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Biochemical characterization of the inhibition of the dengue virus RNA polymerase by beta-D-2′-ethynyl-7-deaza-adenosine triphosphate

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ABSTRACT

Dengue virus (DENV), an emerging pathogen from the *Flaviviridae* family with neither vaccine nor antiviral treatment available, causes a serious worldwide public health threat. In theory, there are several ways by which small molecules could inhibit the replication cycle of DENV. Here, we show that the nucleoside analogue beta-D-2′-ethynyl-7-deaza-adenosine inhibits representative strains of all four serotypes of DENV with an EC_{50} around or below 1 μM. Using membrane-associated native replicase complex as well as recombinant RNA polymerase from each DENV serotype in enzymatic assays, we provide evidence that beta-D-2′-ethynyl-7-deaza-adenosine triphosphate (2′E-7D-ATP) targets viral replication at the polymerase active site by competing with the natural nucleotide substrate with an apparent K_i of 0.060 ± 0.016 μM. In single-nucleotide incorporation experiments, the catalytic efficiency of 2′E-7D-ATP is 10-fold lower than for natural ATP, and the incorporated nucleotide analogue causes immediate chain termination. A combination of bioinformatics and site-directed mutagenesis demonstrates that 2′E-7D-ATP is equipotent across all serotypes because the nucleotide binding site residues are conserved in dengue virus. Overall, beta-D-2′-ethynyl-7-deaza-adenosine provides a promising scaffold for the development of inhibitors of dengue virus polymerase.

1. Introduction

The *Flaviviridae* virus family includes important human pathogens such as hepatitis C virus (HCV), dengue virus (DENV), West Nile virus (WNV), and yellow fever virus (YFV). Dengue virus is spread by the mosquito *Aedes aegypti* and causes an acute fever sometimes followed by hemorrhage (or plasma leakage) and shock syndrome in secondary infections. Dengue virus infects 50–100 million individuals per year through explosive waves of epidemics in the tropical and sub-tropical regions of the world (Halstead, 2007). The genome of DENV contains roughly 11,000 nucleotides of a positive single-stranded RNA that encodes a single polyprotein. The proteolytic cleavage of this precursor results in the releases of 10 structural and non-structural proteins necessary for assembly and release of infectious virus progeny.

The idea of targeting the non-structural proteins of dengue virus with antivirals has recently emerged (Bollati et al., 2009; Ray and Shi, 2006; Sampath and Padmanabhan, 2009). A potentially attractive target for the development of such inhibitors is the non-structural protein 5 (NS5), the largest of the DENV proteins. NS5 is essential for replication of the dengue viral genome. The 900 amino acid protein contains a (guaninine-N7) and (nucleoside-2′-O-) methyltransferase (MTase) domain at its N-terminus and a RNA-dependent-RNA polymerase (RdRp) domain at the C-terminus (Bollati et al., 2009; Brooks et al., 2002; Pryor et al., 2007; Yap et al., 2007). The RdRp domain duplicates the viral genome in a linear and continuous manner, starting from the 3′-end of the single-stranded RNA (ssRNA) template. Initiation of RNA synthesis by polymerases from the *Flaviviridae* family is primer-independent, meaning that the viral enzymes have to generate their own primer prior to reaching the processive elongation mode (Ackermann and Padmanabhan, 2001; van Dijk et al., 2004). As seen in HCV, bovine viral diarrhea virus (BVDV), and WNV, the DENV polymerase adopts a typical right-hand structure featuring three subdomains: fingers, palm, and thumb. (Bressanelli et al., 1999; Choi et al., 2004; Malet

Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; BVDV, Bovine viral diarrhea virus; CPE, cytopathic effect; DENV, dengue virus; HCV, hepatitis C virus; IU, international unit; MTase, methyltransferase; NS5, non-structural protein 5; NS5pol, polymerase domain of NS5; FL, full length; NGC, new Guinea C; ssRNA, single-stranded RNA; YFV, yellow fever virus; WNV, West Nile virus; 2′E-7D-ATP, beta-D-2′-ethynyl-7-deaza-adenosine triphosphate.

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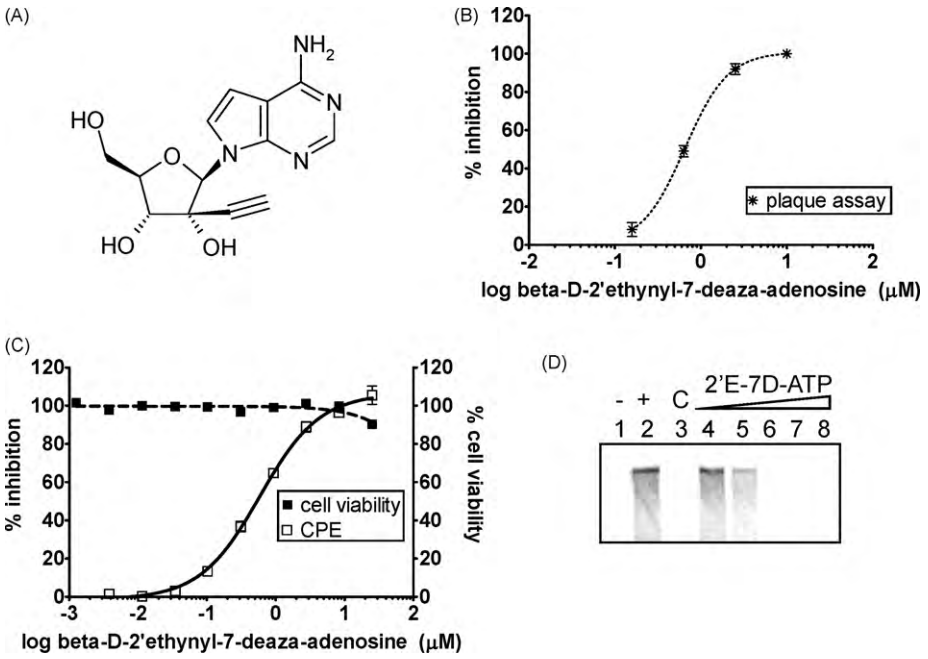

Fig. 1. Antiviral activity of beta-D-2′-ethynyl-7-deaza-adenosine against dengue virus. (A) Chemical structure of beta-D-2′-ethynyl-7-deaza-adenosine. (B) Inhibition of DENV-3 replication by beta-D-2′-ethynyl-7-deaza-adenosine is followed indirectly in a standard plaque assay. Protection of BHK-21 cells from virus-induced cytopathic effect is monitored in the presence of four serial dilutions of beta-D-2′-ethynyl-7-deaza-adenosine (10, 2.5, 0.6, and 0.2 μM). (C) Inhibition of virus-induced cytopathic effect (CPE) in Huh-7 cells, by increasing concentrations of beta-D-2′-ethynyl-7-deaza-adenosine up to 25 μM. Cell viability was monitored with the same compound concentration range in uninfected cells. (D) Inhibition of the polymerase activity of membrane-associated native replicase of DENV by 2′E-7D-ATP. The membrane fraction from Vero cells containing a stable DENV-2 replicon was incubated for 2 h in the presence of all four NTPs including 1 μM ATP and radiolabeled ^{33}P-CTP as tracer (lane 2), and 50 μM of the chain terminator 3'dATP was chosen as inhibitor control of the polymerase activity (lane 3). Lanes 4–8: increasing concentrations of 2′E-7D-ATP were added to the polymerase reaction, lane 4: 0.08 μM, lane 5: 0.4 μM, lane 6: 2 μM, lane 7: 10 μM, and lane 8: 50 μM.

et al., 2007; Yap et al., 2007). In the absence of an elongated primer, the polymerase domain adopts a close conformation where fingers and thumb are connected. Therefore, significant conformational changes are likely to take place within these subdomains in order for the enzyme to transition from the initiation to the elongation step, offering multiple opportunities by which a small molecule can have a pharmacological effect.

Nucleoside analogues are widely validated for antiviral therapies. These molecules have been clinically tested or approved against herpesvirus and cytomegalovirus, hepatitis B and C viruses, HIV, and influenza (De Clercq and Neyts, 2009). Most nucleosides inhibit viral polymerases in the cytoplasm by the process of chain termination, in which the incorporation of the nucleotide analogue stops nucleic acid synthesis. These analogues must be transported into the infected cell and converted to the active triphosphate by cellular kinases in order to inhibit viral polymerases by chain termination.

In this study, we provide a detailed biochemical characterization of the inhibition of dengue virus polymerase by the nucleotide analogue beta-D-2′-ethynyl-7-deaza-adenosine triphosphate (2′E-7D-ATP). First, we investigated at the cell-based level the antiviral potency of beta-D-2′-ethynyl-7-deaza-adenosine (Fig. 1A), a novel 2′-modified nucleoside with anti-dengue virus properties (Yin et al., 2009, 2008). We showed that 2′E-7D-ATP inhibits the polymerase activity of membrane-associated DENV replicase complex, which explains the antiviral potency of the parent nucleoside against representative strains of the four serotypes. In order to further characterize the mode of inhibition of 2′E-7D-ATP, we produced the corresponding recombinant NS5 for each DENV serotype and measured their intrinsic polymerase activity by steady-state kinetics. We observed significant heterogeneity in catalytic efficiency (1) between the polymerase domain and the full-length NS5, and (2) across serotypes. Most importantly, we found that 2′E-7D-ATP targets dengue virus replication at the polymerase level by first competing with the natural substrate for nucleotide incorporation, followed by immediate chain termination. Overall, this makes beta-D-2′-ethynyl-7-deaza-adenosine the most potent chain terminator of dengue virus polymerase reported to date.

2. Materials and methods

2.1. Chemicals and nucleic acid

The RNA template sequence used in single-nucleotide experiments was chemically synthesized by Integrated DNA Technologies: 5′-UCGUGGCCCAAAAGGGCC-3′ (HP5-A18). The underlined base shows the unique site for incorporation of A-analogues. 5′-end labeling was conducted with [γ^{33}P] ATP and T4 polynucleotide kinase according to manufacturer's recommendation (Invitrogen). After inactivation of the kinase by heat, the radiolabeled RNA was purified with G25 spin columns (GE healthcare). The minus strand 3′UTR RNA was synthesized with the T7 Megascript kit (Ambion), using the last 380 bases from the negative strand of the DENV serotype 2 New Guinea C (NGC strain) genome as template. 3′-dATP was purchased from TriLink Biotechnologies. Beta-D-2′-ethynyl-7-deaza-adenosine adenosine was synthesized in-house (94.6% purity) while the corresponding triphosphate was synthesized by TriLink Biotechnologies (95% purity).

2.2. Antiviral assay

Dengue virus representative strains of the four serotypes DENV-1 (Th-Sman), DENV-2 (Th-36), DENV-3 (H-87) and DENV-4 (H-241) were all obtained from the ATCC (Manassas, VA). Virus titers were measured on BHK-21 cells, using a standard plaque assay procedure. For the determination of EC_{50} of nucleoside in the antiviral assay, Huh-7 cells were plated in white 96-well plates in MEM media supplemented with 10% FBS and 1% penicillin/streptomycin.