4**) moieties on the ADAM rings oriented trans to the side chains show similar stacking interactions with the aromatic side chain of Phe227. Given the observed stacking interactions between the methyl ester of **3** and Phe227, replacement of this functional group by the cyclic oxazolone in **4** may have the benefit of rigidifying the atoms in the proper planar orientation for pi-stacking with Phe227. This may be one reason for the increased potency of **4**. It is interesting to note that the IC₅₀ of ADAMs **2** and **4** differ by over an order of magnitude with **4** being the more potent of the two. The major difference between **2** and **4** is that **2** retains the methyl ester on the end of its alkenyl arm, while **4** has an oxadiazole in this place. If **2** and **4** bind to RT in a similar fashion, the pi-stacking with Tyr181 (which is also observed in the co-crystal structure of **3**) should be retained. However, **4** has the benefit of a rigidified, planar heterocycle for this stacking interaction while **2** retains the methyl ester.

The aromatic rings of **3** and **4** that are oriented cis to the side chains are cradled by the hydrophobic residues Leu100, Val106, Val179, and Leu234. The protein-ligand interactions described for both structures are primarily hydrophobic in nature and help explaining why the carboxylate analogues, derived from hydrolysis of the ADAM esters, are biologically inactive.

With regard to the point mutation data for ADAMs **1** and **4**, the application of information gleaned from the two ADAM-RT crystal structures leads to several interesting observations and conclusions on the data. In the case of ADAM **4**, given the significant edge-to-face and van der Waals contacts between the inhibitor and Tyr181 residue, it is unsurprising that mutation of this residue significantly impacts the antiviral activity of **4**. However, the crystal structure shows no specific interactions between **4** and Lys103, suggesting conformational changes in the binding pocket residues lead to attenuation of **4**'s antiviral activity. If one were to assume ADAM **1** binds to RT in a manner similar to **3**, then ADAM **1** would exhibit many of the same binding interactions with RT as are observed for ADAM **4**. Yet, the point mutation data indicate that modulating the shape of the NNRTI binding pocket via replacement of the Tyr181 and Lys103 residues results in small to moderate changes in the affinity of **1** for HIV-1 RT. This observation can be rationalized in two ways: 1) ADAM **1** binds to RT in an orientation vastly different from **3** and **4**, making analyses based on the two ADAM-RT crystal structures impossible; 2) because **1** possesses more conformational flexibility relative to **4**, the former analogue is capable of adopting a favorable, alternative binding conformation. In the absence of additional crystallographic or accurate modeling data for ADAM **1**, validation of either scenario is problematic.

Conclusion

Described herein are the first crystal structures of HIV-1 RT in complex with two ADAM NNRTIs. Of the two inhibitors discussed, ADAM **4** has been identified as a sub-nanomolar inhibitor of HIV-1 RT enzymatic activity in vitro, and the synthesis of this compound has been outlined. Interestingly, the crystal structures for the ADAM complexes reveal that inhibitors **3** and **4**, which differ in enzymatic inhibitory activity by at least two orders of magnitude, exhibit nearly identical binding orientations and interactions with the NNRTI-binding pocket. Moreover, the binding interactions observed in the protein-inhibitor complexes are exclusively hydrophobic in nature with additional pi-stacking attractions. The deep binding exhibited by **3** and **4** allows the inhibitors to protrude through the back of the NNRTI binding pocket and into the polymerase active site, making the ADAMs unique among other published NNRTI-RT complexes. The former observation has led to the hypothesis that it may be possible to tether an ADAM to a traditional nucleoside/nucleotide inhibitor (ex: AZT, tenofovir), generating a dual inhibitor that may simultaneously bind to the NNRTI and NtRTI and polymerase binding-sites. Such a compound would undoubtedly be an extremely potent RT inhibitor and potential antiviral agent.

Experimental

All ^1H NMR spectra were obtained at 300 MHz in CDCl_3 , using the residual CHCl_3 resonance as the internal standard for chemical shifts. A Perkin-Elmer 1600 series FT-IR spectrometer was used to record infrared spectra of all compounds. Preparative TLC separations utilized Analtech Uniplates with glass-supported silica (20×20 cm, 2000 micron thickness) and UV indicator (254 nm). The progress of reactions was monitored with Baker-flex silica gel IB2-F plates (0.25 mm thickness). Unless specifically mentioned, chemicals and solvents were of minimum reagent grade and used as obtained from commercial sources without further purification. Anhydrous tetrahydrofuran was prepared by distillation from sodium-benzophenone ketal radical. The hydrolytic stability assay utilized lyophilized rat plasma (LOTs 065K7555) from Sigma Chemical Co., St. Louis, MO. Elemental analyses were performed at the Purdue University Microanalysis Laboratory. All yields reported refer to isolated yields. Elemental analysis established >95% purity of ADAM **4**.

(*E*)-*S*-Methyl 5-(1-(3,7-Dimethyl-2-oxo-2,3-dihydrobenzo[d]oxazol-5-yl)-5-(5-methyl-1,3,4-oxadiazol-2-yl)pent-1-enyl)-2-methoxy-3-methylbenzothioate (**4**)

An oven-dried flask was charged with stannane **8**²⁰ (202 mg, 0.319 mmol) and iodide **9**¹⁹ (140 mg, 0.533 mmol). Anhydrous DMF (3 mL) was added via a syringe and the resulting mixture was sparged with argon for 10 min. Cesium fluoride (195 mg, 1.28 mmol), $\text{Pd}(\text{PPh}_3)_4$ (38 mg, 0.033 mmol), and copper(I) iodide (16 mg, 0.084 mmol) were quickly weighed and added to the flask in the order listed. The reaction mixture was allowed to stir at 60 °C, under an argon atmosphere, for 14 h. The system was allowed to cool to room temperature and the reaction mixture was diluted with ethyl acetate (5 mL), water (1 mL), and methanol (1 mL). The mixture was sonicated at room temperature (30 sec) and filtered through a pad of silica (10 mL). The filter pad was washed with ethyl acetate (50 mL) and the filtrate was washed with 1.0 M ethylenediaminetetraacetic acid solution (pH = 10, 2×20 mL) and sat. ammonium chloride solution (2×20 mL). The phases were separated and the organic phase was dried over magnesium sulfate, filtered, and condensed in vacuo. The crude products were purified by preparative thin layer chromatography using ethyl acetate as the eluant. The desired product was further purified, two more times, by preparative thin layer chromatography for which 66% ethyl acetate-hexanes was used as the developing solution for the first plate and 80% ethyl acetate-toluene was used for the second. The desired product was isolated as an opaque oil (6 mg, 4%). IR (neat) 2929, 2862, 1777, 1675, 1641, 1595, 1570, 1472, 1367, 1333, 1303, 1245, 1227, 1149, 1123, 1044, 1002 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ 7.37 (d, $J = 2.1$ Hz, 1 H),

7.08 (d, $J = 2.1$ Hz, 1 H), 6.74 (s, 1 H), 6.57 (s, 1 H), 5.97 (t, $J = 7.5$ Hz, 1 H), 3.88 (s, 3 H), 3.35 (s, 3 H), 2.82 (t, $J = 7.5$ Hz, 2 H), 2.47 (s, 3 H), 2.45 (s, 3 H), 2.33 (s, 6 H), 2.25 (q, $J = 7.5$ Hz, 2 H), 1.95 (p, $J = 7.5$ Hz, 2 H); ESIMS m/z (relative intensity) 530 (MNa^+ , 24), 508 (MH^+ , 3), 478 ($MH^+ - SCH_3 + H_2O$, 100). Anal. calcd for $C_{27}H_{29}N_3O_5S$: C, 63.89; H, 5.76; N, 8.28. Found: C, 63.60; H, 5.96; N, 7.91.

Crystallography

All crystallography experiments utilized an RT construct (RT52A) specifically optimized to co-crystallize with NNRTIs.³² RT was purified as described previously, concentrated to 40 mg/mL and then flash frozen and stored at -80 °C. Prior to use, RT was allowed to thaw on ice and then diluted to a final concentration of 20 mg/mL in 10 mM Tris pH 8.0 and 75 mM NaCl. ADAM inhibitors were added to RT at a stoichiometry of 1:1.5 protein:inhibitor from stock solutions previously dissolved in DMSO. Inhibitor and protein were allowed to incubate at room temperature for 10 min and then returned on ice. Crystals were grown using the vapor diffusion method by mixing 1 μ L of protein-inhibitor complex and 1 μ L of mother liquor sealed over a 500 μ L reservoir solution. The best crystals appeared at 4 °C using a mother liquor consisting of 50 mM imidazole pH 6.4, 10% PEG 8000, 100 mM ammonium sulfate, 10 mM spermine and 30 mM magnesium sulfate. Crystals were harvested and flash cooled in liquid nitrogen after transferring to mother liquor supplemented with 25% ethylene glycol. Data were collected at the Cornell High Energy Synchrotron Source (CHESS) F1 beam line. The program HKL2000³³ was used to index, scale, and merge reflection data. The phase problem was solved by molecular replacement using the program Molrep and the protein coordinates of the RT52A/10 complex (PDB ID 2ZD1) as the starting model.³⁴ Upon initial examination of the difference Fourier density, the presence of each respective inhibitor was clear. Refinement of protein coordinates proceeded by iterative rounds of manual fitting of electron density using the program COOT³⁵ followed by reciprocal space refinement using CNS.³⁶ In the final stages of model refinement, as the R_{work} and R_{free} converged, the respective inhibitors were built into the electron density and subject to coordinate and B-factor minimization.

RT Inhibition Assay

The ability of ADAM 4 to inhibit the enzymatic activity of recombinant HIV-1 RT (p66/51 dimer) was evaluated as previously described.^{24,37} Briefly, inhibition of purified HIV-1 reverse transcriptase was determined by the amount of ^{32}P labeled GTP incorporated into a nascent DNA strand, with a poly(rC)·oligo(dG) homopolymer primer, in the presence of increasing concentrations of the target compounds.

In Vitro Anti-Viral Assays

The anti-viral activity of ADAM 4 was determined for the HIV strains: HIV-1_{RF}, HIV-1_{IIIB}, and HIV-2_{ROD}. The anti-viral activity against HIV-1_{RF} and NNRTI-resistant strains (Y181C/K103N) was determined in infected CEM-SS cells using the XTT cytoprotection assay, as previously described.³⁷ Evaluation of anti-viral activity against the HIV-1_{IIIB} and HIV-2_{ROD} strains was performed in infected MT-4 cells using the previously described MTT assay.^{18,38}

In Vitro Hydrolytic Stability Assay Utilizing Rat Plasma

ADAM 4 was tested for its hydrolytic stability in solutions of reconstituted rat plasma using methods that have been previously reported.¹⁸ The internal standard used was 1,1-diphenylethylene and rat plasma from Sigma was utilized for the experiments (LOT# 065K7555). The aliquot supernatants were analyzed using a Waters binary HPLC system (Model 1525, 20 μ L injection loop) and a Waters dual wavelength absorbance UV detector (Model 2487) set for 254 nm. Data were collected and processed using the Waters Breeze software (version 3.3) on a Dell Optiplex GX280 personal computer. The mobile phase

consisted of 8:2 (v/v) acetonitrile-water and the Symmetry[®] HPLC column (4.6 mm × 150 mm) was packed with C₁₈ silica from Waters. The column was maintained at room temperature during the analyses. The reported half-life is an average calculated from two replicates. Half-lives for the individual replicates were calculated from regression curves fitted to plots of the compound concentration versus time.

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Abbreviations

ADAM, alkenyldiarylmethane; HAART, highly active antiretroviral therapy; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NtRTI, nucleotide reverse transcriptase inhibitor; TI, therapeutic index.

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Figure 1.

An overlay of the backbone tube structures of unliganded apo-RT (1HMV, yellow), the RT/**3** complex (red), and the RT/**4** complex (green). The crystal structure obtained for the RT/**3** and RT/**4** complexes show hyperextension of the thumb region and displacement of the fingers, relative to the structure of unliganded apo-RT. In areas of very close overlap, the red and green tubes appear as either red or green.

Figure 2.

(Top) Depiction of the NNRTI-binding pocket obtained from the RT-ADAM 3 crystal structure; (Bottom) depiction of the NNRTI-binding pocket obtained from the RT-ADAM 4 crystal structure. The view for each panel is from the 'front side' of the binding pocket, from the point of view of the putative solvent channel through which NNRTIs are believed to enter. The stereoview is programmed for wall-eyed viewing.

Figure 3. Overlay of the crystal structures of RT bound to ADAM **4** (red), nevirapine (blue), and delavirdine (green). The NNRTI binding pocket is more compact when nevirapine is bound in contrast to a more open pocket when delavirdine or ADAM **4** are present; $\beta 12$, $\beta 13$ and $\beta 14$ are rearranged to make room for the larger inhibitors.

1 **2** **3** **4**

Scheme 1a.

^aReagents and conditions: (a) cat. $\text{PdCl}_2(\text{PPh}_3)_2$, cat. CuI , Et_3N , THF; (b) cat. $\text{Pd}(\text{PPh}_3)_4$, Bu_3SnH , THF, 0 °C; (c) cat. $\text{Pd}(\text{PPh}_3)_4$, cat. CuI , CsF , DMF, 60 °C.

Table 1

Anti-viral activity and hydrolysis half-lives of ADAMs 1-4.

Compound	IC ₅₀ (μM) ^a	EC ₅₀ (μM) ^b			CC ₅₀ (μM) ^c		Half-Life t _{1/2} ± SD (min) ^d
		HIV-1 _{RF}	HIV-1 _{IIB} 1	MV-2 _{ROD}	CEM-SS	MT-4	
117	0.30	0.1	0.3	N.A. ^e	13	91	6.2 ± 0.4
219	0.02	0.03	0.09	N.A. ^e	5.1	17	1.3 ± 0.09
318	0.38	0.7	0.8	N.A. ^e	17	> 100	12.4 ± 0.7 ^f
4	<0.001	0.07	0.18	N.A. ^e	3.0	3.9	238 ± 8.5

^a Inhibitory activity versus HIV-1 RT with poly(rC)-oligo(dG) as the template primer.

^b EC₅₀ is the 50% inhibitory concentration for inhibition of the cytopathic effect of HIV-1_{RF} in CEM-SS cells, HIV-1_{IIB} in MT-4 cells, or HIV-2_{ROD} in MT-4 cells.

^c CC₅₀ is the cytotoxic concentration required to induce cell death for 50% of the mock infected CEM-SS or MT-4 cells.

^d Metabolic half-life of the compound when it was incubated with rat plasma; determined from a minimum of two replicates.

^e Not Active.

^f Previously reported in reference 18.

Table 2Anti-viral activity of ADAMs **1** and **4** against NNRTI-resistant HIV-1 strains.

Compound	EC ₅₀ (μM) ^a		
	WT ^b	K103N ^c	Y181C ^d
1 ^f	0.1	0.42	6.1
4	0.7	>100	>100
Nevirapine	N.T. ^e	0.6	0.8

^aEC₅₀ is the 50% inhibitory concentration for inhibition of the cytopathic effect of HIV-1_{RF} in CEM-SS cells.^bCytoprotective activity against HIV-1_{RF} bearing wild-type reverse transcriptase.^cCytoprotective activity against HIV-1 bearing reverse transcriptase containing the K103N mutation.^dCytoprotective activity against HIV-1 bearing reverse transcriptase containing the Y181C mutation.^eNot Tested.^fData were taken from reference ¹⁶.

Table 3

Crystallographic Data Collection and Structural Refinement Statistics.

	RT/ADAM 3	RT/ADAM 4
Crystallographic Data		
Space Group	C2	C2
a,b,c (Å)	162.82, 73.73, 108.69	164.64, 75.39, 110.66
β (°)	99.55	100.08
Resolution range (Å)	50-2.55	50-2.8
Total number of reflections	486 221	362 418
Number of unique reflections	41 074	28 872
$R_{\text{merge}}^{a,b}$	.086(.512)	.091 (.368)
I/σ^d	18.3 (3.0)	10.1 (1.8)
Completeness (%) ^a	98.7 (100.0)	87.2 (92.6)
Refinement		
Number of Reflections in R_{free} set	1 677	1 414
R_{work}^c	25.34	23.62
R_{free}^d	28.27	28.86
RMSD bond lengths (Å)	0.008	0.010
RMSD angles (°)	1.281	1.325
Average B factor of protein atoms (Å ²)	82	91
Average B factor of inhibitor atoms (Å ²)	84	74
No of protein atoms	7 933	7 941
No of inhibitor atoms	37	36
No of water atoms	126	0

^a Number in parentheses denote statistics for last resolution shell.^b $R_{\text{merge}} = \sum_h \sum_i |I_{h,j} - I_h| / \sum_h \sum_i I_{h,i}$, where I_h is the mean intensity of symmetry-related reflections^c $R_{\text{work}} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.^d $R_{\text{free}} = \sum ||F_o| - |F_c|| / \sum |F_o|$, for reflections in the test set