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Targeting flavivirus RNA dependent RNA polymerase through a pyridobenzothiazole inhibitor

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ABSTRACT

RNA dependent RNA polymerases (RdRp) are essential enzymes for flavivirus replication. Starting from an *in silico* docking analysis we identified a pyridobenzothiazole compound, HeE1-2Tyr, able to inhibit West Nile and Dengue RdRps activity *in vitro*, which proved effective against different flaviviruses in cell culture. Crystallographic data show that HeE1-2Tyr binds between the fingers domain and the priming loop of Dengue virus RdRp (Site 1). Conversely, enzyme kinetics, binding studies and mutational analyses suggest that, during the catalytic cycle and assembly of the RdRp-RNA complex, HeE1-2Tyr might be hosted in a distinct binding site (Site 2). RdRp mutational studies, driven by *in silico* docking analysis, allowed us to locate the inhibition Site 2 in the thumb domain. Taken together, our results provide innovative concepts for optimization of a new class of anti-flavivirus compounds.

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1. Introduction

The genus Flavivirus belongs to the *Flaviviridae* family and consists of over seventy related members, mostly transmitted by arthropods, and pathogenic to humans (Mackenzie et al., 2004). Among them, Dengue virus (DENV), West Nile virus (WNV), Japanese Encephalitis virus (JEV), Yellow fever virus (YFV), and lately Zika virus, are considered important pathogens that occupy a special area within the RNA virus world (Daep et al., 2014). Flaviviral infections are classified by the World Health Organization (WHO) as neglected tropical diseases (NTDs); nevertheless, a well-established body of environmental, demographic, and ecological evidences suggest that known and/or novel flavivirus are likely to spread to other regions of the word in the near future (http://www.who.int/csr/don/21-october-2015-zika/en/).

Flaviviruses are small, enveloped, RNA viruses whose genome consists of a 11 kb 5'-capped (+)*ss*-RNA that encodes for a single polyprotein, which is processed to yield three structural and seven non-structural (NS) proteins. The expression of NS proteins induces modifications on the host endoplasmic reticulum where viral replication takes place (Welsch et al., 2009). NS5 is a multi-domain protein endowed with N-terminal methyltransferase activity and C-terminal RNA-dependent RNA polymerase (RdRp) activity. RdRp replicates (+)RNA into (-)RNA that is later used as a template to produce large excess of the viral genome. It has been shown that NS5 initiates RNA synthesis *de novo* (*i.e.* primer-independent) using a conserved loop (the priming loop) that protrudes from the RdRp

Abbreviations: RdRp, RNA dependent RNA polymerase; **HeE1-2Tyr**, N-{[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl] carbonyl}-L-tyrosinate; DENV, Dengue virus; WNV, West Nile virus; JEV, Japanese encephalitis virus; YFV, yellow fever virus; HCV, hepatitis C virus.

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thumb domain toward the fingers domain to assist formation of the initiation complex (Selisko et al., 2012).

An effective and specific therapy against flaviviruses is currently not available; even the broad-spectrum antiviral compound ribavirin appears ineffective for treating flavivirus infections (Leyssen et al., 2000; Malinoski et al., 1990). In the search for new antivirals, a promising strategy is to target the viral polymerase, as shown by the results obtained *versus* HIV reverse transcriptase, and with the HCV NS5B inhibitors, currently in clinical use. Although a number of DENV (and to less extent of WNV) NS5 RdRp inhibitors have been reported, to date none of these passed efficacy/safety tests in animals or in clinical trials (Lim et al., 2015; Xu et al., 2015).

In the context of a drug discovery program targeting flavivirus RdRp, we firstly analyzed *in silico* a focused library consisting of 203 published (Manfroni et al., 2014a,b; Barreca et al., 2013; Manfroni et al., 2012) and unpublished compounds, designed to target specifically HCV NS5B RdRp. The results of such *in silico* screening vs. DENV RdRp brought us to the biochemical and crystallographic characterization of one selected compound, **HeE1-2Ty**, that proved to be a micromolar inhibitor of flavivirus RdRp. Importantly, this pyridobenzothiazole-based compound, showed specific antiviral activity (in the micromolar range) in cell cultures against a panel of different flaviviruses. Here we report the chemical synthesis of **HeE1-2Tyr**, together with the crystal structure of the DENV (serotype 3, DENV3) RdRp/**HeE1-2Tyr** complex, and describe its biochemical characterization as a DENV3 RdRp inhibitor. To exert its RdRp inhibitory activity, **HeE1-2Tyr** exploits an inhibition and binding mechanism, new to RdRp, which might be extended to the design of a new class of inhibitory compounds.

2. Materials and methods

2.1. Preparation of N-{[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1H-pyrido[2,1-b][1,3]benzothiazol-4-yl]carbonyl}-L-tyrosinate (HeE1-2Tyr)

For general methods: see Supplementary material.

A solution of methyl ester **6** (150 mg, 0.3 mmol) and aq 1N LiOH (0.9 mmol) in 1,4-dioxane (5 mL) was stirred for 0.5 h at room temperature. The mixture was poured into ice/water, acidified with 2N HCl (pH = 3) and extracted three times with EtOAc. The combined organic layers were washed two times with brine, dried, and evaporate under vacuum to give a crude solid which was purified by flash column chromatography eluting with $CHCl_3$/MeOH (90:10) affording the title compound **HeE1-2Tyr** (87 mg, 50%) as yellow solid: mp 260–262 °C; TLC ($CHCl_3$/MeOH, 80:20 v/v): $R_f = 0.40$; $[\alpha]_D = -0.36$ (0.5% p/v, MeOH); ^1H NMR (400 MHz, DMSO-d_6): δ 12.80 (brs, 1H, Tyr-CO_2H), 9.20 (brs, 1H, Tyr-OH), 8.90–8.80 (m, 2H, Tyr-NH and H-9), 8.50 (s, 1H, H-3), 7.90 (d, $J = 8.7$ Hz, 1H, H-6), 7.80–7.75 (m, 2H, Ar-H), 7.50–7.45 (m, 2H, Ar-H), 7.40–7.35 (m, 1H, Ar-H), 7.20 (dd, $J = 2.2$ and 8.7 Hz, 1H, H-7), 7.15–7.10 (m, 2H, Tyr Ar-H), 6.65–6.60 (m, 2H, Tyr Ar-H), 4.70–4.60 (m, 1H, Tyr-CH), 4.45–4.35 (m, 1H, Cy-CH), 3.10 (dd, $J = 4.7$ and 13.9 Hz, 1H, Tyr-CH_AH_B), 2.90 (dd, $J = 10.5$ and 13.9 Hz, 1H, Tyr-CH_AH_B), 2.00–1.85 and 1.75–1.60 (each m, 2H, Cy-CH_2), 1.55–1.20 (m, 6H, Cy-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.81, 164.49, 161.32, 156.31, 156.29, 151.89, 138.98, 136.77, 133.92, 130.41, 129.36, 128.47, 127.83, 127.89, 123.13, 122.39, 120.90, 116.50, 115.45, 107.52, 105.37, 75.66, 55.13, 36.09, 31.57, 25.52, 23.48; HRMS (*m/z*): $[M]^+$ calcd. for $C_{33}H_{30}N_2O_6S$, 583.1902; found 583.1908; LC-MS: ret. time 7.35 min. Chiral HPLC: ret time$_{Zwix\text{-}(+)}$ 5.16 min; ret time$_{Zwix\text{-}(-)}$ 12.12 min, ee: 100%.

2.2. In silico docking

The AutoDock 4.2 software package (Morris et al., 2009) was used for a docking search on a library with 203 compounds developed as potential HCV RdRp inhibitors (Manfroni et al., 2014a,b; Barreca et al., 2013; Manfroni et al., 2012). Briefly, the atomic coordinates of the DENV3 RdRp (pdb-id: **2J7U**; (Yap et al., 2007)) were chosen as a rigid protein model, removing all water molecules. The protein was prepared using the program Python Molecule Viewer 1.4.5 (http://mgltools.scripps.edu/packages/pmv), adding hydrogen atoms and Kollman charges.

2.2.1. In silico docking around the protein active site

The RdRp protein model was employed to build a discrete grid within a box of 22.1 × 29.6 × 22.1 Å^3, centered near the side chain of Trp795 (Autogrid 4.2; grid step size: 0.375 Å) as the explored volume for the docking search. All the compound formulas from the focused library (2D sdf format) were processed with MolConvert (ChemAxon Ltd) to generate 3D sdf format; the program Open Babel (O'Boyle et al., 2011) then converted all sdf into pdb files. After the addition of Gasteiger charges, and of the rotational constraints on every molecule in the library (MGLTools package: http://mgtools.scripps.edu/), a 'docking parameter file' was generated for each compound, to perform 30 independent genetic algorithm runs in the docking procedure. The results were analyzed with Python Molecule Viewer 1.4.5. The best two molecules (ΔG values −10.05 and −9.52 kcal/mol), were selected to be tested in *in vitro* activity assays.

2.2.2. In silico docking around the thumb domain

For the purpose of a second docking analysis (see below), the priming loop of the protein (from His786 to Asp808) was removed from the pdb, leaving an open gap in the polypeptide chain. The protein was then prepared using the program Python Molecule Viewer 1.4.5 as described before. The protein model was employed to build a discrete grid within a box of 22.9 × 22.9 × 22.9 Å^3 dimensions, centered near the thumb domain residue Ala757. A search for the best docking sites of **HeE1-2Tyr** was performed using AutoDock 4.2 (60 genetic algorithm runs).

2.3. Cloning, expression and purification of the DENV3 RdRp domain

DENV3 RdRp (amino acids 272-900) was cloned into pET15b with a His6-tag at the N-ter, and expressed in *E. coli* BL21pLys cells (Noble et al., 2013). Cells were grown at 37 °C to an OD_{600} 0.8, induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) and further incubated O/N at 15 °C. Cells were harvested by centrifugation and the pellet was resuspended in 20 mM Na HEPES at pH 7.0, 300 mM NaCl, 5 mM imidazole, DNase I (2 μg/ml), 20 mM $MgSO_4$ and EDTA-free complete protease inhibitors (Roche), and lysed using a cell disruptor (Basic Z Bench top 0.75 KW; Constant System). The lysate was clarified by centrifugation at 18,000 rpm for 1 h at 4 °C. The supernatant was purified through a 5 ml bed volume HiTrap nickel immobilized metal ion affinity chromatography column (G.E. Healthcare) connected to a FPLC system (G.E. Healthcare), after washing unbound protein with buffer A (20 mM Na HEPES at pH 7.0, 300 mM NaCl, EDTA-free complete protease inhibitors (Roche)) with 60 mM imidazole; RdRp was then eluted with buffer A + 255 mM imidazole. To remove the N-terminal His6-tag, 500 U of thrombin (Sigma) was added to the pooled fraction containing the RdRp, and the mixture was dialyzed O/N at 4 °C against 1 l of buffer A supplemented with 5 mM TCEP. DENV3 RdRp was further purified by size exclusion chromatography (Superdex 200) using the same buffer. The purity of the resulting RdRp was

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