Bioorganic & Medicinal Chemistry Letters 22 (2012) 4064–4067

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Pre-steady state kinetic analysis of cyclobutyl derivatives of 2′-deoxyadenosine 5′-triphosphate as inhibitors of HIV-1 reverse transcriptase

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ARTICLE INFO

Article history:
Received 1 March 2012
Revised 13 April 2012
Accepted 16 April 2012
Available online 24 April 2012

Keywords:
HIV-1 RT
Cyclobutyl adenosine analogs
Tenofovir
K65R mutant of RT
Pre-steady state kinetics

ABSTRACT

Pre-steady state kinetic analysis was utilized for biochemical evaluation of a series of cyclobutyl adenosine nucleotide analogs with HIV-1 RT^{WT}. The phosphonyl-diphosphate form of the cyclobutyl nucleotide, **5**, was the most efficiently incorporated of the series. Nucleotide **5** was fourfold more efficiently incorporated than the FDA approved TFV-DP by RT^{WT}. The kinetics of incorporation for **5** using the drug resistant mutant enzyme K65R was also determined. Compound **5** was threefold more efficiently incorporated compared to TFV-DP with RT^{K65R}. These results demonstrate cyclobutyl adenosine analogs can act as substrates for incorporation by HIV-1 RT and be a potential scaffold for HIV inhibitors.

Human immunodeficiency virus (HIV) affects millions of people around the world. Currently, therapeutics are available for the treatment of HIV-1 with nucleoside analogs continuing to be the backbone for the majority of persons infected. This class of drugs, in their triphosphate form target the enzyme reverse transcriptase (RT). This polymerase is essential for viral replication and is responsible for the conversion of viral RNA into the double stranded proviral DNA which will be subsequently be integrated into the host cell's genome. One class of therapeutics available is the nucleoside reverse transcriptase inhibitors (NRTIs). These nucleoside analogs, which are phosphorylated to the triphosphate form by cellular kinases act as a substrate for HIV-1 RT and are incorporated into the growing strand of viral DNA. Due to the lack of the 3′-hydroxyl group, these analogs act as chain terminators and do not allow for further elongation of the viral DNA.

Although there are many NRTIs available, there is still a need for novel and more potent inhibitors. Clinical use of FDA approved NRTIs give rise to drug resistant mutations in RT that render these inhibitors ineffective. One such mutation is K65R, which causes resistance against tenofovir (TFV or PMPA).[1,2] The emergence of such drug resistant viruses gives the need for new NRTIs with different scaffolds to be explored as potential therapeutics.

Successful cyclobutyl nucleosides have been developed as antiviral agents, such as Lobucavir against HBV. A cyclobutyl analog of cytosine triphosphate was found to be effective against WT and the M184V drug resistant form of HIV-1 RT.[3] In this Letter, we report the ability of cyclobutyl adenosine derivatives to be incorporated by HIV-1 RT. In order to examine the analogs' ability to act as a substrate, we utilized pre-steady state kinetics. With the use of a DNA/DNA primer-template containing the primer binding site (PBS) and its complementary sequence, we examined the kinetics to determine the incorporation efficiency for the natural substrate, dATP, as well as the analogs.

Three different compounds were synthesized in this series—cyclobutyl-adenosine triphosphate **3**, 2′-fluoro-cyclobutyl adenosine triphosphate **4**, and cyclobutyl-adenosine-phosphonyl-diphosphate **5**.[§] Details on the synthesis have been reported sepa-

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§ Details on the synthesis of these compounds have been reported separately. (Li, Y.; Hager, M. W.; Mao, S.; Bluemling, G. R.; Wang, L.; Kim, J.; Schinazi, R. F.; Anderson, K. S.; Liotta, D. C. *ACS Med. Chem. Lett.* **2012**, submitted for publication) A brief synopsis of the synthesis is included in the Supplementary data.

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http://dx.doi.org/10.1016/j.bmcl.2012.04.078

rately, a brief synopsis of the synthesis is included in the Supplementary data. These compounds, along with the natural substrate 2′-deoxyadenosine 5′-triphosphate (dATP) **1**, and the acyclic FDA approved 2′-deoxyadenosine nucleotide tenofovir diphosphate (TFV-DP) **2**, were tested for their ability to elongate a radiolabeled primer-template under the conditions of 250 nM HIV-1 RT (active site concentration), 50 nM primer-template, in the presence of 10 mM $MgCl_2$, and various concentrations of the nucleotides as previously described.[4]

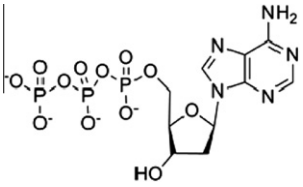
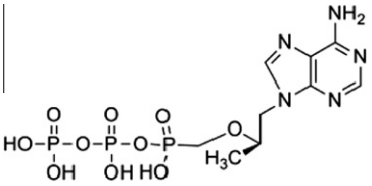
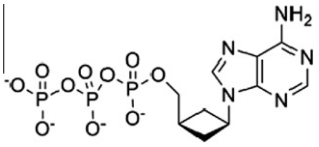
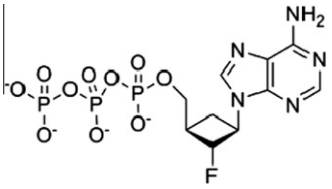
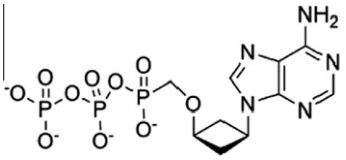
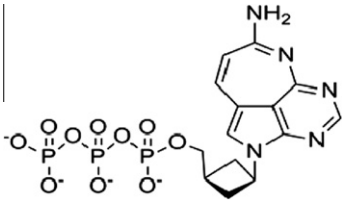
The natural substrate was most efficiently incorporated, compared to the derivatives as shown in Table 1. Tenofovir diphosphate **2**, was 56-fold less efficient for incorporation compared to the natural substrate. This decrease in efficiency was largely due to the 15-fold decrease in the maximum rate of incorporation (k_{pol}) and a fourfold decrease in the binding affinity of the nucleotide as observed by the increase in the binding affinity constant (K_d). Previous kinetic work has been performed with TFV-DP **2** and demonstrates a decrease in incorporation efficiency of the analog versus dATP with varying degrees, which may be due to the use of different primer-template sequences.[1,5,6]

The presence of a cyclobutyl ring, **3**, decreased the incorporation efficiency by over 200-fold (2.7 s^{-1} μM^{-1} for **1** versus 0.011 s^{-1} μM^{-1} for **3**), compared to the natural substrate. This difference in efficiency was imparted by a 53-fold decrease in the k_{pol} and a 4.5-fold decrease in the binding affinity. From an acyclic nucleotide **2**, to the cyclobutyl ring **3**, the efficiency was decreased by fourfold, which was due to a fourfold decrease in the k_{pol}.

Many studies involving the presence of fluorine in nucleotide analogs have the substituent at the base of the nucleotide. Incorporation studies of these fluorine substituted analogs using a DNA/DNA primer-template have demonstrated an improvement in incorporation efficiency.[7] Lobucavir contains a hydroxy methyl substituent at the 2′ position of the cyclobutyl ring. The fluorine present at the 2′ position of the cyclobutyl ring, **4**, negatively affected the incorporation compared to its parent compound, **3**, and was twofold less efficient (0.0052 s^{-1} μM^{-1} for **4**) as a substrate for incorporation. This difference in efficiency was observed through a twofold decrease in k_{pol}. Although the rate of incorporation was affected, the K_d of **3** and **4** were within error.

A phosphonyl-diphosphate form of the cyclobutyl adenosine analog was synthesized, **5**. This compound was clearly a better substrate for incorporation by RT compared to the triphosphate form **3**. The efficiency of incorporation for the phosphonyl diphosphate **5** was 20-fold higher (0.21 s^{-1} μM^{-1}) compared to the

Table 1
Structures and pre-steady state kinetic data for incorporation of deoxyadenosine triphosphate, tenofovir diphosphate, and cyclobutyl analogs using HIV-1 RT^{WT}

Compound	Structure	k_{pol} (s^{-1})	K_d (μM)	Efficiency (s^{-1} μM^{-1})	Discrimination
1		12.3 ± 1.0	4.6 ± 1.6	2.7	
2		0.79 ± 0.08	16.5 ± 4.6	0.048	56
3		0.23 ± 0.02	20.4 ± 4.6	0.011	245
4		0.13 ± 0.01	25.5 ± 3.8	0.0052	519
5		1.3 ± 0.1	6.5 ± 2.0	0.21	13
6		0.16 ± 0.01	22.4 ± 2.8	0.007	386

triphosphate **3**. This increase in efficiency was imparted by a sixfold increase in the k_{pol} as well as the threefold tighter binding affinity. A phosphonate group has several advantages over a phosphate. The phosphorous–carbon bond is not susceptible to hydrolysis by phosphatases. Also, once the prodrug form of a nucleoside has entered a cell, the presence of the phosphonate group bypasses the first phosphorylation step, which is considered to be the rate limiting step in the conversion of the triphosphate form of a nucleoside by cellular kinases. A recent review discusses these advantages and gives examples of other antiviral phosphonate candidates.[8] Interestingly, compound **5** was 4.4-fold more efficiently incorporated by RT^{WT} compared to the tenofovir diphosphate **2** (Fig. 1, solid bars). Compound **5** had a twofold faster k_{pol} and an almost threefold tighter binding affinity compared to the FDA approved nucleotide analog. Compound **5** was able to act as a chain terminator by inhibiting incorporation of the next correct nucleotide, as shown in Supplementary Figure 1.

Another cyclobutyl containing compound **6**, was synthesized as an adenosine analog to base-pair with dTTP. This compound was not a good substrate for incorporation by RT, and was almost twofold less efficient (0.007 s^{-1} μM^{-1}) compared to the parent compound **3**. This difference was due to the almost twofold decrease in the k_{pol}.

The HIV-1 RT's ability to discriminate a nucleotide analog was determined, which is the ratio of the efficiency of incorporation for the natural substrate **1** to the efficiency of incorporation for the analog. The RT^{WT} was fourfold less discriminative against compound **5** versus compound **2**. The enzyme was 4- to 7-fold more discriminative against the other cyclobutyl analogs (**3, 4, 6**) versus compound **2**, and 19- to 40-fold more discriminative versus compound **5**.

Tenofovir-diphosphate **2**, is an acyclic adenosine analog that also contains a phosphonate group, similarly to compound **5**. Upon administration of tenofovir to infected persons, a drug resistant form of HIV-1 RT may arise containing a K65R mutation. We determined the pre-steady state kinetic parameters for the natural substrate dATP, **1**, tenofovir diphosphate **2**, and the most promising cyclobutyl compound, **5** using the K65R mutant form of HIV-1 RT, as shown in Table 2.

The RT^{K65R} was fourfold less efficient in incorporating the natural substrate **1**, and 14-fold less efficient in incorporating the cyclobutyl compound **5**. The large decrease in incorporation efficiency using the mutant RT was observed through a decrease in the k_{pol}, compared to the wild type enzyme. Compound **2** was ninefold less efficiently incorporated by the mutant enzyme compared to the wild type which was imparted by a fivefold decrease in the k_{pol},

Table 2
Pre-steady state kinetic data for incorporation of deoxyadenosine triphosphate (dATP), tenofovir diphosphate (TFV-DP) and cyclobutyl-adenosine-phosphonyl-diphosphate (**5**) using HIV-1 RT^{K65R}

Compound	k_{pol} (s^{-1})	K_d (μM)	Efficiency (s^{-1} μM^{-1})	Discrimination
dATP	0.4 ± 0.3	6.6 ± 1.2	0.61	
TFV-DP	0.15 ± 0.01	29.1 ± 6.0	0.005	122
5	0.07 ± 0.004	4.7 ± 1.0	0.015	41

and a twofold decrease in the binding affinity. The RT^{K65R} was able to incorporate the cyclobutyl compound **5** more efficiently compared to the tenofovir diphosphate **2** (Fig. 1, cross hatch bars) by threefold. The discrimination of the two nucleotide analogs was also determined. The RT^{K65R} mutant was threefold less discriminative against compound **5** versus compound **2**.

Recent work has been performed to solve the crystal structures of wild type and the K65R mutant of RT in the presence of a primer-template and dATP or TFV-DP.[9,10] Three major residues are involved in the interactions with the incoming dNTP in both polymerases—K(R)65, R72, and Y115. R72 interacts with the base, sugar, and α-phosphate of the incoming nucleotide. When K65 is mutated to an R, guanidinium stacking occurs with this amino acid and R72, causing steric restriction on R72 and therefore affecting the incorporation rate by RT. The amino acid Y115 stacks against the ribose ring of the natural substrate, which is partially lost with TFV-DP due to its acyclic structure as shown in Figure 2a. In order to analyze potential binding interactions of **5**, the compound was docked into the RT active site bound to primer-template (Tuske et al. Ref. 10, PDB code 1T05). The cyclobutyl ring of compound **5** may be able to stack with Y115, or form favorable Van der Waals contacts, which may explain its better binding affinity compared to TFV-DP as shown in Figure 2a and b. Similar to the natural substrate and TFV-DP, the K65R mutation greatly decreases the rate of incorporation perhaps in the same manner, through the restriction of the R72 and its interaction with the phosphonate group present in the cyclobutyl compound. It is interesting to note that from the wild type RT structure with TFV-DP, the side chains of Lys65 and Lys219 are positioned to make potential salt bridges with the α-phosphate of the phosphonate. Based on the binding predictions from our model, potential salt bridges may be formed between compound **5**, Lys65, and Lys219 (Fig. 2b). This differs from dNTP in which only Lys65 is positioned to make a potential salt bridge with the α-phosphate. The additional salt bridge formed with the phosphonate may give rise to potency of the compound. The docking model and structural data may also help explain the increased

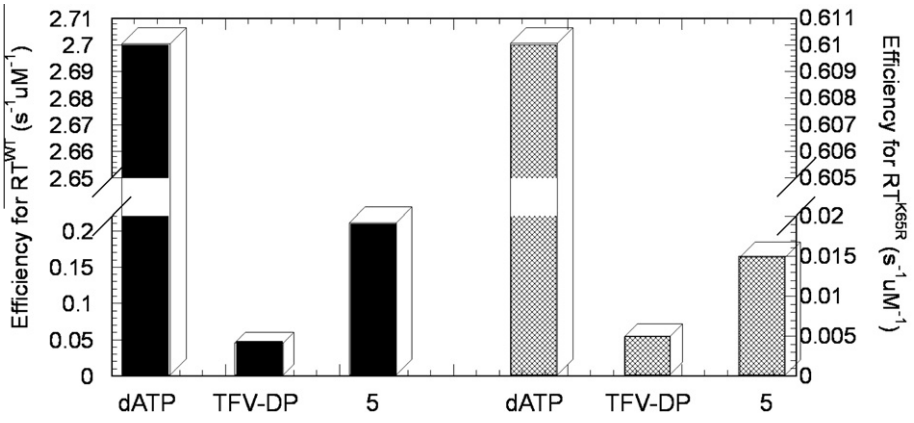


Figure 1. Incorporation efficiency for deoxyadenosine triphosphate (dATP), tenofovir diphosphate (TFV-DP), cyclobutyl adenosine phosphonyl disphosphate (**5**) for wild type (solid black) and K65R (cross hatch) HIV-1 RT. The natural substrate, dATP, is the most efficiently incorporated. The cyclobutyl analog, **5**, is more efficiently incorporated by both forms of RT compared to the FDA approved TFV-DP

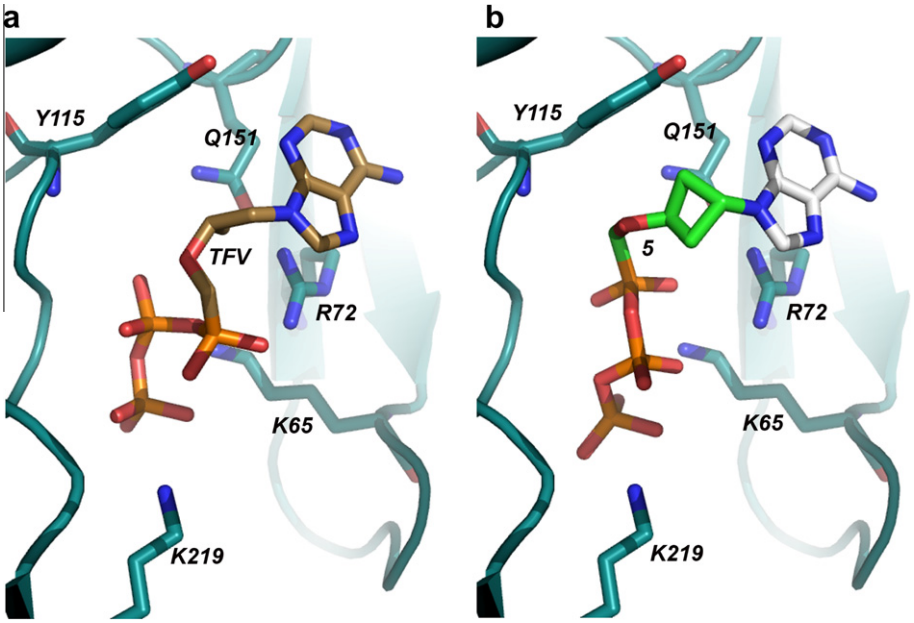


Figure 2. Tenofovir and compound **5** in the active site of HIV-1 RT. (a) Crystal structure of HIV-1 RT cross-linked to template-primer and tenofovir-diphosphate bound as the incoming nucleotide substrate (PDB code: 1T05). (b) Docked model of HIV-1 RT with compound **5** bound as the incoming nucleotide substrate using Autodock Vina. Information on the docking model is in the Supplementary data.

catalytic efficiency of HIV-1 RT observed with the phosphonate compound **5** compared to the triphosphate compound **3**.

Several cyclobutyl adenosine triphosphate analogs were evaluated for their ability to be incorporated by HIV-1 RT using pre-steady state kinetics. These data indicate the potential for cyclobutyl compounds to be a substrate for the enzyme. The phosphonyl diphosphate cyclobutyl adenosine analog **5** was most efficiently incorporated with a similar K_d to the natural substrate **1**. Compound **5** was also more efficiently incorporated compared to the clinically available tenofovir diphosphate **2** with both the wild type and K65R mutant forms of HIV-1 RT. We also demonstrated the ability of compound **5** to act as chain terminator by inhibiting incorporation of the next incoming nucleotide. Future work will further explore the phosphonyl group and use tenofovir disoproxil fumarate and the compound GS7340, an isopropylalaninyl monoamidate phenyl monoester prodrug of TFV[11] as models for a prodrug strategy of compound **5**.

Acknowledgments

We would like to thank Drs. Stephen Hughes, Paul Boyer and Andrea Ferris (NIH) for the HIV-1 RT^{WT} clone. We would also like to acknowledge Sierra Bioresearch (Tucson, AZ) for synthesizing compound **4**. We would also like to thank the National Institutes of Health for support to GM49551 to K.S.A. RFS is supported by CFAR NIH Grant 2P30-AI-050409 and the Department of Veterans Affairs.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl. 2012.04. 078.

References and notes

1. Deval, J.; White, K. L.; Miller, M. D.; Parkin, N. T.; Courcambeck, J.; Halfon, P.; Selmi, B.; Boretto, J.; Canard, B. *J. Biol. Chem.* **2004**, *279*, 509.
2. White, K. L.; Margot, N. A.; Wrin, T.; Petropoulos, C. J.; Miller, M. D.; Naeger, L. K. *Antimicrob. Agents Chemother.* **2002**, *46*, 3437.
3. Li, Y.; Mao, S.; Hager, M. W.; Becnel, K. D.; Schinazi, R. F.; Liotta, D. C. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3398.
4. Kati, W. M.; Johnson, K. A.; Jerva, L. F.; Anderson, K. S. *J. Biol. Chem.* **1992**, *267*, 25988.
5. Frangeul, A.; Barral, K.; Alvarez, K.; Canard, B. *Antimicrob. Agents Chemother.* **2007**, *51*, 3162.
6. Suo, Z.; Johnson, K. A. *J. Biol. Chem.* **1998**, *273*, 27250.
7. Ray, A. S.; Schinazi, R. F.; Murakami, E.; Basavapathruni, A.; Shi, J.; Zorca, S. M.; Chu, C. K.; Anderson, K. S. *Antiviral Chem. Chemother.* **2003**, *14*, 115.
8. De Clercq, E. *Biochem. Pharmacol.* **2011**(2), 99.
9. Das, K.; Bandwar, R. P.; White, K. L.; Feng, J. Y.; Sarafianos, S. G.; Tuske, S.; Tu, X.; Clark, A. D., Jr.; Boyer, P. L.; Hou, X.; Gaffney, B. L.; Jones, R. A.; Miller, M. D.; Hughes, S. H.; Arnold, E. *J. Biol. Chem.* **2009**, *284*, 35092.
10. Tuske, S.; Sarafianos, S. G.; Clark, A. D., Jr.; Ding, J.; Naeger, L. K.; White, K. L.; Miller, M. D.; Gibbs, C. S.; Boyer, P. L.; Clark, P.; Wang, G.; Gaffney, B. L.; Jones, R. A.; Jerina, D. M.; Hughes, S. H.; Arnold, E. *Nat. Struct. Mol. Biol.* **2004**, *11*, 469.
11. Lee, W. A.; He, G. X.; Eisenberg, E.; Cihlar, T.; Swaminathan, S.; Mulato, A.; Cundy, K. C. *Antimicrob. Agents Chemother.* **2005**, *49*, 1898.