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Discovery of conjugated thiazolidinone-thiadiazole scaffold as anti-dengue virus polymerase inhibitors

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ABSTRACT

Dengue virus (DENV) infection is a significant health threat to the global population with no therapeutic option. DENV NS5 RNA-dependent RNA polymerase (RdRp) is the key replicating protein of the virus and thus an attractive target for drug development. Herein, we report on the synthesis and biological evaluation of a series of hybrid thiazolidinone-thiadiazole derivatives as a new class of DENV-2 NS5 RdRp inhibitors. This yielded compounds **12** and **21** with IC_{50} values of 2.3 μM and 2.1 μM, respectively, as promising leads. Limited SAR analysis indicated 3-fluorobenzylidene as the optimal substituent at C5-position of the thiazolidinone core, whereas both 2-chlorophenyl and 3-fluorophenyl substituents were equally effective at C5-position of the 1,3,4-thiadiazole core. Biophysical characterization and molecular docking studies conferred the binding site of this scaffold on DENV NS5 polymerase. Binding mode of compound **21** in Thumb pocket-II of DENV-2 NS5 polymerase will form the basis for future structure-activity relationship optimization.

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1. Introduction

Dengue virus (DENV) an emerging pathogen infects ~390 million people each year and poses a global public health threat. Although, a vast majority of DENV infections get resolved within 7–10 days, in cases where it persists, dengue leads to life threatening condition such as dengue hemorrhagic fever or dengue shock syndrome. These account for nearly 50 million symptomatic DENV cases every year resulting in hospitalization of over half a million infected individuals and ~25,000 deaths, mainly among children [1]. Despite the significant burden of DENV infections on economy, there is neither a protective drug nor any approved medicine available to treat this illness. Furthermore, no vaccine against DENV has been licensed yet. Thus, there exists an urgent demand for therapy to treat this infection [2].

DENV, a member of *Flavivirus* genus of Flaviviridae family, is a single-stranded RNA virus of positive polarity which encodes three structural (capsid, prM, envelope) and seven non-structural (NS: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins upon translation [3]. The structural and non-structural proteins play a unique role in facilitating virus replication and evading the host immune response during DENV infection. NS5 is the most conserved DENV protein amongst the five DENV serotypes and has been shown to play critical role in RNA capping and DENV genome replication during the infection. The 104 kDa NS5 encodes the methyl transferase domain (30 kDa) at its N terminus and the polymerase domain at its C-terminus (74 kDa) [4]. Both of these domains are connected by an adaptable linker. Unlike other viral polymerases such as bovine viral diarrhea virus (BVDV) and hepatitis C virus (HCV), DENV polymerase has been shown to be able to perform RNA synthesis by both primer-independent (de novo) and primer-dependent manner [5,6].

Given the clinical success in targeting HIV and HCV viral polymerases by small molecule inhibitors, DENV NS5 polymerase appears an attractive target for therapeutic intervention [7,8]. Several small molecules such as geneticin [9], castanospermine [10], mycophenolic acid [11] ribavirin [12] and nucleoside analogs [13,14] have been reported to inhibit DENV replication. Intervention

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strategies with siRNAs [15], antisense oligomers [16], peptidomimetic inhibitors [17,18], and therapeutic antibodies [19] have also been explored as DENV replication inhibitors. However, most of these molecules exhibited limitations of high doses and lacked pharmacological properties for development as clinical molecules. Thus, there exists an urgent and unmet clinical need to develop novel and potent therapeutics to combat DENV infection.

In the present study, we examined the 4-thiazolidinone scaffold for its anti-DENV NS5 RNA-dependent RNA polymerase (RdRp) potency. A total of 39 structural analogs of hybrid thiazolidinone-thiadiazole were screened in vitro in RdRp, cytotoxicity and DENV replicon reporter based assays. Further, the interactions of 3 representative NS5 inhibitors was examined by fluorescence quenching (FQ) and the binding site of the 4-thiazolidinone scaffold was predicted using molecular docking studies.

2. Materials and methods

2.1. BHK-D2-Fluc reporter assay and cellular cytotoxicity

The BHK-D2-Fluc replicon reporter cells have been described earlier [20]. To estimate the anti-DENV activity of the compounds, DENV replicon reporter cells (1×10^4 cells/well) were seeded in 96 well plate. After 6 h post seeding, cells were treated either with compounds or DMSO (control). After 48 h, cells were washed twice with PBS and firefly luciferase activities were measured according the manufacturer's protocol (Promega, USA). Compound cytotoxicity was evaluated in parental BHK-21 cells employing MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation kit, Promega).

2.2. Expression and purification of DENV-2 NS5

DENV-2 NS5 protein was purified with some modification of the procedure described previously [21]. Briefly, the Rosetta (DE3) cells transformed with pET30a DENV-2-NS5 were grown until O.D. 0.8 (595 nm) and the protein was induced by adding 0.4 mM IPTG with vigorous shaking at 18 °C for 6 h. Cells were harvested and homogenized in lysis buffer containing 20 mM Na_2PO_4, pH 7.0, 0.5 M NaCl, 5 mM β-Mercapto ethanol, 0.5% Triton-X-100, Protease inhibitor cocktail and 10% glycerol. The cells were gently sonicated and centrifuged to separate supernatant. The supernatant was quickly passed through Ni-sepharose-6 fast flow column and several fractions of proteins were collected. The fractions containing NS5 were pooled together and concentrated using 50 kDa cut-off membrane (Millipore) and washed successively using buffer-1 (50 mM Tris. HCl (pH 7.8), 750 mM NaCl, 5 mM β-mercaptoethanol and 5% glycerol). The concentrated protein was applied on Suprerdex 200 10/300-GL column loaded on Pharmacia UPC-900. The fractions showing the correct protein size and most purity were pulled together and dialyzed in the dialysis buffer (50 mM Tris. HCl (pH 7.8), 100 mM NaCl, 5 mM β-Mercaptoethanol and 10% glycerol) and stored at −80° C in dialysis buffer. The protein equivalent of 1 ng was western blotted against anti-His antibody to confirm the presence of His-tagged DENV-2 NS5 protein.

2.3. DENV NS5 RNA dependent RNA polymerase (RdRp) activity assay

The RdRp assays were carried out in a reaction mixture containing 50 mM Tris HCl (pH 7.8), 10 mM KCl, 0.1 mg/ml BSA, 10 μM cold UTP, 1μCi/assay α^{32}P-UTP, 250 nM pre-annealed PolyrA/rU_{12} and 48 nM purified DENV NS5 in a final volume of 20 μl. The compounds or DMSO (control) were incubated in the reaction mixture for nearly 5 min at room temperature and polymerase reaction was started by adding $MnCl_2$ (0.5 mM). After 1h incubation at 30° C, the reactions were stopped by adding 5% ice chilled TCA to precipitate the radio-labeled RNA products. The precipitated acid insoluble materials were filtered on G/FB filters and successively washed three times with TCA, water and 50% ethanol. The filters were dried and counted for radioactivity using Tri-Carb scintillation

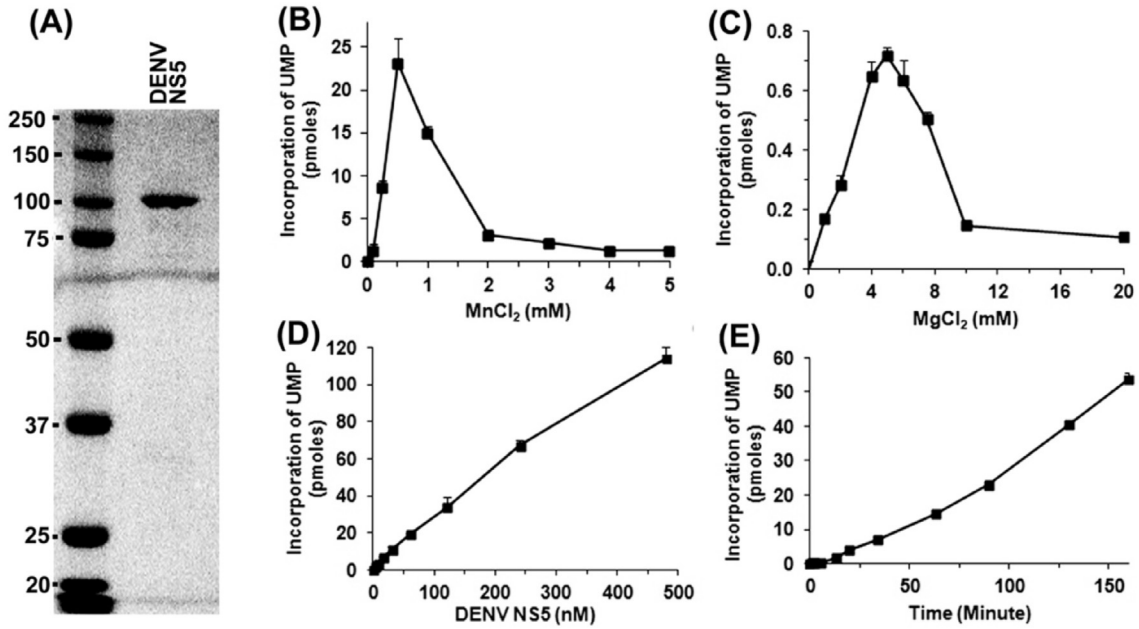

Fig. 1. Purification and characterization of DENV NS5 polymerase. (A) Purified full length DENV-NS5 (1 μg) was resolved on 8% SDS-PAGE and visualized by coomassie blue staining. The left panel indicates the size of protein molecular weight marker. (B and C) RdRp reactions were performed in the presence of 250 nM polyrA/U_{12}, 10 μM UTP, 1 μCi α^{32}P-UTP at the indicated concentrations of $MnCl_2$ and $MgCl_2$. Polymerase reactions were carried out using 48 nM and 480 nM DENV-2 NS5 for $MnCl_2$ and $MgCl_2$, respectively. (D) Dose-response profile of DENV RdRp reaction as a function of DENV-2 NS5 protein with 0.5 mM $MnCl_2$ as divalent cation at 30 °C for 1h. (E) Time-course of DENV RdRp reaction at 30 °C with 0.5 mM $MnCl_2$. Each point represents a mean of three independent experiments in triplicates with ±S.D.

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