Antiviral Research 99 (2013) 301–306

Contents lists available at SciVerse ScienceDirect

Antiviral Research

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

Short Communication

Nuclear localization of dengue virus (DENV) 1–4 non-structural protein 5; protection against all 4 DENV serotypes by the inhibitor Ivermectin

M.Y.F. Tay [a,1], J.E. Fraser [b,1], W.K.K. Chan [a], N.J. Moreland [a], A.P. Rathore [a], C. Wang [b], S.G. Vasudevan [a,2], D.A. Jans [b,*,2]

[a] Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School, Singapore, Singapore
[b] Nuclear Signaling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Victoria, Australia

ARTICLE INFO

Article history:
Received 10 March 2013
Revised 15 May 2013
Accepted 4 June 2013
Available online 14 June 2013

Keywords:
Dengue
Dengue virus
Ivermectin
Nonstructural protein-5
Nuclear import
Flavivirus

ABSTRACT

Infection by one of the 4 distinct serotypes of dengue virus (DENV) threatens >40% of the world's population, with no efficacious vaccine or antiviral agent currently available. DENV replication through the virus-encoded nonstructural protein (NS) 5 protein occurs in the infected cell cytoplasm, but NS5 from DENV2 has thus far been shown to localize strongly in the nucleus throughout infection. Here we use specific antibodies cross-reactive with NS5 from DENV1–4 to demonstrate nuclear localization of NS5 from all DENV serotypes for the first time in both infected as well as transfected cells, although to differing extents. The small-molecule inhibitor Ivermectin was inhibitory towards both DENV 1 and 2 NS5 interaction with its nuclear transporter importin α/β *in vitro*, and protected against infection from DENV1–4. Ivermectin thus has potential in the clinical setting as a dengue antiviral.

1. Introduction, M&M, Results and Discussion

Dengue virus (DENV), a mosquito-borne human pathogen encompassing 4 distinct but closely related serotypes, is a threat to >40% of the world's population. It contains a positive-sense, single-stranded RNA genome that is translated and replicated in the host cell cytoplasm, encoding three structural (capsid, pre-membrane/membrane, and envelope) and seven nonstructural (NS) proteins (Lindenbach et al., 2007). The multifunctional NS5 is the most conserved amongst DENV1–4, comprising an N-terminal methyltransferase (amino acids, aa1–272) (Egloff et al., 2002; Yap et al., 2010) and C-terminal RNA-dependent RNA polymerase (RdRp) (aa273–900) domains (Ackermann and Padmanabhan, 2001; Bartholomeusz and Wright, 1993; Tan et al., 1996; Yap et al., 2007). The basis of the finding that hyperphosphorylated NS5 appears strongly in the infected cell nucleus as well as in the cytoplasm (Kapoor et al., 1995) is that there are functional signals for nuclear import (nuclear localization sequence, NLS) and export (nuclear export signal, NES) within aa320–405 of NS5 RdRp (Fig. 1A), conserved in DENV1–4 (Brooks et al., 2002; Forwood et al., 1999; Pryor et al., 2007; Rawlinson et al., 2009). We previously showed that the NS5 NLS and NES mediate nucleocytoplasmic transport through conferring specific recognition by the cellular nuclear transporters importin (IMP) heterodimer IMPα/β1 and CRM1 (exportin 1), respectively (Alvisi et al., 2008; Caly et al., 2012; Wagstaff and Jans, 2009); importantly, mutational studies with infectious clones show that a loss of nuclear localization of DENV2 NS5 strongly correlates with restricted virus production (Kumar et al., 2013; Pryor et al., 2007; Rawlinson et al., 2009).

DENV2 NS5 nuclear localization has been documented in infected as well as transfected cells (Brooks et al., 2002; Forwood et al., 1999; Pryor et al., 2007) but little information is available for the other DENV serotypes. We derived cross-reactive, NS5-specific, human antibody fragments using phage display (Zhao et al., manuscript in preparation), and expressed full IgG versions of the RdRp reactive 5R3 and MTase-reactive 5M1 antibodies in HEK293T cells (Chan et al., 2012; Moreland et al., 2012); the antibodies recognize full-length NS5 from all four DENV serotypes with nM affinity. The antibodies were subsequently employed to show that NS5 can be detected in DENV1–4 infected (MOI = 10) Huh-7 cells fixed 24 h post-infection (p.i.) by immunostaining/confocal laser scanning microscopy (CLSM) (Fig. 1B). Results for the ratio of nuclear to cytoplasmic fluorescence (Fn/c), determined from image analysis (Collins, 2007) of digitized CLSM images (such as those in Fig. 1B), confirmed that DENV3 NS5 was predominantly nuclear like DENV2 NS5 (Fn/c of 11 and 12, respectively), whereas

* Corresponding authors.
E-mail addresses: subhash.vasudevan@duke-nus.edu.sg (S.G. Vasudevan), david.jans@monash.edu (D.A. Jans).
[1] These authors contributed equally as first authors to this work.
[2] These authors contributed equally as senior authors to this work.

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.06.002

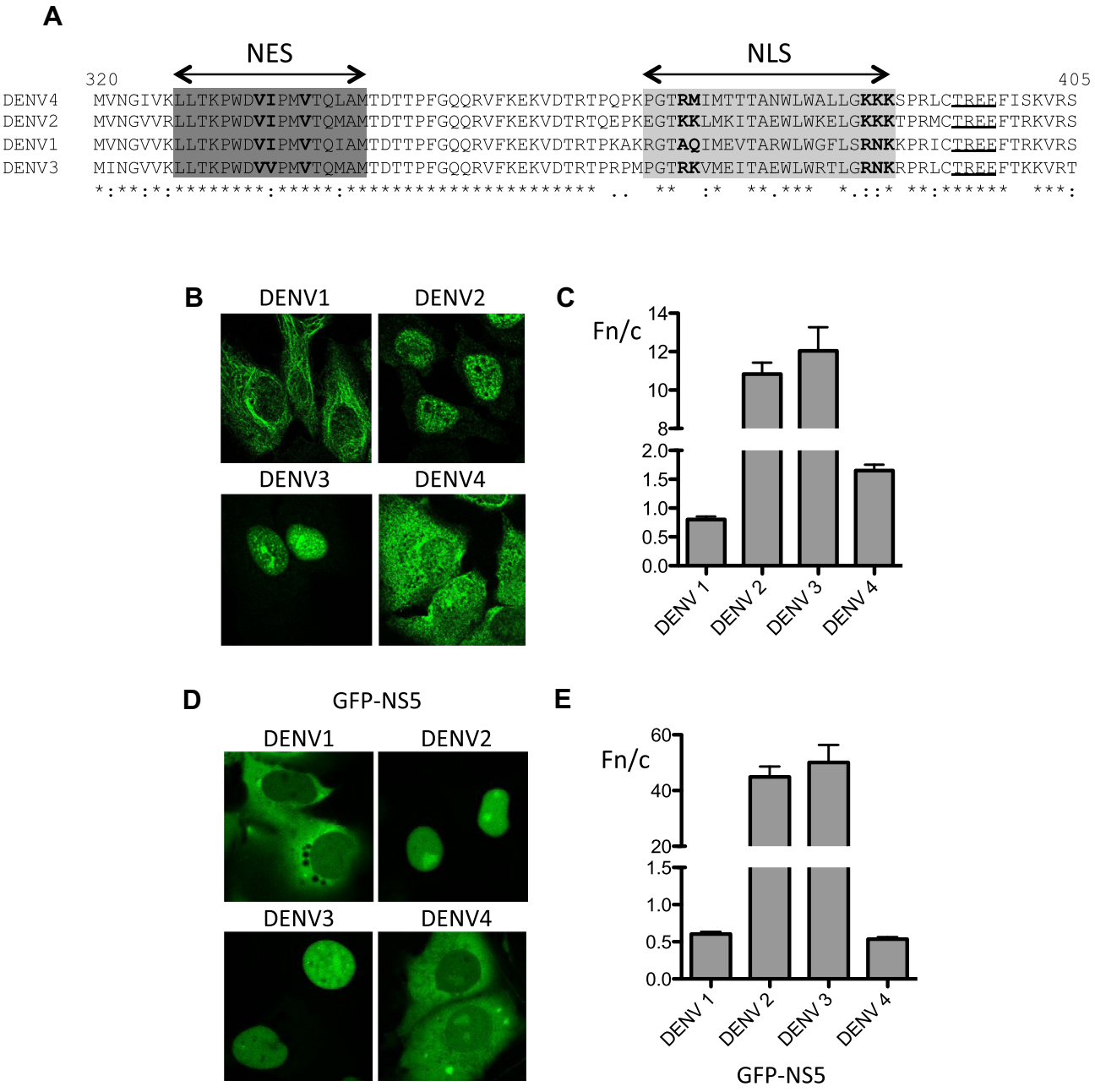

Fig. 1. NS5 from DENV1–4 exhibits nuclear localization, but to differing extents. (A) Alignment of aa320–405 of NS5 RdRp domain in four DENV serotypes reveals conserved NLS and NES sequences. The NS5 NLS (aa368–389; highlighted in grey), is characterized by two clusters of basic residues (in bold) separated by a 10–12 aa spacer (Fontes et al., 2003; Robbins et al., 1991). The NS5 NES (aa327–343; highlighted in dark grey) is characterized by the presence of hydrophobic/leucine residues (in bold) (Fornerod et al., 1997; la Cour et al., 2004). Phosphorylation of threonine within TREE region (underlined) by protein kinase CK2 is believed to reduce nuclear import (Forwood et al., 1999). GenBank™ accession numbers for the relevant sequences are: DENV1 (EU081230), DENV2 (EU081177), DENV3 (EU081190) and DENV4 (GQ398256). (B) Huh-7 cells were mock-infected or infected with DENV1–4 (GenBank™ accession numbers are as per A) at an MOI of 10 and fixed at 24 h p.i., and anti-NS5 and -envelope (not shown) antibodies used for immunostaining. Images were captured by confocal laser scanning microscopy (CLSM) using a Zeiss LSM 710 upright confocal microscope. (C) Image analysis was performed on digitized images such as those in B to determine nuclear to cytoplasmic ratio (Fn/c) for NS5 for each serotype using ImageJ software. Data represent the mean ± SEM ($n > 25$ cells), background fluorescence subtracted, from a single assay, representative of two independent experiments. (D) Cos-7 cells were transfected with GFP or GFP-NS5 (serotypes 1–4; sequences as in (A) except DENV2 accession number is AY037116) and imaged live at 24 h post–transfection by CLSM using a Nikon C1 invert microscope. (E) Image analysis was performed on digitized images such as those in (D) to determine the Fn/c ratio. Data are the mean ± SEM ($n > 40$ cells), background fluorescence subtracted, from a single assay, representative of three independent experiments.

DENV1 and DENV4 NS5 were strikingly less so (Fn/c of 0.8 and 1.7, respectively) (Fig. 1C). Uniquely in the case of DENV1, NS5 also exhibited localization in the perinuclear region in infected cells (Fig. 1B). Similar results were observed for DENV1–4 infected BHK-21 cells (not shown); see also Fig. 2A and B. Thus, although NS5 is clearly present in the nucleus of DENV1–4 infected cells, the amount of nuclear protein varies widely.

The nuclear targeting ability of NS5 from the different DENV serotypes was further assessed by fusing the NS5 coding sequences to that of green fluorescent protein (GFP), and expressing the encoded proteins in transfected Cos-7 cells. Live cell CLSM gave results similar to those observed for NS5 in infected cells (Fig. 1D and E); all GFP-NS5 proteins showed nuclear localization, with DENV2 and 3 NS5 showing higher accumulation. DENV1 NS5

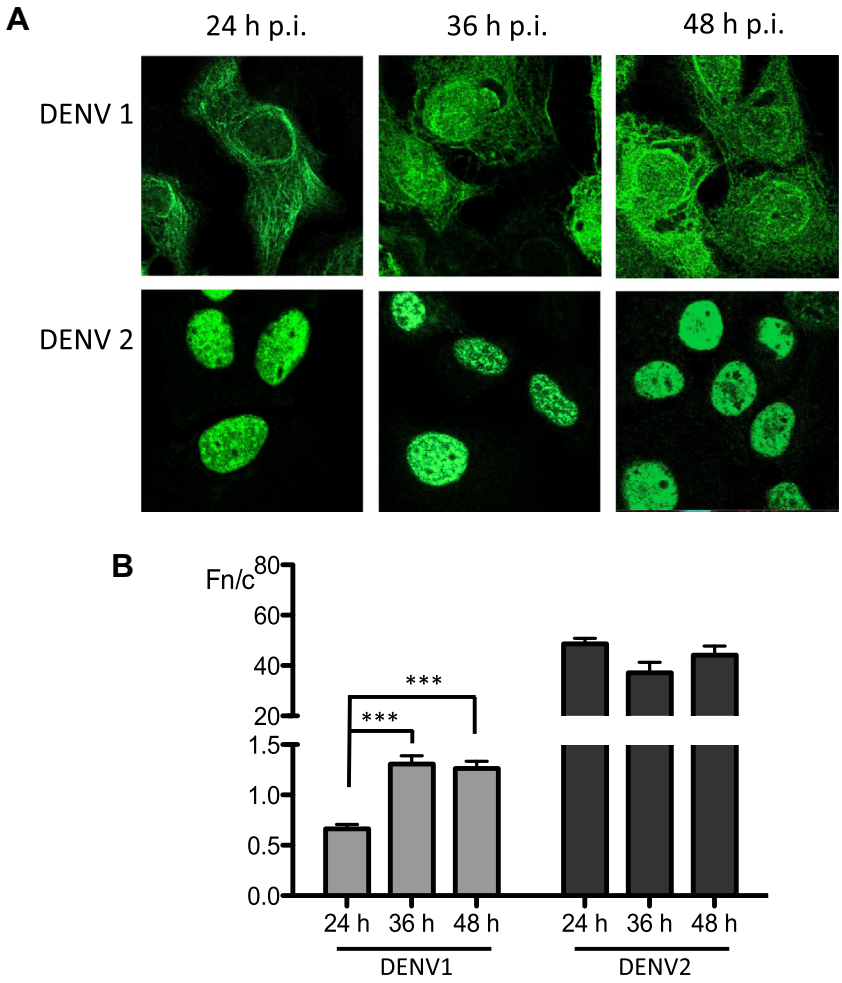

Fig. 2. DENV1 NS5 shows increased nuclear localization over time in infected cells. (A) Huh-7 cells were mock-infected or infected with DENV1 or 2 at an MOI of 10 and fixed at various times p.i. (B) Image analysis was performed on digitized images such as those in (A) to determine the Fn/c ratio. Data are the mean ± SEM ($n > 25$ cells), from a single assay, representative of two independent experiments. Statistical analysis (Student's t test) was performed using GraphPad Prism software (***$p < 0.0001$).

showed the lowest level of accumulation (Fn/c of 0.5), but this could be increased significantly by treatment with the CRM1 specific inhibitor leptomycin B (LMB; see Supplementary Fig. 1), indicating for the first time the contribution to DENV1 NS5 cytoplasmic localisation of CRM1-dependent nuclear export, previously documented for DENV2 (Pryor et al., 2007; Rawlinson et al., 2009). Importantly, in the absence of LMB DENV1 NS5 accumulates in the nucleus to a c. 3-fold higher ($p < 0.0001$) extent (Supplementary Fig. 1) than the NLS/CRM1-recognized NES-containing control Rabies P protein (Moseley et al., 2009), indicative of significant nuclear targeting ability on the part of DENV1 NS5.

We focussed on DENV1- and DENV2-infected cells, NS5 being able to be detected as early as 12 h post-infection (not shown). DENV2 NS5 was predominantly nuclear at all subsequent time points (Fig. 2A and B), whereas DENV1 NS5 was more cytoplasmic at 24 h (Fn/c of 0.7), but displayed significant nuclear localization at 36 and 48 h post-infection (Fn/c reaching almost 1.5, well in excess of a value of 1 which denotes equimolar concentrations in nucleus and cytoplasm). The slower kinetics of nuclear localization of DENV1 NS5 could not be attributed to a significantly slower infection rate in DENV1 compared to that of DENV2 (see Supplementary Fig. 2); data were similar for the DENV1 prototype Hawaii strain (not shown). Clearly, NS5 localizes in the nucleus in DENV1-infected cells, but to a lower extent than in DENV2-infected cells.

We confirmed DENV1 NS5's nuclear targeting ability by testing whether it may be recognized by IMPα/β1 (Fig. 3A), in similar fashion to DENV2 NS5 (Pryor et al., 2007; Rawlinson et al., 2009; Wagstaff et al., 2012) in an AlphaScreen® binding assay (Wagstaff and Jans, 2006; Wagstaff et al., 2011, 2012). Both DENV1 and 2 His_6-NS5 were found to be recognized by IMPα/β1 with high affinity (apparent dissociation constant of 0.2–0.4 nM).

We recently established that the small molecule Ivermectin, identified in a screen for inhibitors of interaction of viral proteins with IMPα/β1 that specifically targets the host IMPα protein (Wagstaff et al., 2011), is also able to block interaction of IMPα/β1 but not of IMPβ1 alone with DENV2 NS5 (Wagstaff et al., 2012). Importantly, we found that Ivermectin inhibited recognition of both DENV1 and DENV2 NS5 by IMPα/β1 with similar half-maximal inhibitory concentration (IC_{50}) values of 2.3 and 1.5 μM, respectively (Fig. 3B); the specificity of the effects are indicated by the fact that Ivermectin did not inhibit binding of NS5–NS3 (Takahashi et al., 2012) (see Supplementary Fig. 3), further supporting the specificity of Ivermectin's action being towards IMPα rather than NS5 (Wagstaff et al., 2012).

To examine the implications of the above with respect to Ivermectin's antiviral potential (Wagstaff et al., 2012), we tested its ability to protect against infection by DENV1–4 in BHK-21 and Huh-7 cells, commonly used for DENV infectious assays. BHK-21 cells were mock-infected or infected with DENV1–4 at an MOI of 0.3 in the presence or absence of increasing concentrations of Ivermectin, followed by fixation/quantitation of virus envelope protein at 48 h p.i. The half-maximal effective concentration (EC_{50}) values

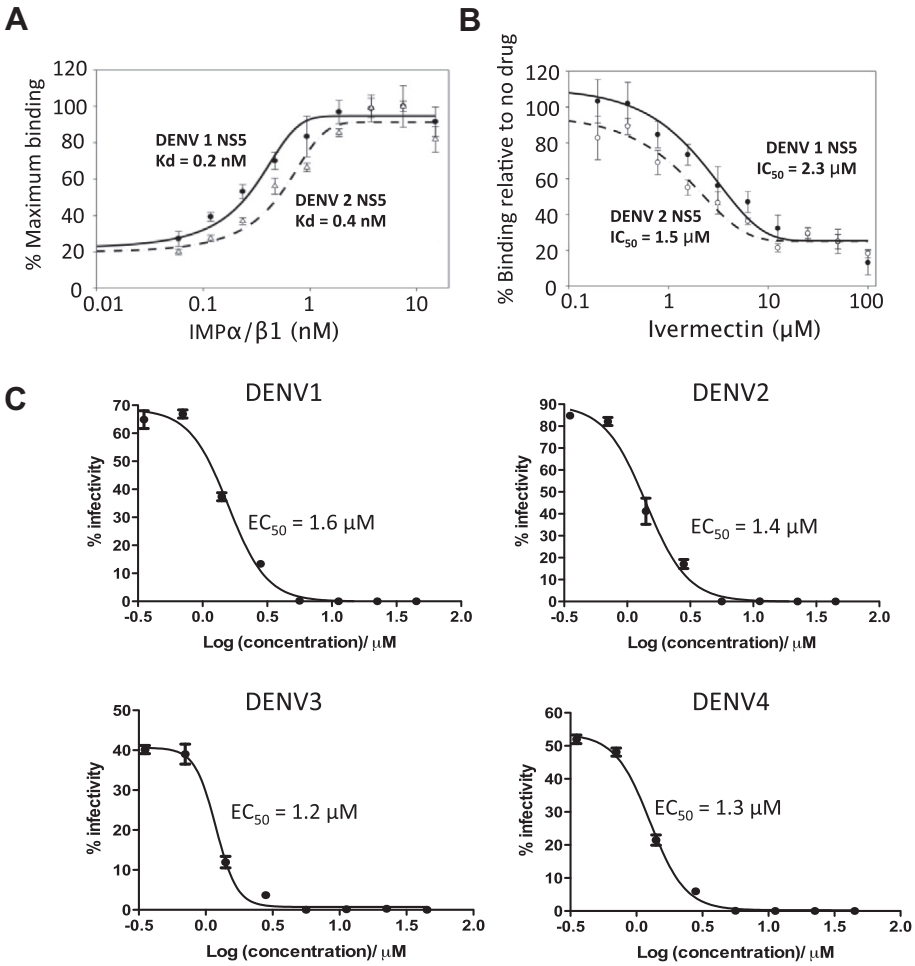

Fig. 3. Ivermectin can block association of DENV 1 and 2 NS5 with IMPα/β1 and inhibits DENV1–4 replication. (A) The binding affinities of recognition of DENV1/2 NS5 by IMPα/β1 were determined using an AlphaScreen® assay (Wagstaff and Jans, 2006; Wagstaff et al., 2011) whereby 30 nM His_6-NS5 was incubated with increasing concentrations of IMPα/β1-B (Impα/β heterodimer containing biotinylated IMPβ1; β1-B). Data represent the mean ± standard deviation (SD) with background signal for the assay in the absence of IMPα/β1-B subtracted, for a single typical experiment performed in quadruplicate, representative of a series of 3 independent experiments. (B) Binding of His_6-NS5 to IMPα/β1-B was validated by including increasing concentrations of the nuclear import inhibitor Ivermectin, a compound that disrupts NS5-IMPα/β1 interaction (Wagstaff et al., 2012), in the assay, performed using 30 nM NS5 and 10 nM IMPα/β1-B. Data are the mean ± SD as per (A). (C) DENV1–4 infected BHK-21 cells were treated with various concentrations of Ivermectin (0.4–45 μM) and the EC_{50} values ($n = 3$) determined by CFI assay 48 h p.i. Data are representative of a series of two independent experiments; see Table 1 for pooled data.

(Fig. 3C), estimated using an established cell-based flavivirus immunodetection assay (Rathore et al., 2011; Wang et al., 2009), were similar for DENV1–4 (1.6–2.3 μM; see Table 1). Ivermectin's antiviral action was confirmed in Huh-7 cells for DENV1/2 using an established plaque assay (Table 1) (Wang et al., 2009), underlining the fact that Ivermectin's effects are not cell-type specific. Ivermectin was not toxic at concentrations near the EC_{50} value (see footnote to Table 1; Wagstaff et al., 2012). That the EC_{50} values for all 4 DENV serotypes were comparable and close to the values for inhibition of IMPα/β1 binding to NS5 *in vitro* (Fig. 3B) is consistent with the idea that Ivermectin targets an important host protein (IMPα – see above); thus, even though the level of NS5 nuclear accumulation is markedly different for DENV1–4, in part due to the balance of the distinct nuclear import and export targeting activities of each (see Figs. 1 and 2; Supplementary Fig. 1), the respective level of nuclear accumulation of NS5 evident in infected cells appears to be of critical importance to the viral life cycle. Despite this, it cannot be excluded that the antiviral effects of Ivermectin are due to inhibition of IMPα/β1-mediated nuclear import of a cellular factor(s) important for DENV infection, rather than of NS5 itself. Ivermectin did not inhibit DENV NS5 RdRp activity *in vitro* (not shown) assessed as previously (Yap et al., 2007), further consistent with its antiviral activity being due to action on IMPα rather than NS5 (Wagstaff et al., 2011, 2012).

It should also be noted in this context that Ivermectin has recently been reported to have inhibitory activity towards dengue NS3 (Mastrangelo et al., 2012), meaning that it is not formally possible to interpret the results for the antiviral activity of Ivermectin reported here for the first time as attributable purely to inhibition of NS5 nuclear import. Our preliminary results (manuscript in preparation) for a newly identified, distinct inhibitor of IMPα/β1 binding to NS5, retinoic acid p-hydroxyanilide (4-HPR; Fraser et al., 2013), however, are strongly consistent with this idea. What the present study clearly shows for the first time is that NS5 of DENV1–4 localize in the nucleus in both infected and transfected cells to differing extents, and that the IMPα targeting inhibitor Ivermectin can inhibit infection by DENV1–4, implying that NS5 nuclear localization may be critical to infection by all 4 DENV serotypes. Although NS5's precise nuclear role in pathogenesis remains to be delineated in detail, it presumably relates in part to transcriptional modulation of expression of the antiviral response, including induction of the cytokine interleukin-8 (Medin et al., 2005; Pryor et al., 2007; Rawlinson et al., 2009).

Table 1
Summary of antiviral activities of Ivermectin and other agents towards DENV serotypes 1–4.

Viral titre (μM) (EC_{50})			
Serotype	Agent	BHK-21[a]	Huh-7[b]
DENV1	Ivermectin[c]	2.32 ± 1.06	2.97 ± 0.07
	Celgosivir[d]	0.26 ± 0.00	N.D.[e]
	NITD008[d]	1.37 ± 0.41	2.53 ± 0.08
DENV2	Ivermectin	2.08 ± 0.94	1.74 ± 0.15
	Celgosivir	0.32 ± 0.05	N.D.[e]
	NITD008	1.32 ± 0.56	0.22 ± 0.05
DENV3	Ivermectin	1.66 ± 0.66	N.D.[e]
	Celgosivir	0.09 ± 0.02	N.D.[e]
	NITD008	0.50 ± 0.19	N.D.[e]
DENV4	Ivermectin	1.90 ± 0.89	N.D.[e]
	Celgosivir	0.08 ± 0.01	N.D.[e]
	NITD008	0.65 ± 0.28	N.D.[e]

[a] Data are for the mean ± SD (2 independent experiments performed in triplicate) as determined by CFI assay on mock or DENV1–4 infected BHK-21 cells treated with 0.4–45 μM of the indicated agents as per Fig. 3C.
[b] Data are for the mean ± SD (single experiment performed in duplicate), as determined by plaque assay on BHK-21 cells using media supernatant at 48 h post-infection of mock-infected or DENV1–2 infected Huh-7 cells treated with 0.16–5 μM of the indicated agents.
[c] CC50 for BHK-21 and Huh-7 for Ivermectin are 9.8 and 7.3 μM, respectively (48 h treatment).
[d] The established inhibitors of DENV Celgosivir (Rathore et al., 2011) and NITD008 (Yin et al., 2009) were included in the assays as controls; CC50 values of > 100 μM.
[e] N.D., Not determined.

Ivermectin has been in clinical use for almost 25 years as a broad-spectrum antiparasitic drug against helminths, offering important opportunities for repurposing as an antiviral agent for dengue (Omura, 2008; Mastrangelo et al., 2012). Since Ivermectin is widely used in Papua New Guinea communities with frequent dengue infections (Omura, 2008), an intriguing possibility in this context may be to conduct prospective serosurveys on cohorts of patients to confirm Ivermectin's protective efficacy.

Acknowledgements

We thank Lee Chin Chin, Sabrina Koh and Elfin Lim for assistance with phage display and preparation of NS5 antibodies, Zhao Yong Qian and Brendon Hansen for NS5 and IgG expression plasmids, respectively, and Amy Rogers and Cassandra David for technical assistance. This work was supported by the National Health and Medical Research Council Australia (Project Grant ID#606409 and Senior Principal Research Fellowship APP1002486), DUKE-NUS Signature Research Program (funded by the Ministry of Health, Singapore), 60° Pharmaceuticals, LLC (USA) and the National Medical Research Council, Singapore (http://www.nmrc.gov.sg), under grant NMRC/1315/2011.

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.antiviral.2013.06.002.

References

Ackermann, M., Padmanabhan, R., 2001. De novo synthesis of RNA by the dengue virus RNA-dependent RNA polymerase exhibits temperature dependence at the initiation but not elongation phase. J. Biol. Chem. 276, 39926–39937.
Alvisi, G., Avanzi, S., Musiani, D., Camozzi, D., Leoni, V., Ly-Huynh, J.D., Ripalti, A., 2008. Nuclear import of HSV-1 DNA polymerase processivity factor UL42 is mediated by a C-terminally located bipartite nuclear localization signal. Biochemistry 47, 13764–13777.
Bartholomeusz, A.I., Wright, P.J., 1993. Synthesis of dengue virus RNA in vitro: initiation and the involvement of proteins NS3 and NS5. Arch. Virol. 128, 111–121.
Brooks, A.J., Johansson, M., John, A.V., Xu, Y., Jans, D.A., Vasudevan, S.G., 2002. The interdomain region of dengue NS5 protein that binds to the viral helicase NS3 contains independently functional importin beta 1 and importin alpha/beta-recognized nuclear localization signals. J. Biol. Chem. 277, 36399–36407.
Caly, L., Wagstaff, K.M., Jans, D.A., 2012. Nuclear trafficking of proteins from RNA viruses: potential target for antivirals? Antiviral Res. 95, 202–206.
Chan, A.H., Tan, H.C., Chow, A.Y., Lim, A.P., Lok, S.M., Moreland, N.J., Vasudevan, S.G., MacAry, P.A., Ooi, E.E., Hanson, B.J., 2012. A human PrM antibody that recognizes a novel cryptic epitope on dengue E glycoprotein. PLoS One 7, e33451.
Collins, T.J., 2007. ImageJ for microscopy. Biotechniques 43, 25–30.
Egloff, M.P., Benarroch, D., Selisko, B., Romette, J.L., Canard, B., 2002. An RNA cap (nucleoside-2′-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. EMBO J. 21, 2757–2768.
Fontes, M.R., Teh, T., Jans, D., Brinkworth, R.I., Kobe, B., 2003. Structural basis for the specificity of bipartite nuclear localization sequence binding by importin-alpha. J. Biol. Chem. 278, 27981–27987.
Fornerod, M., Ohno, M., Yoshida, M., Mattaj, I.W., 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell 90, 1051–1060.
Forwood, J.K., Brooks, A., Briggs, L.J., Xiao, C.Y., Jans, D.A., Vasudevan, S.G., 1999. The 37-amino-acid interdomain of dengue virus NS5 protein contains a functional NLS and inhibitory CK2 site. Biochem. Biophys. Res. Commun. 257, 731–737.
Fraser, J.E., Wagstaff, K.M., Jans, D.A., 2013. Method of viral inhibition. Provisional patent filed 16 April, 2013, Australia. 2013901525.
Kapoor, M., Zhang, L., Ramachandra, M., Kusukawa, J., Ebner, K.E., Padmanabhan, R., 1995. Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. J. Biol. Chem. 270, 19100–19106.
Kumar, A., Buhler, S., Selisko, B., Davidson, A., Mulder, K., Canard, B., Miller, S., Bartenschlager, R., 2013. Nuclear localization of dengue virus nonstructural protein 5 does not strictly correlate with efficient viral RNA replication and inhibition of type I interferon signaling. J. Virol. 87, 4545–4557.
la Cour, T., Kiemer, L., Molgaard, A., Gupta, R., Skriver, K., Brunak, S., 2004. Analysis and prediction of leucine-rich nuclear export signals. Protein Eng. Des. Sel. 17, 527–536.
Lindenbach, B.D., Thiel, H., Rice, C.M., 2007. Flaviviridae: The Viruses and Their Replication. Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia.
Mastrangelo, E., Pezzullo, M., De Burghgraeve, T., Kaptein, S., Pastorino, B., Dallmeier, K., de Lamballerie, X., Neyts, J., Hanson, A.M., Frick, D.N., Bolognesi, M., Milani, M., 2012. Ivermectin is a potent inhibitor of flavivirus replication specifically targeting NS_3 helicase activity: new prospects for an old drug. J. Antimicrob. Chemother. 67, 1884–1894.
Medin, C.L., Fitzgerald, K.A., Rothman, A.L., 2005. Dengue virus nonstructural protein NS5 induces interleukin-8 transcription and secretion. J. Virol. 79 (17), 11053–11061.
Moreland, N.J., Tay, M.Y., Lim, E., Rathore, A.P., Lim, A.P., Hanson, B.J., Vasudevan, S.G., 2012. Monoclonal antibodies against dengue NS2B and NS3 proteins for the study of protein interactions in the flaviviral replication complex. J. Virol. Methods 179, 97–103.
Moseley, G.W., Lahaye, X., Roth, D.M., Oksayan, S., Filmer, R.P., Rowe, C.L., Blondel, D., Jans, D.A., 2009. Dual modes of rabies P-protein association with microtubules: a novel strategy to suppress the antiviral response. J. Cell Sci. 122, 3652–3662.
Omura, S., 2008. Ivermectin: 25 years and still going strong. Int. J. Antimicrob Agents 31 (2), 91–98.
Pryor, M.J., Rawlinson, S.M., Butcher, R.E., Barton, C.L., Waterhouse, T.A., Vasudevan, S.G., Bardin, P.G., Wright, P.J., Jans, D.A., Davidson, A.D., 2007. Nuclear localization of dengue virus nonstructural protein 5 through its importin alpha/beta-recognized nuclear localization sequences is integral to viral infection. Traffic 8, 795–807.
Rathore, A.P., Paradkar, P.N., Watanabe, S., Tan, K.H., Sung, C., Connolly, J.E., Low, J., Ooi, E.E., Vasudevan, S.G., 2011. Celgosivir treatment misfolds dengue virus NS1 protein, induces cellular pro-survival genes and protects against lethal challenge mouse model. Antiviral Res. 92, 453–460.
Rawlinson, S.M., Pryor, M.J., Wright, P.J., Jans, D.A., 2009. CRM1-mediated nuclear export of dengue virus RNA polymerase NS5 modulates interleukin-8 induction and virus production. J. Biol. Chem. 284, 15589–15597.
Robbins, J., Dilworth, S.M., Laskey, R.A., Dingwall, C., 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell 64, 615–623.
Takahashi, H., Takahashi, C., Moreland, N.J., Chang, Y.T., Sawasaki, T., Ryo, A., Vasudevan, S.G., Suzuki, Y., Yamamoto, N., 2012. Establishment of a robust dengue virus NS3-NS5 binding assay for identification of protein-protein interaction inhibitors. Antiviral Res. 96, 305–314.
Tan, B.H., Fu, J., Sugrue, R.J., Yap, E.H., Chan, Y.C., Tan, Y.H., 1996. Recombinant dengue type 1 virus NS5 protein expressed in *Escherichia coli* exhibits RNA-dependent RNA polymerase activity. Virology 216, 317–325.
Wagstaff, K.M., Jans, D.A., 2006. Intramolecular masking of nuclear localization signals: analysis of importin binding using a novel AlphaScreen-based method. Anal. Biochem. 348, 49–56.
Wagstaff, K.M., Jans, D.A., 2009. Importins and beyond: non-conventional nuclear transport mechanisms. Traffic 10, 1188–1198.

Wagstaff, K.M., Rawlinson, S.M., Hearps, A.C., Jans, D.A., 2011. An AlphaScreen(R)-based assay for high-throughput screening for specific inhibitors of nuclear import. J. Biomol. Screen. 16, 192–200.

Wagstaff, K.M., Sivakumaran, H., Heaton, S.M., Harrich, D., Jans, D.A., 2012. Ivermectin is a specific inhibitor of importin alpha/beta-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus. Biochem. J. 443, 851–856.

Wang, Q.Y., Patel, S.J., Vangrevelinghe, E., Xu, H.Y., Rao, R., Jaber, D., Schul, W., Gu, F., Heudi, O., Ma, N.L., Poh, M.K., Phong, W.Y., Keller, T.H., Jacoby, E., Vasudevan, S.G., 2009. A small-molecule dengue virus entry inhibitor. Antimicrob. Agents Chemother. 53, 1823–1831.

Yap, L.J., Luo, D., Chung, K.Y., Lim, S.P., Bodenreider, C., Noble, C., Shi, P.Y., Lescar, J., 2010. Crystal structure of the dengue virus methyltransferase bound to a 5′-capped octameric RNA. PLoS One, 5.

Yap, T.L., Xu, T., Chen, Y.L., Malet, H., Egloff, M.P., Canard, B., Vasudevan, S.G., Lescar, J., 2007. Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution. J. Virol. 81, 4753–4765.

Yin, Z., Chen, Y.L., Schul, W., Wang, Q.Y., Gu, F., Duraiswamy, J., Kondreddi, R.R., Niyomrattanakit, P., Lakshminarayana, S.B., Goh, A., Xu, H.Y., Liu, W., Liu, B., Lim, J.Y., Ng, C.Y., Qing, M., Lim, C.C., Yip, A., Wang, G., Chan, W.L., Tan, H.P., Lin, K., Zhang, B., Zou, G., Bernard, K.A., Garrett, C., Beltz, K., Dong, M., Weaver, M., He, H., Pichota, A., Dartois, V., Keller, T.H., Shi, P.Y., 2009. An adenosine nucleoside inhibitor of dengue virus. Proc. Natl. Acad. Sci. U.S.A. 106, 20435–20439.