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Short Communication

Antiviral activities of 15 dengue NS2B-NS3 protease inhibitors using a human cell-based viral quantification assay

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ABSTRACT

The dengue virus is a mosquito-borne pathogen responsible for an estimated 50–100 million human dengue infections annually. There are currently no approved drugs against this disease, resulting in a major unmet clinical need. The dengue viral NS2B-NS3 protease has been identified as a plausible drug target due to its involvement in viral replication in mammalian host cells. In the past decade, at least 20 dengue NS2B-NS3 protease inhibitors have been reported in the literature with a range of inhibitory activities in protease assays. However, such assays do not shed light on an inhibitor's ability to penetrate human cell membranes where the viral protease resides. In this study, we investigated the antiviral activities of 15 small-molecule and peptide-based NS2B-NS3 inhibitors on dengue serotype 2-infected HuH-7 human hepatocarcinoma cells. Experimental results revealed anthraquinone ARDP0006 (compound **5**) to be the most potent inhibitor which reduced dengue viral titer by more than 1 log PFU/mL at 1 μM in our cell-based assays involving HuH-7 and K562 cell lines, suggesting that its scaffold could serve as a lead for further medicinal chemistry studies. Compound **5** was also found to be non-cytotoxic at 1 μM over 3 days incubation on HuH-7 cells using the Alamar Blue cellular toxicity assay.

The dengue virus is a mosquito-borne pathogen transmitted by *Aedes* mosquitoes and is the most widely distributed mosquito-borne virus of humans (Solomon and Mallewa, 2001). Dengue infection symptoms range from general malaise and fever to the life-threatening dengue hemorrhagic fever and dengue shock syndrome (Gubler, 1998; Simmons et al., 2012). Although dengue infections affect an estimated 50–100 million people annually, there is presently no drug treatment for this disease, resulting in significant drug research in this area (Lim et al., 2013; Noble et al., 2010; Stevens et al., 2009; Tomlinson et al., 2009a).

Upon entry into a mammalian host cell *via* receptor-mediated endocytosis (Pastorino et al., 2010), the viral genome encodes for three structural and seven non-structural (NS) proteins, of which NS3 and its NS2B co-factor bind together to form a serine protease complex (Arias et al., 1993). This complex is involved in proteolytic processing of the viral polyprotein to produce functional viral proteins needed for viral replication (Clum et al., 1997). Due to its unusual specificity for two consecutive basic residues (e.g. lysine and arginine) not found in humans, the dengue virus NS2B-NS3 protease complex is deemed a plausible drug target (Lescar et al., 2008; Melino and Paci, 2007; Noble and Shi, 2012; Yusof et al., 2000). Indeed, substantial efforts have been expended in the screening, identification, design and synthesis of dengue NS2B-NS3 inhibitors. Publications between 2003 and 2013 revealed at least 20 papers on small-molecule and peptide-based inhibitors with varying inhibitory activities against the dengue virus NS2B-NS3 protease. These experiments involved non-cell-based biochemical assays which, although relatively convenient to perform, did not shed light on the compound's ability to penetrate infected human cells where the viral protease is found. Herein, we report the antiviral activities of 15 reported inhibitors using a cell-based

Abbreviations: ATCC, American type culture collection; BHK, baby hamster kidney; CHIKV, Chikungunya virus; DENV2, dengue virus serotype 2; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; HuH, human hepatocarcinoma; KUNV, Kunjin virus; MOI, multiplicity of infection; NMR, nuclear magnetic resonance; NS, non-structural; PFU, plaque forming units; RPMI, Roswell Park Memorial Institute.

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assay involving dengue virus serotype 2 (DENV2)-infected human hepatocarcinoma cells (HuH-7) and followed by infectious virus quantification using a virus plaque assay.

The 15 inhibitors reported herein (Table 1), in chronological publication order, were either purchased or synthesized; compounds **1**, **11** and **12** were purchased from GL Biochem (China). Compounds **2** and **8** were purchased from Maybridge (UK). Compound **3** was purchased from Mimotopes (Australia). Compound **4** was purchased from Otava (Ukraine). Compounds **5**, **13** – **15** were purchased from Sigma–Aldrich (USA). Compound **6** was synthesized in-house (see supplementary data for synthetic protocol and spectral data). Compounds **7**, **9** and **10** were purchased from Chembridge Screening Libraries (USA). Compound purities were >95% with the exception of **3** (62%) due to intramolecular cyclization.

Cell lines used in this study were C6/36 cells, a continuous mosquito cell line isolated from *Aedes albopictus* embryonic tissue, HuH-7 human hepatocarcinoma cells (kind gift from Dr. Priscilla Yang, Harvard Medical School), K562 human monocytic leukemia cells (ATCC) and BHK-21 baby hamster kidney cells (ATCC). C6/36 cells were maintained in L-15 medium (Life Technologies) containing 10% FCS (GE Healthcare) at 28 °C and were used for the propagation of DENV2 New Guinea C strain, Kunjin virus (KUNV) and Chikungunya virus (CHIKV). The CHIKV strain used in this study, D1225Y08, was a kind gift from the Environmental Health Institute, National Environment Agency, Singapore. D1225Y08 was isolated from the serum of a febrile patient during the 2007 to 2008 Chikungunya outbreak in Singapore. HuH-7 cells were cultured in DMEM medium (Sigma–Aldrich) with 10% FCS (GE Healthcare) and K562 and BHK-21 cells were cultured in RPMI 1640 medium (Sigma–Aldrich) with 10% FCS (GE Healthcare) at 37 °C in a 5% CO_2 incubator. HuH-7 cells were first infected with DENV2, KUNV or CHIKV at an MOI of 1.0 for an hour and then treated with test compounds or with the control (0.1% DMSO in cell culture medium). Test compounds were introduced at 1 and 100 μM concentrations. Toxic compounds which decreased HuH-7 cell viability to <70% at 1 μM were excluded from this assay. The supernatant containing the test compound and virus-infected cells were harvested 72 h post-infection and subjected to infectious virus quantification using a viral plaque assay. Briefly, BHK-21 cells were seeded in 24-well plates and incubated overnight. Virus samples were serially diluted ten-fold using DMEM containing 2% FCS. 100 μL of the diluted virus was then added to the 24-well plates in triplicate. The cells were incubated for 1 h in a 37 °C, 5% CO_2 incubator with gentle rocking. After 1 h, the cells were washed twice and overlaid with 1% CMC in RPMI medium containing 2% FCS. The cells were then fixed and stained with 10% paraformaldehyde/1% crystal violet solution after 4 days for virus plaque visualization. The crystal violet solution was removed after 1 h and the plates were washed and left to dry in the incubator. Virus titer was quantified as plaque forming unit per milliliter (PFU/mL).

A cell viability assay was conducted for all test compounds to ensure they were non-cytotoxic to HuH-7 cells. Briefly, HuH-7 cells were incubated with the test compounds at 1 and 100 μM concentrations in DMEM containing 10% FCS for 3 days using 0.1% DMSO as control. Cell viability was then measured using Alamar Blue® (Resazurin) reagent (Invitrogen). 10 μL of Alamar Blue® reagent was added to the cells in 100 μL of the supernatant and incubated for 2 h at 37 °C. The fluorescence measurements were measured at excitation wavelength of 570 nm and emission wavelength of 585 nm as recommended by the manufacturer using the plate reader (Thermo Scientific). The % cell viability was determined using the 0.1% DMSO control as reference and plotted with respect to the test compounds (Supplementary data Fig. S1). A compound was considered toxic if it exhibited >20% decrease in signal when compared to the 0.1% DMSO control with $p < 0.05$ as determined by a one-tailed student's *t*-test. From Table 1, cellular toxicity were observed for compounds **14** and **15** (Ivermectin and Selamectin respectively) at 1 μM and were thus excluded from the viral quantification assay. Lastly, test compounds were subjected to a parallel artificial membrane permeability assay (PAMPA) to shed light on their membrane-penetrating capabilities at pH 7.4 (Di et al., 2003). The PAMPA assay was outsourced to GVK BIO (India).

Antiviral and PAMPA results are summarized in Table 1. Compound **1**, a hexapeptide with a reported K_i of 12.4 μM in a protease inhibition assay (Chanprapaph et al., 2005) failed to exhibit any cell-based antiviral activity. This could be due to its inability to permeate cell membranes, a well-known shortcoming of using peptides as drugs (Vlieghe et al., 2010). Indeed, our PAMPA results suggested it lacked membrane-penetrating capabilities (Table 1). Compound **2**, a biguanidine with a reported K_i of 44 ± 5 μM in a protease inhibition assay (Ganesh et al., 2005) did not show any cell-based activity. We believe this compound was unable to penetrate cell membranes due to its high polarity imbued by the two positively-charged guanidines as compounds containing basic moieties often result in poor cell membrane permeability (Meanwell, 2011; Peterlin-Mašič and Kikelj, 2001). Indeed, like compound 1, PAMPA results suggested it was not cell membrane permeable (Table 1). Peptide aldehyde **3**, consisting three cationic residues with a reported K_i of 5.3 μM in a protease inhibition assay (Yin et al., 2006) was also found to be inactive in our cell-based assay (Table 1). A reason could be due to the highly charged nature of the compound preventing cell penetration. Like the first two compounds, the PAMPA data suggested it was not membrane-permeable (Table 1). Quinolinol **4** with a reported K_i of 17 ± 4.3 μM in a protease inhibition assay (Mueller et al., 2008) was found inactive in our cellular assay (Table 1). Interestingly, PAMPA results revealed it to be highly membrane-permeable ($P = 4.12 \pm 0.45 \times 10^{-6}$ cm s^{-1}; Table 1). A DENV2 NS2B-NS3 protease IC_{50} assay conducted on **4** revealed its IC_{50} to be 67 ± 7 μM (see Supplementary data Fig. S2), which may explain its lack of potency in our cell-based assay. Anthraquinone **5** (ARDP0006) was found to be the most potent inhibitor among the inhibitors tested, reducing viral titer more than 1 log PFU/mL at 1 μM in HuH-7 cells (Table 1, Fig. 1) and monocytic K562 cells (Fig. 2), suggesting it did not suffer from cell-permeability issues. Indeed, the PAMPA data revealed it to be highly membrane-permeable ($P = 4.46 \pm 0.06 \times 10^{-6}$ cm s^{-1}; Table 1). The CC_{50}, EC_{50} and SI of compound **5** on both the cell lines are shown in Table 2 and the data suggests that this compound was highly effective in inhibiting the dengue virus up to 10 μM without significant cellular cytotoxicity. Compound **5** was originally identified from virtual screening of a Mayo Clinic compound library and further validated by a biochemical DENV2 NS2B-NS3 protease inhibition assay and molecular docking studies (Tomlinson et al., 2009b). Interestingly, compound **5** had no effect on the viral titer on the KUNV (Fig. 3A) and CHIKV (Fig. 3B) -infected HuH-7 cells at 1 μM, suggesting that compound **5** was DENV2 specific as it was not active toward KUNV (a flavivirus of the same family) and CHIKV (an alphavirus).

Biguanidine **6** was one of the most potent compounds reported in the literature with a K_i of 2 μM based on a protease inhibition assay (Knehans et al., 2011). However, in our cell-based assay, it did not exhibit any activity which could be due to poor cell-permeation because of its high polarity due to its two positively-charged guanidines (Meanwell, 2011; Peterlin-Mašič and Kikelj, 2001), a postulation supported by PAMPA results (Table 1). Compound **7** is an analog of **6** with a guanidine moiety substituted for a nitro group. It was originally identified as a West Nile Virus NS2B-NS3 inhibitor from docking studies and a viral protease assay (Ekonomiuk et al., 2009) and was later shown to also bind to the