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Assessment of Dengue virus helicase and methyltransferase as targets for fragment-based drug discovery

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ABSTRACT

Seasonal and pandemic flaviviruses continue to be leading global health concerns. With the view to help drug discovery against Dengue virus (DENV), a fragment-based experimental approach was applied to identify small molecule ligands targeting two main components of the flavivirus replication complex: the NS3 helicase (Hel) and the NS5 mRNA methyltransferase (MTase) domains. A library of 500 drug-like fragments was first screened by thermal-shift assay (TSA) leading to the identification of 36 and 32 fragment hits binding Hel and MTase from DENV, respectively. In a second stage, we set up a fragment-based X-ray crystallographic screening (FBS-X) in order to provide both validated fragment hits and structural binding information. No fragment hit was confirmed for DENV Hel. In contrast, a total of seven fragments were identified as DENV MTase binders and structures of MTase-fragment hit complexes were solved at resolution at least 2.0 Å or better. All fragment hits identified contain either a five- or six-membered aromatic ring or both, and three novel binding sites were located on the MTase. To further characterize the fragment hits identified by TSA and FBS-X, we performed enzymatic assays to assess their inhibition effect on the N7- and 2′-*O*-MTase enzymatic activities: five of these fragment hits inhibit at least one of the two activities with IC_{50} ranging from 180 μM to 9 mM. This work validates the FBS-X strategy for identifying new anti-flaviviral hits targeting MTase, while Hel might not be an amenable target for fragment-based drug discovery (FBDD).

This approach proved to be a fast and efficient screening method for FBDD target validation and discovery of starting hits for the development of higher affinity molecules that bind to novel allosteric sites.

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1. Introduction

Flavivirus infections, such as those caused by mosquito-borne Dengue, West Nile, Kunjin, Japanese encephalitis and Yellow fever viruses, can provoke life-threatening diseases of epidemic proportions with a devastating economic impact. The Dengue virus (DENV) itself causes 50–100 million human infections annually, leading to about 30,000 deaths; its transmission has increased dramatically and has become a major international public health concern. Despite recent studies in vaccine development (Thomas and Endy, 2013), there are currently neither a truly cross-protective vaccine nor approved antiviral compounds to treat DENV infections. In such a context, the deployment of an innovative drug discovery approach towards the development of potent antiviral compounds to prevent or treat DENV infections represents an urgent complement to any vaccination campaign.

A natural target is the flaviviral replication machinery, many steps of which have been characterized, leading to the identification of enzymes whose inhibition could block viral replication (Bollati et al., 2010). Flavivirus are single-stranded positive RNA viruses carrying a cap-1 structure ($^{7Me}GpppA_{2'OMe}$-RNA) at their

Abbreviations: DENV, Dengue virus; NS, non-structural protein; MTase, methyltransferase; Hel, helicase; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; FBDD, fragment-based drug discovery; FBS, fragment-based screening; FBS-X, FBS by X-ray crystallography; LE, ligand efficiency; MW, molecular weight; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance; TSA, thermal-shift assay; MS, mass spectrometry; NMR, nuclear magnetic resonance; HCS, high-concentrated screening bioassay; AMPPNP, Adenosine 5′-(β,γ-imido) triphosphate; DTT, dithiothreitol; PEG, polyethylene glycol; SPA, scintillation proximity assay; HTS, high-throughput screening; IC_{50}, inhibitory concentration that causes 50% reduction in enzyme activity; SD, standard deviation.

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5′-end. The replication of the genome is ensured by the viral replication/transcription complex, composed of five non structural proteins (NS1–NS5) carrying the polymerase, RNA unwinding and capping activities. The flaviviral proteins NS3 and NS5 are carrying two essential enzymatic activities, namely helicase (Hel) and methyltransferase (MTase) that are strictly required for the replication of flaviviruses, and therefore constitute promising targets for the development of anti-flaviviral compounds.

DENV Hel, located at the C-terminal part of NS3, carries nucleotide and RNA triphosphatase (respectively named NTPase and RTPase) as well as RNA unwinding activities (Benarroch et al., 2004b; Yon et al., 2005) involved in RNA capping and viral genome replication, respectively. The inhibition of these functions limits viral replication (Byrd et al., 2013; Daffis et al., 2010; Mastrangelo et al., 2012). DENV NS5 MTase catalyses two consecutive methylation reactions involved in the synthesis of the cap structure: methylation of the cap guanine at its N7-position to yield ^{7Me}GpppA-RNA and methylation of the first transcribed nucleotide at its 2′-*O*-position to yield ^{7Me}GpppA$_{2'OMe}$-RNA (Dong et al., 2008, 2010). Recent biochemical studies coupled to reverse genetic analysis have demonstrated that the N7-MTase activity is essential for the replication of flavivirus (Dong et al., 2010; Zust et al., 2011). By contrast, 2′-*O*-MTase defective viruses can replicate but are highly attenuated (Zust et al., 2013). Thus, both N7- and 2′-*O*-MTase activities are important on the context of antiviral research.

The identification of suitable new target sites and/or starting-point compounds for the development of new anti-flavivirals remains a major challenge (Lim et al., 2013b). In such a perspective, we decided to use a promising drug discovery strategy, namely fragment-based drug discovery (FBDD), now established as a powerful method for the rapid identification of starting hits and their subsequent elaboration into quality lead compounds (Congreve et al., 2008; de Kloe et al., 2009; Erlanson, 2012; Hajduk and Greer, 2007; Murray and Rees, 2009; Scott et al., 2012). This methodology offers an attractive alternative to conventional screening approaches such as HTS. Indeed, the fragment-based screening (FBS) approach has advantages over other lead discovery methods, including the screening of substantially fewer compounds (usually several hundred to a thousand), higher hit rates, a more efficient coverage of the chemical space and a faster fragment hit-to-lead optimization phase. Because of their small size (MW < 300 Da), fragments normally bind with low affinity, however binding is often of high quality as estimated by ligand efficiency (LE) (Abad-Zapatero, 2007; Bembenek et al., 2009; Hopkins et al., 2004). A major challenge in establishing fragment-based screening has been the development of sufficiently sensitive methods to detect weakly binding hits which have typically milli- to micromolar binding affinities (Ciulli and Abell, 2007; Kuo, 2011; Lundqvist, 2005; Siegal et al., 2007). Biophysical methods, such as isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), thermal-shift assay (TSA), mass spectrometry (MS) and nuclear magnetic resonance (NMR), are frequently employed as a first-pass screen before detailed structural characterization by X-ray crystallography (FBS-X). Knowledge of exactly how the fragments bind to the protein target allows the hits to be extended into neighboring pockets to create higher affinity and more specific compounds.

In the past decade, several crystal structures of NS3 DENV Hel (Luo et al., 2008, 2010; Xu et al., 2005) and NS5 DENV MTase (Benarroch et al., 2004a; Egloff et al., 2002, 2007; Geiss et al., 2009; Lim et al., 2011; Yap et al., 2010) have been reported. Crystal forms diffracting to better than 2 Å resolution have been identified (Lim et al., 2011; Luo et al., 2008) and are suitable for determining the co-crystal structures that would provide the molecular basis for downstream ligand optimization. We have therefore applied an FBS approach targeting the NS3 DENV Hel and NS5 DENV MTase domains to discover starting-point compounds for further antiviral development. In this study, we set up a multistep fragment-based screening strategy involving both interaction and enzymatic inhibition analyses. A library of 500 fragments was successively screened by thermal-shift assay allowing the initial selection of fragments that bind to NS3 DENV Hel and NS5 DENV MTase domains, with some of them showing inhibition effect on activity assays. We then successfully identified novel binding sites for NS5 DENV MTase using X-ray crystallography.

2. Materials and methods

2.1. Materials

The 500-compound general fragment library was purchased from Maybridge (RO3 library 2009). ATP was purchased from New Englands BioLabs Inc. [^{3}H]AdoMet (80.7 Ci/mmol) was purchased from PerkinElmer. The fluorescent dye SYPRO Orange was purchased from Life Technologies. All other chemicals were purchased from Sigma–Aldrich.

2.2. Production of recombinant proteins

The DNA fragments coding for DENV4 Hel (amino acid region 172–618) and DENV3 MTase (amino acid region 1–277) were synthesized by Geneart (Life Technologies). The coding sequences were cloned in pMcox20A by Gateway recombination, downstream a cleavable Hexahistidine–Thioredoxin tag using a two step PCR protocol. *Escherichia coli* Rosetta (DE3) pLysS (Novagen) were co-transformed with the two plasmids and cultured in Terrific Broth at 37 °C. When OD_{600nm} reached 0.5, expression was induced by adding 500 μM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) in the culture media and temperature was shifted to 25 and 17 °C for DENV3 MTase and DENV4 Hel, respectively. Protein purification and tag removal was performed in non denaturing conditions as previously described (Lantez et al., 2011). A final Size Exclusion Chromatography step was performed in 20 mM Tris–HCl, 200 mM NaCl, glycerol 10%, 2 mM DTT, pH 7.5 for DENV3 MTase and 10 mM Hepes 300 mM NaCl pH7.5 for DENV4 Hel. Dialysis could then be performed to exchange the buffer depending on the downstream experiment.

2.3. Thermal-shift assays (TSA) and data analysis

The thermal-shift assays were conducted in 96-well thin-wall PCR plates (Bio-Rad) sealed with Optical-Quality Sealing tape (Bio-Rad) and heated with an iCycler iQ real Time Detection System (Bio-Rad) from 20 to 90 °C in increments of 0.2 °C. Thermal denaturation was monitored using SYPRO Orange (Life Technologies) and the fluorescence intensity was measured at 490/530 nm excitation/emission wavelengths, respectively. The denaturation of the proteins was monitored by following the increase of the fluorescence emitted by the probe that binds exposed hydrophobic regions of the denatured protein. The melting temperature (T_m) was calculated as the mid-log of the transition phase from the native to the denatured protein using a Boltzmann model (Origin software). The reference unfolding temperature of proteins in 2% DMSO (T_{mref}) was subtracted from the values in the presence of fragment (T_{mfrag}) to obtain thermal shifts, $\Delta T_m = T_{mfrag} - T_{mref}$. Fragments were considered to be hits when $\Delta T_m > 0.5$ °C.

2.3.1. Fragment-based screening against DENV4 Hel by TSA

TSA plates were prepared by dispensing into each well the DENV4 Hel (final concentration of 20 μM in 50 mM HEPES, 150 mM NaCl, 2 mM $MnCl_2$, pH 7.4) which was mixed with 0.5 μL of fragments (from 100 mM stock in 100% DMSO, to attain

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