Biochemical and Biophysical Research Communications 453 (2014) 539–544

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Suramin inhibits helicase activity of NS3 protein of dengue virus in a fluorescence-based high throughput assay format

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ARTICLE INFO

Article history:
Received 15 September 2014
Available online 2 October 2014

Keywords:
Dengue virus
NS3
RNA helicase
ATP hydrolysis
Suramin
Counter screen

ABSTRACT

Dengue fever is a major health concern worldwide. The virus encoded non-structural protein 3 (NS3) is a multifunctional protein endowed with protease, helicase, nucleoside triphosphatase (NTPase) and RNA 5′ triphosphatase (RTPase) activities. Helicase activity of NS3 catalyzes the unwinding of double stranded polynucleotides by utilizing the energy released from ATP hydrolysis. As this activity is essential for replication, NS3 helicase represents an attractive drug target for developing a dengue antiviral drug.

Here, we report fluorescence based molecular beacon helicase assay using a duplex RNA substrate that contains a fluorophore on the 5′ end and a quencher on the 3′ end of one of the strands. The assay was optimized with respect to several parameters and adapted to 384-well high-throughput screening format, with an average *Z′* factor of 0.65. Assay validation with a small diverse set library of 1600 compounds identified, suramin as a significant inhibitor of the helicase activity of NS3. Helicase activity deficient NS3 K199A was used in a counter-screen to identify compounds interfering with the assay. Suramin inhibited DENV (dengue virus) NS3 helicase activity with a K_i of 0.75 ± 0.03 μM as a non-competitive inhibitor. The molecular beacon helicase assay together with the counter screen and suramin as a tool compound can be used to identify novel inhibitors of DENV helicase.

1. Introduction

Dengue fever, one of the most important *Aedes aegypti* borne infection is endemic in tropical and subtropical countries. It poses a threat to human population as there are no specific and effective therapeutics to treat the disease on the market [1]. Thus, there is an urgent and unmet need for the discovery and development of small molecule inhibitors as potential therapeutics.

The RNA genome of dengue virus is positive stranded and contains an ~11 kb open reading frame which is translated directly by the host machinery into a long poly-protein composed of three structural (C-prM-E) and seven non-structural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5). The polyprotein is then co- and post-translationally processed by host and viral proteases [2]. NS3 is a multifunctional protein endowed with protease, helicase, ATPase and RTPase activities sharing 67% amino acid sequence identity across the four dengue serotypes, which plays an important role in viral polyprotein processing and genome replication [3,4].

NS3 helicase of dengue virus resides within residues 170–618 and belongs to the superfamily 2 of RNA helicases/NTPases comprising Walker A, GK(S/T) and Walker B, DEx(D/H) motifs along with other superfamily characteristic sequence motifs present in domains I and II towards the N-terminal end. It catalyzes the unwinding of the duplex RNA in the 3′–5′ direction releasing the single stranded RNA available to NS5 as a template for replication in the presence of a divalent cation and the energy derived from the hydrolysis of ATP [5]. Functional activity of helicase and NTPase of NS3 has been very well characterized for several members of the family Flaviviridae [6–9]. Unlike other viral protein drug targets, relatively few compounds that target helicases have entered clinical trials [10,11] owing to the complexity associated with developing non-radioactive HTS screening assays and the similarity to wide range of host ATP-utilizing enzymes. However the availability of detailed structural information for DENV helicase [12] opens opportunities to optimize compounds identified by screening assays for greater specificity.

Several of the assays that monitor helicase activity are cumbersome and time-consuming and some of them use radiolabeled substrates which limits their application in high-throughput screening

Abbreviations: DENV, dengue virus serotype; HCV, hepatitis C virus; NS3, non-structural protein 3; FL, full length; H, helicase domain; Trx, thioredoxin; RFU, relative fluorescence unit.

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http://dx.doi.org/10.1016/j.bbrc.2014.09.113
0006-291X/

campaigns [13–16]. Even though, improved next generation fluorescence-based assays have been developed, they have their own set of disadvantages [17].

Here, we present the development of an improved functional assay to monitor NS3 helicase activity of dengue virus for screening compound libraries. This assay was validated using a library of 1600 small molecule compounds with a Z' factor of 0.65 demonstrating its suitability for high-throughput screening. Here, we show for the first time that suramin identified during screening inhibits NS3 helicase activity of dengue virus with an inhibitory constant K_i of 0.75 ± 0.03 μM in a non-competitive manner.

2. Materials and methods

The sequences of oligonucleotides used in this study are as follows: 5′-AGACUAACAACUGACGUC(U)20-3′,5′-CY5-GACGUCAGUUGUUAGUCUACGUC-Dab-3′,5′-CY5-GACGUCAGUUGUUAGUCUACGUC-IABkFQ-3′,5′-CY5-GACGUCAGUUGUUAGUCUACGUC-IAbRQSp-3′ and 5′-CY5-GACGTCAGTTGTTAGTCTACGTC-BH Q2-3′ were purchased from Integrated DNA Technologies (Coralville, IA, USA) in purified form as lyophilized solids.

2.1. Cloning, protein expression and purification of DENV4 NS2B$_{18}$NS3

The DENV3/4 NS2B$_{18}$NS3 full length (NS3FL) and DENV3/4 NS3helicase (NS3H) constructs were generated as described previously [18].

Cells resuspended in buffer A (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM β-mercaptoethanol and 20 mM imidazole, pH 8.0) were lysed by French pressure cell and the cell lysate was clarified. The supernatant was purified using a HisTrapFF column (GE Healthcare LifeSciences) equilibrated with buffer A. Proteins were eluted using a linear gradient of imidazole from 40 mM to 300 mM. Fractions containing Trx-His$_6$-NS2B$_{18}$NS3 were dialyzed against dialysis buffer (20 mM Tris.HCl, pH7.5, 150 mM NaCl, and 1 mM β-mercaptoethanol) while the thioredoxin (Trx) tag was cleaved by thrombin (5U/mg) at 4 °C for 16 h. The cleavage mixture was loaded onto a HisTrap FF column equilibrated with buffer B (20 mM Tris–HCl, pH 7.5, 3 mM β-mercaptoethanol, 150 mM NaCl and 10% glycerol) to remove Trx-His$_6$ tag from the mixture. Peak fractions containing the protein without the tag were pooled and concentrated protein was stored at −80 °C.

2.2. Duplex RNA preparation

The duplex RNA substrate was prepared by mixing 20 μM of the top strand oligonucleotide with 20 μM complementary bottom strand oligonucleotide in buffer containing 10 mM Tris–HCl, pH 8.5 by brief heating at 95 °C, followed by slow cooling to room temperature over 1hr.

2.3. Molecular beacon helicase assay (MBHA)

Assay optimization was performed in a volume of 50 μl in a half-area flat bottom black microtiter plate (Corning, NY, USA) to identify the optimum concentration for enzyme, duplex RNA substrate, ATP as well as buffer composition. The final assay format used for screening compound was carried out in a total volume of 25 μl. Enzyme activity was estimated through the fluorescence decrease monitored in a kinetic mode to obtain a reading every 40 s for 60 min at an excitation/emission wavelength of 640/670 nM in a Tecan Infinite M200 microplate reader (Tecan, Durham, NC). In all cases, initial velocity was measured and expressed as relative fluorescence unit (RFU).

2.4. Screening of a chemical library

A diverse set compound library from Experimental Therapeutics Centre (ETC, Singapore) containing 1600 compounds at a stock concentration of 10 mM dissolved in 100% DMSO was used for screening in a volume of 25 μl in 384-well black plates. The test compounds (25 nl) were pre-dispensed in the plate using a robotic liquid handling system in order to achieve a final concentration of 10 μM. Each assay plate had a 0% negative reaction controls on columns 1 and 24 and 100% positive reaction controls on columns 2 and 23. The compounds were distributed in the middle 3–22 columns. An initial reading was taken after the addition of duplex RNA at an excitation/emission wavelength of 640/670 nM in the Tecan plate reader. The instrument was paused for the addition of ATP to initiate the reaction and readings were continued for 30 min at 30 °C in the kinetic mode.

2.5. Data analysis

Kinetic parameters of ATP and duplex RNA were determined by fitting the data to the Hanes–Woolf plot equation:

$$[S]/v = 1/V_{max} * [S] + K_m/V_{max} \quad (1)$$

and to the Michaelis–Menten equation:

$$v = V_{max} * [S]/K_m + [S] \quad (2)$$

respectively using GraphPad Prism (GraphPad Prism version 5.00, GraphPad Software, San Diego,CA). IC_{50} values were obtained by fitting the data to a non-linear regression variable slope performed using GraphPad algorithm. Z' factor was calculated using the formula:

$$Z = 1 - \{(3SD_{max} + 3SD_{min})/(mean_{max} - mean_{min})\} \quad (3)$$

[19] where SD is the standard deviation. Compound activity was calculated from the formula:

$$\% \text{ inhibition} = \{100 - [(\text{compound signal} - mean_{min})/(mean_{max} - mean_{min} \times 100)]\} \quad (4)$$

where "min" is the minimum reaction with all the components except protein and "max" is the maximum reaction with all the components.

3. Results

3.1. Principle of the molecular beacon helicase assay (MBHA)

A fluorescence-based assay was developed to carry out a high-throughput screening of dengue virus NS3 helicase. The principle of the assay as depicted in Fig. 1A contains a duplex RNA substrate with a (U20 nucleotide) overhang on the 3′ side of one strand to provide a binding site for helicase while the complementary strand carried a fluorophore on the 5′ end and a quencher on the 3′ end. During the enzyme catalyzed unwinding reaction, the complementary strands of the fluorescent substrate are separated and the strand carrying the fluorophore/quencher forms a molecular beacon which is quenched. The formation of a stem-loop structure prevents strand re-annealing and eliminates the need for the addition of single stranded oligonucleotide trap molecules to the reaction mixture [20].

3.2. Selection of an optimal duplex RNA substrate and optimization of protein concentration

Different combinations of fluorophore and quencher were evaluated as substrates in order to obtain an optimal substrate combi-

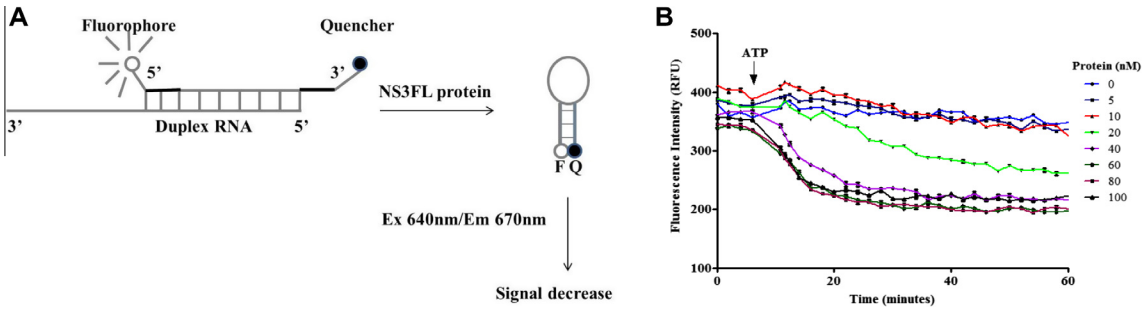

Fig. 1. (A) A schematic representation of molecular beacon helicase assay. The helicase catalyzed unwinding of duplex RNA carrying a fluorophore on the 5′ end and a quencher on the 3′ end on one of the strands results in a molecular beacon which quenches the fluorescence. (B) Helicase reaction progress curves on Cy5 fluorophore and IABkFQ quencher. Reactions were carried out with NS3 at 5–100 nM with 1 mM ATP and 1 nM duplex RNA. Arrows indicate when ATP was added to initiate the reaction ($n = 2$).

nation that shows minimum background activity with maximum fluorescence activity. This was combined with optimization of protein.

Three RNA duplex substrates with Cy5 as a common fluorophore attached to Dab, IABkFQ or IAbRQSp were tested along with a DNA–RNA heteroduplex consisting of fluorophore Cy5 at the 5′ end and BHQ2 at the 3′end. When the molecular beacon is not annealed to a complementary RNA molecule, no fluorescence is observed. The fluorescence increases ~20 fold above baseline when the beacon is annealed to complementary RNA.

Simultaneous optimization of fluorophore and quencher combination and protein was carried out by titrating different concentrations of protein ranging from 5 to 100 nM at a single fixed concentration of 1 nM of four different combinations of the duplex RNA substrate in an assay buffer containing 20 mM Tris–HCl, pH 7.4 and 3 mM $MgCl_2$ with 1 mM ATP in a volume of 50 μl. A reaction lacking the protein was used as a blank. The fluorescence intensities at 100 nM protein are 54, 43, 103 and 48 RFU for Cy5/Dab (Supplementary Fig. 1A), Cy5/BHQ2 (Supplementary Fig. 1B), Cy5/IABkFQ (Fig. 1B) and Cy5/IAbRQSp (Supplementary Fig. 1C) respectively at 60 minutes. Since the Cy5/IABkFQ combination of fluorophore and quencher yielded the best result, this combination was used for further assay development experiments. We selected 20 nM of protein for carrying out the next set of experiments as the reaction was considerably linear for longer time at this concentration for Cy5/IABkFQ combination.

To further improve the fluorescent signal obtained with the helicase catalyzed unwinding, a concentration of up to 5 nM of the duplex RNA (Cy5/IABkFQ) was processed which indicated a linearity up to 3nM (Supplementary Fig. 2) with a fluorescent signal of ~200 RFU and this concentration was used throughout assay development.

3.3. Optimization of assay conditions

After fixing the protein and substrate concentrations at 20 nM and 3 nM duplex RNA respectively, the assay was optimized with respect to different parameters such as temperature, pH and cofactor requirement in order to obtain an optimal signal that can be used for screening purposes. A protein blank was used in all these experiments to account for compound interference. A temperature of 30 °C, buffer Tris–HCl at pH7.4, and cofactor $MgCl_2$ which yielded optimum signal were chosen for all subsequent experiments (Supplementary Fig. 3A–C).

Omitting ATP or duplex RNA from the experiment one at a time, showed that there is no change in the fluorescence implying that the enzyme requires ATP and duplex RNA for the helicase activity of NS3. Furthermore, EDTA at 50 mM was able to stop the reaction by completely inactivating NS3 as demonstrated by the unchanged fluorescence. In reactions incubated with the substrate and helicase alone, the fluorescence did not change. However, when ATP was added to the reaction, a rapid decrease in fluorescence was observed suggesting that this assay is indeed monitoring the ATP dependent helicase catalyzed unwinding activity of NS3 of dengue virus (Fig. 2A).

The helicase reaction kinetics was determined for both ATP and duplex RNA substrates with the optimized assay conditions. The assay was carried out by varying concentrations of ATP ranging from 330 to 4000 μM in the presence of a single fixed concentration of 3 nM duplex RNA for calculating kinetic parameters of ATP. The kinetic constants of duplex RNA were computed similarly using concentrations of the duplex RNA from 0.5 to 5 nM with fixed concentration of 1 mM ATP. From these studies an apparent K_m for ATP and duplex RNA were found to be 228 μM and 5 nM respectively with apparent V_{max} of 6.3 RFU/min (Fig. 2B and C). The assay conditions were further tweaked in order to achieve high sensitivity during screening. We decided to use 500 μM ATP, 2 nM duplex RNA and 200 nM protein in the final assay.

Next, we sought to check the effect of DMSO on the NS3 helicase activity which indicated it did not have any significant adverse effect on the assay up to a concentration of 10% (Supplementary Fig. 4). Hence all subsequent compound screening assays were performed at 4% DMSO.

3.4. Adaptation of the assay to high-throughput format

After optimizing the assay conditions in the 96 well plate format, the assay was adapted to a 384 well plate format to screen potential inhibitors in a high-throughput format by reducing the reaction volume from 50 μl to 25 μl and keeping the final reagent concentrations the same for all other components.

The plate uniformity was tested by including minimum reactions in the first 12 columns and maximum reactions in the last12 columns in the 384 well plates (Fig. 3A). Next, the assay was performed in 384 well plates on two different days to check the robustness of the assay. The Z' greater than 0.5 indicates that this assay is robust for screening of compounds (Fig. 3B) with a signal to noise ratio of 6. As there are no standard reference inhibitors for helicase activity of DENV NS3, we tested if polyU which is known to stimulate the ATPase activity of DENV4 NS3FL or H may be used as an inhibitor. Indeed, we were able to detect inhibitory effects of polyU with an IC_{50} of 0.8 μg/ml indicating that it inhibits the helicase activity of NS3 (Fig. 3C).

3.5. Screen with diverse compound library

A compound library of diverse structures containing 1600 bioactive compounds obtained from ETC was tested at a final concentration of 10 μM. The screening was carried out in five 384 black well plates in a kinetic mode for 30 minutes along with "min"

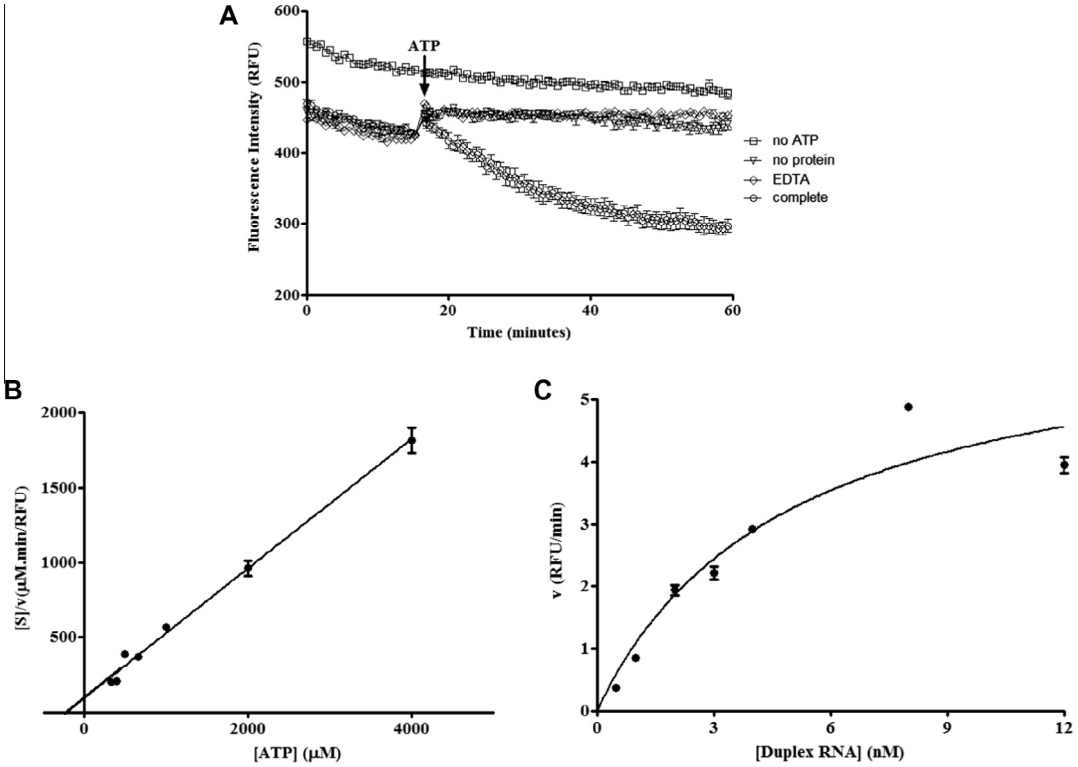

Fig. 2. Optimization of helicase activity of NS3 (A) Control reactions with 50 mM EDTA, "no protein" and "no ATP" reaction conditions contain all the assay components except protein and ATP respectively and complete reaction condition contains all the assay components (B) Apparent kinetic constants for ATP. Experiment was performed at different concentrations of ATP up to 4 mM at a fixed concentration of 3 nM duplex RNA with 20 nM protein. (C) Apparent kinetic constants for duplex RNA. Experiment was performed at different concentrations of duplex RNA up to 12 nM at a fixed concentration of 1 mM ATP with 20 nM protein. Error bars represent the standard error of mean of triplicate measurements.

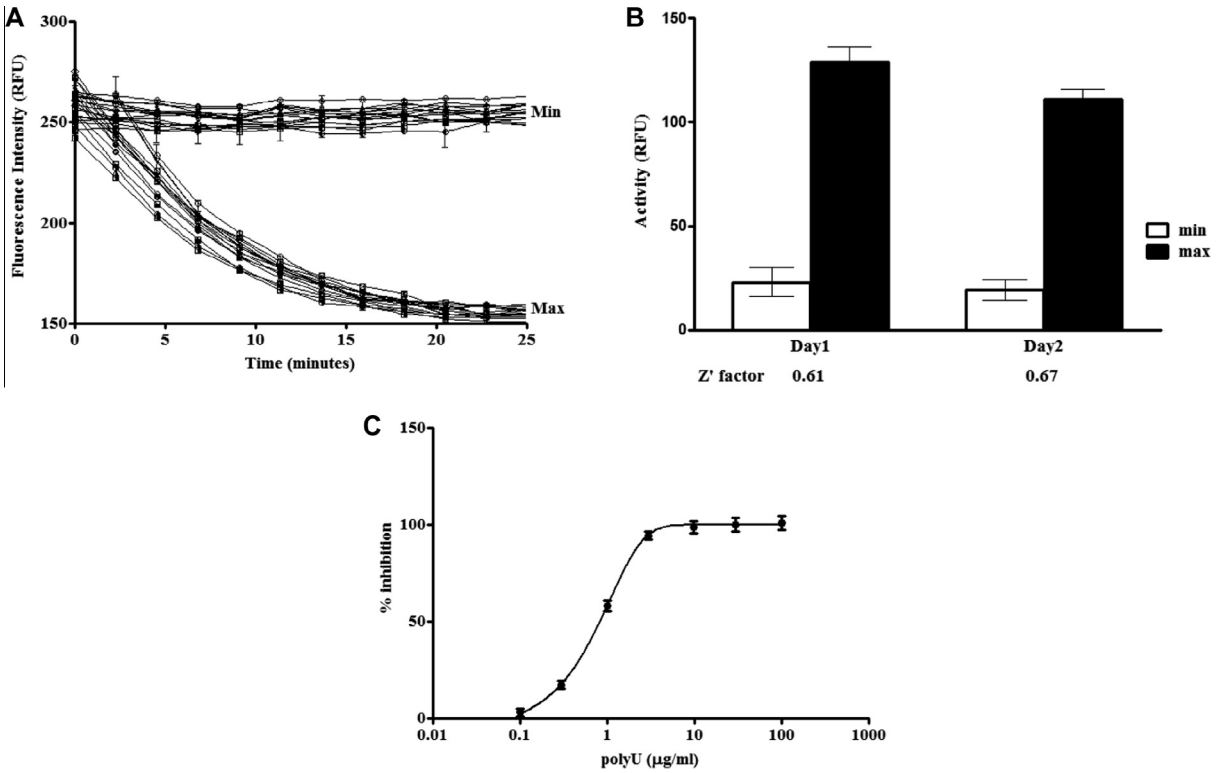

Fig. 3. Assay validation, *Z′* factor and IC_{50} of polyU (A) "min" reactions and "max" reactions in 384 well plates (B) *Z′* factors with 200 nM NS3, 500 μM ATP and 2 nM duplex RNA in 384 well plates (C) IC_{50} dose response curve for polyU. Error bars represent the standard error of mean of triplicate measurements.

and "max" control reactions. The screening results are plotted as percent inhibition in Fig. 4A. In total, 3.5% (56 compounds) of the compounds screened, showed greater or equal to 30% inhibition (Fig. 4B). To validate the assay further, it was decided that a 50% inhibition cut-off would be used as a criterion for confirmation of the hits. Solid compounds were procured for 21 potential inhibitors from Microsource (MI, USA). A dose response validation experiment was performed in the concentration range from 0.1 to 300 μM under the same conditions as those used in the screening. Only one compound confirmed as a potential inhibitor of NS3 helicase from the solid and it was identified as suramin hexasodium (Supplementary Fig. 5). It showed 100% inhibition in the primary screen and reproducible dose dependent inhibition with an IC_{50} of 0.4 μM (Fig. 4C).

3.6. Counter Screen with NS3K199A protein

In any HTS campaign, it is important to have an appropriate counter screen to identify and eliminate non-specific compounds in addition to the controls. A counter-screen was carried out during confirmation of helicase activity on solids for the 21 compounds showing >50% inhibition with NS3FL carrying a K199A mutation which has been shown previously to be essential for helicase, NTPase and RTPase activities [6]. The experiment was performed at a single concentration of 300 μM under the same conditions as those used in the screening. NS3 K199A showed 5% fluorescence of wild type protein indicating it is inactive as a helicase and compound #18 corresponding to suramin showed no fluorescence. Twenty compounds showed significant level of fluorescence (Supplementary Fig. 6).

3.7. Characterization of suramin

We then analyzed the kinetic mechanism of inhibition of suramin by conducting the experiment at six concentrations of suramin ranging from 0.1 to 2.5 μM with 200 nM protein, 500 μM ATP and three concentrations of duplex RNA (5, 10 and 15 nM) in a volume of 25 μl in a kinetic mode in triplicate. The Dixon plot indicates a non-competitive inhibition mechanism relative to the duplex RNA substrate with an inhibition constant (K_i) of 0.75 ± 0.03 μM indicating that the inhibitor is able to bind to the free form of the enzyme (Fig. 4D).

4. Discussion

Helicases play a major role in the life cycle of many viruses, but they are not extensively exploited as drug targets. The main goal of this study is to exploit helicase of dengue virus as a drug target by developing an assay which can be used to screen compounds in a high-throughput manner to identify potential dengue helicase inhibitors. In order to achieve this goal, an improved fluorescence based molecular beacon assay has been developed and validated by screening 1600 compounds.

Suramin was identified as a potent inhibitor from the validation screen with an IC_{50} of 0.4 μM using DENV4NS3FL protein suggesting that the screening assay developed in this work is sensitive

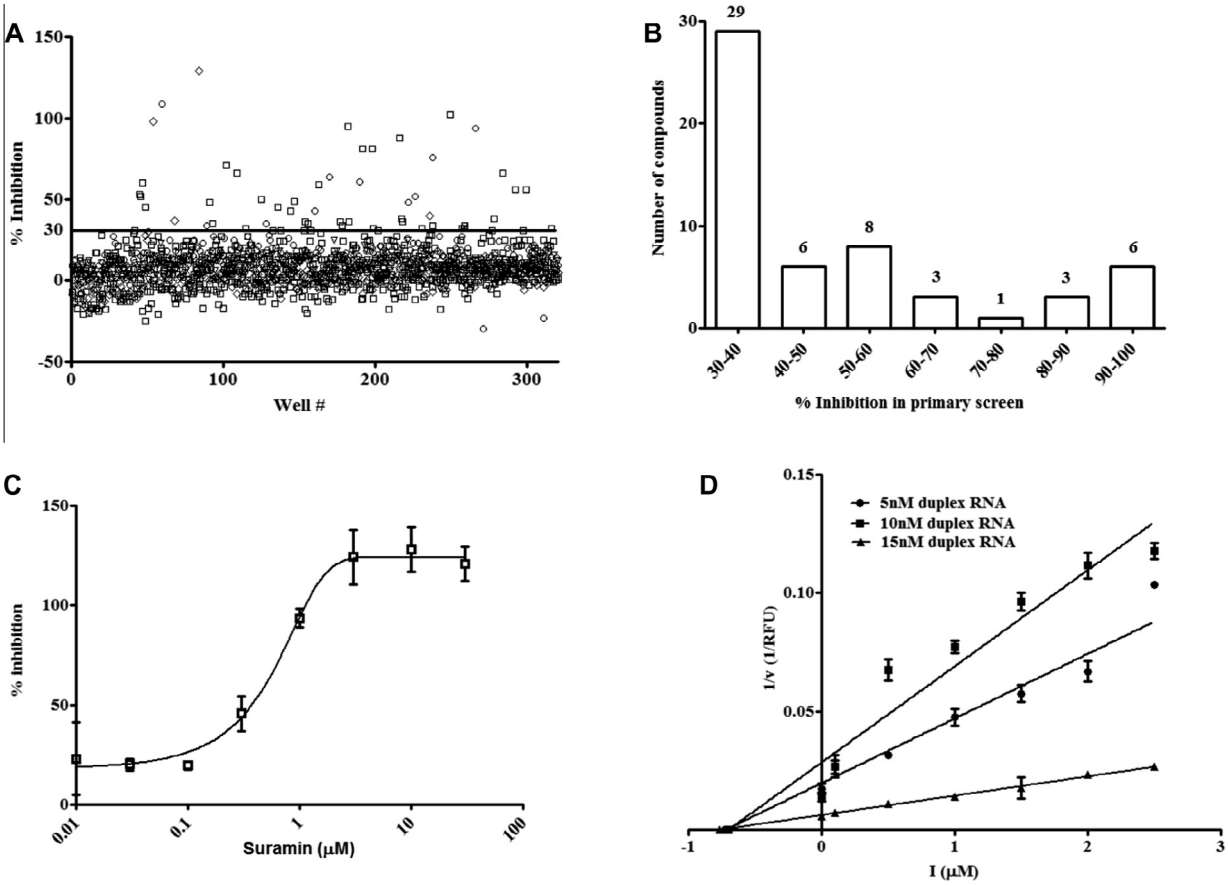

Fig. 4. Screening of compound library (A) Inhibition plot of 1600 compounds. Compounds showing negative percent inhibition values are likely due to signal interference (B) Distribution of hits from the primary screening according to the percent inhibition. The numbers on top of the bars refer to the number of hits in each category (C) Dose response curve of suramin in helicase assay with 200 nM NS3, 500 μM ATP and 2 nM duplex RNA in 384 well plate (D) Dixon plot of suramin in helicase assay. The assay was carried out at different concentrations of duplex RNA at 5, 10 and 15 nM and suramin from 0.1 to 2.5 μM with 200 nM protein. Error bars represent the standard error of mean of triplicate measurements.

enough to detect inhibitors with a high potency. Further investigation of mode of action revealed that suramin is a non-competitive inhibitor of the NS3 helicase activity of DENV. Suramin has been shown to inhibit HCV helicase with an IC_{50} of 8.9 μM [21]. Crystal structure of suramin complexes with NS3 helicase would provide further insight into the understanding of its exact location of binding and interaction with residues on the NS3 protein for the design of compounds to inhibit helicase activity.

Suramin, originally synthesized by Bayer AG [22] is a polysulfonated naphthylurea compound for which many new and therapeutically significant properties have been identified. Recently, it has been reported that suramin inhibits both murine and human norovirus RNA-dependent RNA polymerase (NV-RdRp) and its binding site was identified to be close to the protein active site for murine norovirus through crystal structures [23]. In further studies the same team demonstrated that chemical modifications of suramin can improve the water solubility and logP values while at the same time retaining the potency against NV-RdRp [24].

The adaptability of this assay was assessed by checking the dose-dependent inhibition of suramin using NS3 protein (FL and H) from other dengue serotypes. The inhibition potency of suramin against different serotypes was found to be similar (DENV3NS3FL IC_{50}:0.6 μM, DENV3NS3H IC_{50}:0.8 μM and DENV4H IC_{50}:0.8 μM) indicating that this assay is versatile and can be adapted for helicases of other serotypes of dengue virus (data not shown) to identify pan-serotype inhibitors.

One advantage of using DENV NS3FL protein in this assay over NS3H domain alone is that it may aid in identifying allosteric compounds that would bind to the protease or interdomain regions and inhibit the helicase activity. No such compound that inhibit both NS3 protease and helicase have been reported until now except for a recent HCV NS3/4A protease inhibitor structure showing interaction with residues in both the helicase and protease domains [25].

In conclusion, the molecular beacon helicase assay developed to monitor the NS3 helicase activity of dengue virus has many advantages over the existing assays in that it is continuous, non-radioactive, irreversible, quite robust and versatile. Screens of larger, more diverse libraries might yield potent, specific inhibitors required to study the role of DENV helicase. We show here for the first time that suramin acts as a potent NS3 helicase inhibitor of dengue virus by a non-competitive mode of inhibition.

Author contributions

Conceived the study: S.V., Designed and performed the experiments: B.C.H., Analyzed the data: B.C.H., Wrote the paper: B.C.H., S.V.

Acknowledgments

We thank Experimental Therapeutics Center (A*Star, Singapore) for the gift of the diversity library. Drs. Youichi Suzuki and Nancy Brown are thanked for their comments on the manuscript. This work was supported by the National Medical Research Council, Singapore (http://www.nmrc.gov.sg), under Grant NMRC/1315/2011 to S.V.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.113.

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