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A mechanistic and structural investigation of modified derivatives of the diaryltriazine class of NNRTIs targeting HIV-1 reverse transcriptase

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ABSTRACT

Background: Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are vital in treating HIV-1 infection by inhibiting reverse transcriptase (RT). Drug toxicity and resistance drive the need for effective new inhibitors with improved physiochemical properties and potent antiviral activity. Computer-aided and structure-based drug design have guided the addition of solubilizing substituents to the diaryltriazine scaffold. These derivatives have markedly improved solubility and maintain low nanomolar antiviral activity against RT. The molecular and structural basis of inhibition for this series was determined to facilitate future inhibitor development with improved pharmacological profiles.
Methods: The molecular mechanism of inhibition was investigated using transient-state kinetic analysis. Crystal structures of RT in complex with each inhibitor were obtained to investigate the structural basis of inhibition.
Results: The diaryltriazine and its morpholine derivative have RT inhibition constants of 9 ± 2 nM and 14 ± 4 nM, respectively. They adopt differential binding modes within the non-nucleoside inhibitor binding pocket to distort the catalytic site geometry and primer grip regions. The novel morpholinopropoxy substituent extends into the RT/solvent interface of the NNIBP.
Conclusions: Kinetic and structural analyses show that these inhibitors behave as conventional NNRTIs and inhibit the polymerization step. This study confirms that appending solubilizing substituents on the azine ring of diaryltriazine class of NNRTIs that extend into the RT/solvent interface effectively maintains low nanomolar potency and improves physiochemical properties.
General significance: The modification of NNRTI scaffolds with solubilizing substituents, which extend into the RT/solvent interface, yields potent antivirals and is an effective strategy for developing novel inhibitors with improved pharmacological properties.

1. Introduction

An estimated 35 million people worldwide are infected with HIV-1. The administration of highly active antiretroviral therapy (HAART), a combination of inhibitors targeting essential viral enzymes of the HIV-1 life cycle, has improved patients' quality of life. Key among the enzymes inhibited by HAART is HIV-1 reverse transcriptase (RT), a viral polymerase that transforms single-stranded RNA into double-stranded DNA that is subsequently integrated into the host genome [1]. The error prone nature of RT [2] along with inhibitor side effects and dosing regimens stemming from poor physiochemical properties [3,4] necessitate the development of novel antiretroviral inhibitors with improved resistance and pharmacological profiles to combat this disease.

To this end, our previous efforts utilized computer-aided and structure-based rational drug design to develop non-nucleoside reverse transcriptase inhibitors (NNRTIs) with both potent antiviral activity and improved physiochemical properties [5,6]. A key physiochemical property for an effective orally administered drug is aqueous solubility. The second-generation FDA-approved diarylpyrimidine (DAPY) NNRTIs etravirine and rilpivirine, with reported aqueous solubility of $\ll 1$ [7] and 0.02–0.24 μg/ml [8,9], respectively, have poor solubility, limiting the ease of formulation and bioavailability [7]. Given the poor solubility of approved DAPY NNRTIs, our drug design efforts focused on the addition of solubilizing groups to a structurally related class of NNRTIs, the diaryltriazines (DATAs). The impact of structural modifications of DATAs on antiviral activity and physiological properties has been previously examined but a detailed molecular and structural investigation is lacking [10]. Our previous work showed the addition of a solubilizing morpholinopropoxy substituent yielded a novel, structurally diverse DATA compound with 63- to 700-fold greater aqueous solubility than

Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; NNRTI, non-nucleoside reverse transcriptase inhibitor; DAPY, diarylpyrimidine; DATA, diaryltriazine; NNIBP, non-nucleoside inhibitor binding pocket; PDB, protein data bank

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the parent DATA inhibitor and both FDA-approved NNRTIs etravirine and rilpivirine [6] (Fig. 1).

To understand how the morpholinopropoxy substituent significantly improved solubility while maintaining nanomolar levels of potency against wild-type (WT) HIV-1 [6], we sought to understand the molecular mechanism and structural basis of inhibition of RT by compound **1** and its morpholinopropoxy derivative, compound **2**. We evaluated the mechanism of inhibition by transient kinetic analysis using pre-steady state burst experiments to examine single nucleotide incorporation. These studies illustrate the value of this approach for measuring the inhibition constant (K_i) of low nanomolar small molecule inhibitors of RT, a significant challenge arising from limits in assay sensitivity and stoichiometric binding. Transient kinetic analysis indicates that compounds **1** and **2** bind RT with high affinity and behave as conventional NNRTIs by inhibiting the polymerization reaction. Comparative structural analysis reveals that compounds **1** and **2** have different binding modes, with both inhibiting RT by preventing formation of a catalytically competent complex. Extension of the structural analysis provides possible implications on how resistance mutations within the non-nucleoside inhibitor binding pocket (NNIBP) may impact the antiviral activity of these inhibitors.

2. Materials and methods

2.1. Purification of HIV-1 RT for transient-state analysis

Recombinant HIV-1 RT (p66/p51 heterodimer), a clone kindly provided by Stephen Hughes, Paul Boyer, and Andrea Ferris (Frederick Cancer Research and Development Center, MD), was expressed in *E. coli* BL21(DE3) pLysS cells and purified as described previously [11]. RT concentration was estimated by UV absorbance at 280 nm using an extinction coefficient of 260,450 M^{-1} cm^{-1} as previously described [12]. RT purity as judged by SDS-PAGE analysis with Coomassie staining was >90%. RT active site concentration was determined by pre-steady-state burst experiments as previously described [13] and subsequent transient state biochemical experiments were performed using active site concentrations. RT protein samples were stored at −80 °C.

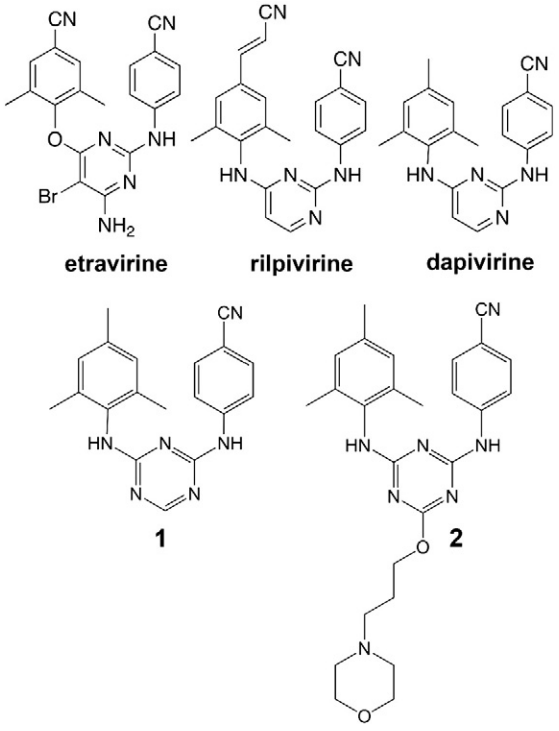

Fig. 1. Structures of diarylazine NNRTIs. Top row: current DAPY NNRTIs approved for the treatment of HIV-1 infection. Compound **1** and compound **2** are DATA derivatives. Compound **2** contains a morpholinopropoxy substituent at the C-6 position of the triazine ring. Drawings were rendered with Chemdraw 12.0.3 (CambridgeSoft).

2.2. Nucleotides and oligonucleotides

Natural 2′-deoxynucleotides were purchased from GE Healthcare Biosciences (Pittsburgh, PA). DNA oligonucleotides were purchased from Integrated DNA Technologies (Coraville, IA) and further purified using 20% polyacrylamide denaturing gel electrophoresis. The sequences of DNA primers and templates used for single nucleotide incorporation experiments were: D21 primer (5′-TCAGGTCCCTGTTCGGGCGCC-3′) and D36 template (5′-TCTCTAGCAGTGGCGCCCGAACAGGGACCTGAAAGC-3′). D21 primer was 5′-^{32}P-labeled and annealed to the D36 template as previously described [12,14].

2.3. In vitro radiolabeled-primer extension assay

Single nucleotide incorporation reactions catalyzed by RT in the absence and presence of compounds **1** and **2** were performed. RT (10 nM active site) and inhibitor concentrations ranging from 0 to 100 nM were pre-incubated in buffer solution (50 mM Tris pH 7.5, 50 mM NaCl) for 15 min at 4 °C. Subsequently, (5′-^{32}P)-labeled D21/D36 (30 nM) was added to this mixture and pre-incubated at 4 °C for an additional 5 min. A RQF-3 rapid chemical quench (KinTek Instruments) was used to rapidly mix the inhibitor·DNA solution with a saturating concentration of dATP (20 μM) in buffer containing 10 mM $MgCl_2$ at 37 °C. The reactions were quenched with 0.5 M EDTA pH 8.0. The concentrations are the final concentrations after 1:1 mixing in the instrument and all samples were performed in duplicate. The reaction mixtures were separated on a 20% polyacrylamide denaturing gel (8 M urea), visualized by phosphorimaging (Bio-Rad Molecular Imager FX), and extension of 5′-^{32}P-labeled D21 to D22-mer was quantified with Quantity One 4.6.9 (Bio-Rad). DMSO concentrations were 0.1% in all reactions.

2.4. Data analysis

Data were fit by nonlinear regression using KaleidaGraph (Synergy Software). Single nucleotide incorporation time courses at each inhibitor concentration tested were plotted and fit to a burst equation $[\text{product}] = A \cdot (1-e^{-k_{obs}t}) + A \cdot k_{ss} \cdot t$, where A is the burst phase amplitude, k_{obs} is the observed single exponential rate, k_{ss} is the steady-state rate, and t is the time. To generate inhibitor K_i values, the burst phase amplitudes were plotted versus inhibitor concentration and fit to a quadratic equation $A = 0.5(K_i + [E] + [D]) - 0.5((K_i + [E] + [D])^2 - 4[E][D])^{1/2}$ where A is the burst phase amplitude, E is the enzyme concentration, D is the primer-template concentration, and K_i is the inhibition constant. The data were fit to a quadratic function because the concentration of RT used in the assay was comparable to the K_i values and thus the assumption that the free concentration of inhibitor was equal to the total concentration of inhibitor added was not valid.

2.5. Chemical synthesis

Details of chemical synthesis of **1** and **2** were previously reported [6].

2.6. Crystallization and structure refinement

Recombinant RT52A enzyme was expressed and purified to homogeneity using methods previously described [15]. Crystals of recombinant RT52A in complex with 0.5 mM of compound **1** or compound **2** were prepared using methods similar to those previously described [15]. Briefly, the final optimized condition for crystal growth was 20.0% (w/v) PEG 8000, 100 mM ammonium sulfate, 15 mM magnesium sulfate, 5 mM spermine-HCl, and 50 mM citric acid pH 5.5 or 50 mM HEPES pH 7.0. Crystals grew in approximately 28–30 days at 4 °C.