



## Antiviral activity of natural and synthetic $\beta$ -carbolines against dengue virus



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### ABSTRACT

Dengue virus (DENV) is the most prevalent mosquito borne viral pathogen worldwide. In this work we first evaluated the antiviral activity of natural and synthetic  $\beta$ -carbolines against DENV-2 multiplication in cell cultures. We determined that the natural  $\beta$ -carboline harmol and a synthetic harmine derivative, 9N-methylharmine, exhibit inhibitory effect on DENV-2 production without virucidal activity. The active compounds were inhibitory of all DENV serotypes, being DENV-2 the more susceptible to their antiviral action. The mode of action of 9N-methylharmine against DENV-2 was further explored. We determined that the derivative neither affects viral adsorption-internalization events nor viral RNA synthesis. The quantification of intracellular and extracellular viral genomes and infectious virus particles indicated that 9N-methylharmine would impair the maturation and release of virus particles to the extracellular medium affecting the spreading of the infection. Furthermore, we also determined that 9N-methylharmine antiviral activity is not related to the ability of the compound to downregulate p38 MAPK phosphorylation.

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### 1. Introduction

Dengue, a mosquito-borne viral infection that prevails in tropical and subtropical regions of the world, is caused by any of the four dengue virus serotypes (DENV 1–4). Dengue is endemic in more than 100 countries and its incidence has increased 30-fold in the past 50 years (Guzman and Harris, 2015; Simmons et al., 2015). DENV infection presents a wide spectrum of manifestations including asymptomatic condition, dengue fever, or severe forms, such as dengue hemorrhagic fever and dengue shock syndrome (John et al., 2015). There is a need for an effective dengue therapeutic that can prevent the development of severe complications and reduce on-going transmission of the virus to mosquitoes and new human hosts. Although several compounds that target virus or host components are in development, only a few agents have been assessed in formal clinical trials and unfortunately the assayed compounds showed no benefit in preventing the outcome of severe

forms of the disease or in reducing plasma viremia (Castilla et al., 2015; Whitehorn et al., 2014).

The use of natural sources is a well-established strategy to obtain new substances with potential therapeutic activity (Cao et al., 2007; Khan et al., 2013). Among alkaloids,  $\beta$ -carbolines (aromatic structures with three 6-5-6-fused rings) are known to present diverse biological properties. For example, harmine and harmane, which constitute between 2 and 6% of *Peganum harmala* seeds, have a wide range of therapeutic applications (Moloudizargari et al., 2013; Nenaah, 2010; Patel et al., 2012). Previous studies performed with natural and synthetic  $\beta$ -carbolines have shown that these compounds display antiviral activity against herpes simplex virus (HSV), poliovirus (PV) and human immunodeficiency virus (Bag et al., 2014; Brahmabhatt et al., 2010; Chen et al., 2015; Nazari Formagio et al., 2009; Wang et al., 2007). The antiviral activity of other structurally related alkaloids against viruses with DNA and RNA genome has also been reported (Ashok et al., 2015; Liu et al., 2014; Sako et al., 2008; Salim et al., 2010; Song et al., 2014; van Maarseveen et al., 1999). Particularly,  $\gamma$ -carbolines have been proved to be highly potent inhibitors of bovine

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viral diarrhea virus (BVDV), a bovine pathogen that belongs to the *Flaviviridae* family (Sako et al., 2008, Salim et al., 2010).

Here we evaluated the antiviral activity of natural and synthetic  $\beta$ -carbolines against DENV and the mode of antiviral action of the active compound 9N-methylharmine was investigated.

## 2. Materials and methods

### 2.1. Virus and cells

The African green monkey kidney cell line Vero (ATCC CCL-81) was grown at 37 °C in Eagle's minimum essential medium (MEM) (GIBCO, USA) supplemented with 5% calf serum (GIBCO, USA) and 50  $\mu$ g/ml gentamycin. For maintenance medium (MM) serum concentration was reduced to 1.5%. DENV-2 strain New Guinea C (NGC), DENV-1 strain Hawaii, DENV-3 strain H87 and DENV-4 strain 8124 were used.

### 2.2. Synthesis of $\beta$ -carboline derivatives

Commercial  $\beta$ -carbolines, harmine, harmone and norharmone purchased from Sigma Aldrich (USA) and harmol from Wako Pure Chemical Industries Ltd. (Japan), were of analytical grade and were used without additional purification.

9N-methylharmine, 9N-methylharmone and 9N-methylnorharmone were synthesized as previously described (Cao et al., 2004, 2005). Harmol aliphatic esters with chains of 2, 3, 4, 5 or 6 carbons were obtained according to Begum et al. (2004).

Drug stocks solutions were prepared in dimethyl sulfoxide and diluted in MM before use.

### 2.3. Cytotoxicity assay

Monolayers of confluent Vero cells were exposed to various concentrations of the assayed compound in MM. After 48 h incubation, cell viability was examined by the MTS colorimetric assay (Promega, USA). The 50% cytotoxic concentration (CC<sub>50</sub>) was defined as the compound concentration required to reduce cell viability by 50%.

### 2.4. Virus yield reduction assay

Vero cells were infected with DENV at a multiplicity of infection (MOI) of 1 or 0.1 PFU/cell. After 1 h adsorption at 37 °C cells were covered with MM containing varying non-cytotoxic concentrations of  $\beta$ -carbolines. At 48 h post-infection (p.i.), supernatant cultures were harvested and virus yields were quantified by plaque assay in Vero cells.

Antiviral activity was expressed as 50% effective concentration (EC<sub>50</sub>), the compound concentration required to reduce virus yield by 50% as compared with the untreated control cultures.

### 2.5. Virucidal assay

Aliquots of DENV-2 stock were incubated for 2 h at 37 °C in the presence or absence of different concentrations of the compounds. Next, samples were diluted in MM and tested for virus survival using a plaque assay.

### 2.6. Time of addition experiment

Vero cells were pretreated with 9N-methylharmine (40  $\mu$ M) for 1 h and then infected with DENV-2 (MOI = 1). In other set of infected cultures 9N-methylharmine was added at 1, 3, 5 or 8 h p.i. and the compound was maintained till 48 h p.i. At this time

extracellular virus titer was determined from culture supernatants and cell monolayers were subjected to two freeze-thaw cycles, followed by low-speed centrifugation in order to quantify intracellular infectivity.

### 2.7. Real time RT-PCR

Cells were infected with DENV-2 (MOI = 1) and after 1 h adsorption at 37 °C cultures were incubated with 9N-methylharmine (40  $\mu$ M) or ribavirin (80  $\mu$ M) till 6 or 24 h p.i. After washing with PBS, total RNA was extracted from cells by using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Then, cDNA was generated by using murine reverse transcriptase M-MLV (Promega, USA) and the antisense primer (5'-CACTACGC-CATGCCGTGTACAGC-3') corresponding to viral 3' UTR as previously described (Talarico and Damonte, 2007). The antisense and the sense primer (5'-CCTGTAGCTCCACCTGAGAAG-3'), which allow the amplification of nucleotides 10,419 to 10,493 within the viral 3' UTR were used to perform real time PCR using SYBRGreen (Promega, USA) detection. Real time PCR was carried out with an initial incubation at 95 °C during 3 min followed by 40 cycles of 15 s at 95 °C, 1 min at 61 °C and 30 s at 72 °C. The cellular gene actin was used as standard for normalization. Average viral RNA Ct values were normalized to the average Ct values of actin and  $\Delta\Delta$ Ct based fold-change calculations were set relative to untreated-virus infected cells using Bio-Rad iQ5 2.1 software. To determine extracellular viral genome copies culture supernatants were concentrated from ultracentrifugation at 100,000g for 2 h and resuspended in TRIzol for RNA extraction, whereas intracellular viral RNA was extracted as described above. A standard curve was generated using *in vitro* transcribed DENV replicon RNA (Alvarez et al., 2005).

### 2.8. Immunofluorescence assay

Vero cells grown on glass coverslips were infected with DENV-2 (MOI = 1). After 1 h of virus adsorption, MM containing 9N-methylharmine was added and cells were further incubated at 37 °C for 48 h. Then cells were fixed with methanol and further stained using mouse monoclonal antibody anti DENV-2 E glycoprotein (Abcam, UK) as primary antibody and anti-mouse immunoglobulins conjugated to isothiocyanate of fluorescein (FITC) (Sigma-Aldrich, USA) as secondary antibody. Cells were mounted and visualized in a fluorescence microscope (Olympus BX51). The percentage of cells expressing viral glycoprotein was determined by counting twenty randomly selected fields.

### 2.9. Western blot analysis

Vero cells, infected or not with DENV-2 (MOI = 1), were treated with different concentrations of 9N-methylharmine during 24 h. Then, supernatants were removed and cells were lysed in sample buffer (BioRad, USA). SDS-PAGE and protein transference to a PVDF membrane (Amersham, UK) was performed as previously described (Maeto et al., 2011). After transference, membranes were incubated in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 3% bovine serum albumin (blocking buffer) and further incubated overnight at 4 °C with rabbit anti P-p38 (Santa Cruz Biotechnology, USA) as primary antibody. After several washes, membranes were incubated for 1 h at room temperature with peroxidase anti-rabbit IgG (Amersham, UK) as secondary antibody. GAPDH, used as loading control, was detected with a mouse antibody (Abcam, UK) and peroxidase-conjugated anti-mouse IgG (Promega, USA) was used as secondary antibody. The intensities of protein bands, visualized by chemiluminescence detection, were quantified using Image J software, and the relative intensity P-p38/

GAPDH was calculated for each sample.

### 2.10. Statistical analysis

The 95% confidence intervals (CI) of virus titers were calculated according to Poisson distribution from replicates of one representative experiment. Statistical significance was determined by ANOVA analysis using data obtained from three independent experiments; \* $p < 0.05$ , \*\* $p < 0.01$ .

## 3. Results

### 3.1. Cytotoxic and antiviral activity of natural and synthetic $\beta$ -carbolines

In order to investigate the ability of natural  $\beta$ -carbolines and their synthetic derivatives to inhibit virus replication in Vero cell cultures we first evaluated the effect of the compounds on cell viability. For this purpose, Vero cells were treated with different concentrations of each compound and after 48 h of incubation at 37 °C cell viability was assessed using the MTS method and the  $CC_{50}$  was determined.

Harmine was the most cytotoxic compound, indicating that the C-7 methoxy group adversely affects cell viability (Table 1). Table 1 shows that the 9N-methylated derivatives of norharmane, harmane and harmine display higher  $CC_{50}$  values than natural compounds indicating that the methylation of the pyrrole ring caused a marked

reduction in the cytotoxicity. A 25-fold, 3.6-fold and more than 1.5-fold decrease in the cytotoxic effect with respect to the non-methylated compounds was determined for 9N-methylharmine, 9N-methylharmane and 9N-methylnorharmane, respectively (Table 1).

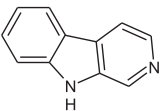
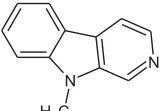
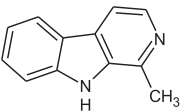
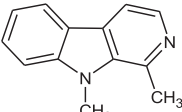
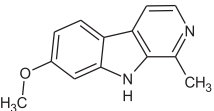
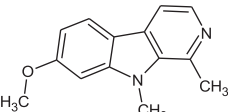
We also evaluated the effect of the natural compound harmol and harmol aliphatic esters with chains of 2, 3, 4, 5 or 6 carbons on Vero cell viability. Harmol was the less cytotoxic natural compound assayed (Table 2) whereas harmol acetate and harmol propionate displayed lower  $CC_{50}$  values than the natural compound (Table 2).

The antiviral activity against DENV-2 of the assayed  $\beta$ -carbolines was evaluated by a virus yield inhibition assay in Vero cells. The  $EC_{50}$  was determined and the selectivity index (SI) was calculated as the ratio  $CC_{50}/EC_{50}$ . 9N-methylharmine and harmol were the most active compounds against DENV-2 with SI values of 56.2 and 61.3, respectively (Table 1). The C-7 substitution present in these compounds seems to be relevant for their inhibitory effect against DENV-2. However, harmol derivatives with aliphatic esters as C-7 substituents do not exhibit important antiviral activity (Table 2).

The analysis of the antiviral activity of 9N-methylharmine and harmol against other DENV serotypes revealed that the compounds were inhibitory of all serotypes but displayed lower efficacy against DENV-1, DENV-3 and DENV-4 in comparison to DENV-2 (Table 3).

As shown in Fig. 1A the inhibitory effect of 9N-methylharmine against DENV-2 was similar when cells were infected either at an MOI of 0.1 or 1 PFU/cell. We further investigated whether the active compounds affect DENV-2 adsorption-penetration into Vero cells.

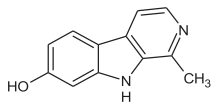
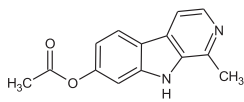
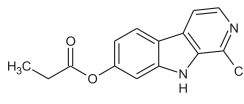
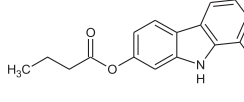
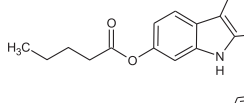
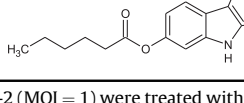
**Table 1**  
Antiviral activity of natural  $\beta$ -carbolines and their 9N-methylated derivatives against DENV-2.

Compound	Structural formula	MW	$CC_{50}$ ( $\mu$ M)	$EC_{50}$ ( $\mu$ M)	SI
Norharmane		168	99.9 $\pm$ 5.7	I	–
9N-methylnorharmane		182	>153.5 $\pm$ 21.3 <sup>a</sup>	I	–
Harmane		182	68.7 $\pm$ 13.2	I	–
9N-methylharmane		196	247.9 $\pm$ 10.4	I	–
Harmine		212	7.1 $\pm$ 0.4	I	–
9N-methylharmine		226	178.6 $\pm$ 11.5	3.2 $\pm$ 0.6	56.2

Vero cells infected or not with DENV-2 (MOI = 1) were treated with different concentrations of each compound for 48 h at 37 °C. Cell viability was assayed by the MTS method and  $CC_{50}$  (compound concentration that reduced cell viability by 50% respect to untreated cultures) was calculated. Antiviral activity was evaluated by a virus yield inhibition assay and  $EC_{50}$  (compound concentration that reduced extracellular virus yield by 50% respect to untreated infected cultures) was determined. Data are mean values from two experiments  $\pm$  SD. SI (selectivity index): ratio  $CC_{50}/EC_{50}$ ; I: inactive compound.

<sup>a</sup> The highest concentration tested reduced cell viability by less than 50%.

**Table 2**  
Antiviral activity of harmol and harmol derivatives against DENV-2.

Compound	Structural formula	MW	CC <sub>50</sub> (μM)	EC <sub>50</sub> (μM)	SI
Harmol		198	203.3 ± 1.2	3.3 ± 0.4	61.3
Harmol acetate		240	62.1 ± 6.4	I	–
Harmol propionate		254	62.1 ± 1.8	I	–
Harmol butyrate		268	153.5 ± 12.2	39.2 ± 1.3	3.9
Harmol valerate		282	580.9 ± 3.9	61.6 ± 1.5	9.4
Harmol caprate		296	220.4 ± 5.5	87.6 ± 1.3	2.5

Vero cells infected or not with DENV-2 (MOI = 1) were treated with different concentrations of each compound for 48 h at 37 °C. Cell viability was assayed by the MTS method and CC<sub>50</sub> (compound concentration that reduced cell viability by 50% respect to untreated cultures) was calculated. Antiviral activity was evaluated by a virus yield inhibition assay and EC<sub>50</sub> (compound concentration that reduced extracellular virus yield by 50% respect to untreated infected cultures) was determined. Data are mean values from two experiments ± SD. SI (selectivity index): ratio CC<sub>50</sub>/EC<sub>50</sub>; I: inactive compound.

**Table 3**  
Antiviral activity of 9N-methylharminol and harmol against other DENV serotypes.

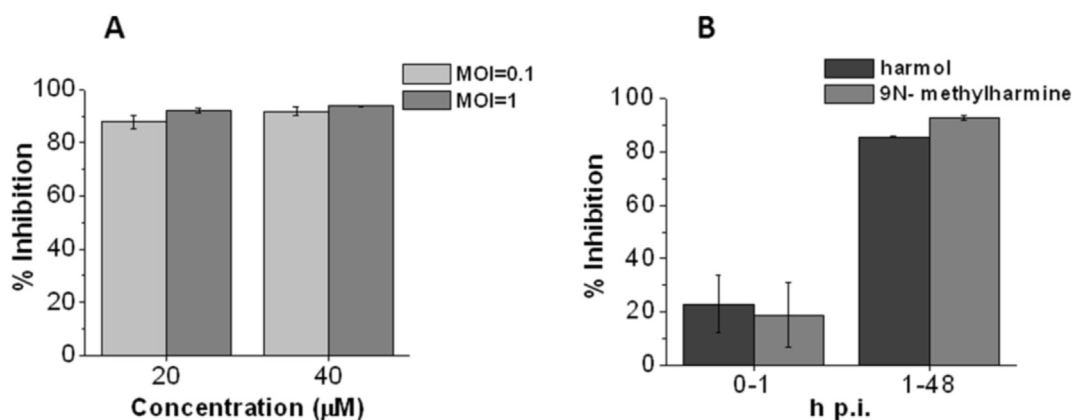
Compound	DENV-1	DENV-3	DENV-4
9N-methylharminol EC <sub>50</sub> (μM)	49.5 ± 2.5	33.7 ± 1.3	16.3 ± 1.2
Harmol EC <sub>50</sub> (μM)	10.1 ± 1.1	7.7 ± 1.1	21.1 ± 1.5

Vero cells infected with DENV-1, DENV-3 or DENV-4 (MOI = 1) were treated with different concentrations of 9N-methylharminol or harmol for 48 h. Then extracellular virus yields were quantified and EC<sub>50</sub> (compound concentration that reduced extracellular virus yield by 50% respect to untreated infected cultures) values were calculated. Data are mean values from two experiments ± SD.

The presence of harmol or 9N-methylharminol during the first hour of infection did not affect virus multiplication (Fig. 1B). By contrast, a marked reduction in virus yields was observed when the compounds were added at 1 h p.i. and maintained for 48 h.

### 3.2. Lack of harmol and 9N-methylharminol virucidal action against DENV-2

In order to determine whether the compounds that display antiviral activity are able to cause a direct inactivating effect on



**Fig. 1. Inhibitory effect of active β-carbolines on DENV-2 multiplication.** (A) Vero cells were infected with DENV-2 at a MOI of 0.1 or 1 PFU/cell and after 1 h adsorption at 37 °C, cells were covered with MM containing 9N-methylharminol (20 or 40 μM). At 48 h p.i. extracellular virus yield was quantified by plaque assay. Data are mean values from two determinations ± SD. (B) Vero cells were infected with DENV-2 (MOI = 1) in the presence of harmol or 9N-methylharminol (40 μM) for 1 h (0–1 h p.i.). Other cultures were infected with DENV-2 (MOI = 1) and after 1 h of infection, harmol or 9N-methylharminol (40 μM) was added and maintained for 48 h (1–48 h p.i.). In all cases culture supernatants were collected at 48 h p.i. and extracellular virus production was determined by plaque assay. Data are mean values from two independent experiments ± standard deviation (SD).

DENV-2 particles we performed a virucidal assay by incubation of viral suspensions with different concentrations of the active compounds. No virucidal activity was detected either for harmol or for 9N-methylharmine (Fig. 2).

### 3.3. Antiviral mode of action of 9N-methylharmine against DENV-2

In order to further dissect the antiviral mode of action of 9N-methylharmine against DENV-2, we performed a time of addition experiment. First, we treated Vero cells with the compound during 1 h at 37 °C (Fig. 3A, Pre) and then monolayers were washed and infected with DENV-2. Both intracellular and extracellular virus production, measured at 48 h p.i., were similar in pre-treated and non-treated cultures. By contrast, when the compound was added at 0, 1, 3, 5 or 8 h p.i. extracellular virus production was reduced by approximately 90% whereas intracellular infectivity exhibited only a minor reduction when the compound was added at 0 or 1 h p.i. and no changes in the intracellular virus titers were detected when the compound was added beyond 1 h p.i. (Fig. 3A). We also confirmed that the addition of 9N-methylharmine at 1 h p.i. did not significantly affect viral RNA synthesis detected at 6 or 24 h p.i., whereas the presence of the reference drug ribavirin caused a marked reduction of DENV RNA synthesis (Fig. 3B).

In order to investigate whether 9N-methylharmine affects viral particle release or alternatively, non-infective viral particles were released to the extracellular medium in 9N-methylharmine treated cultures, we quantified the number of intracellular and extracellular viral genome copies by absolute quantitative RT-PCR. Whereas, non-significant difference in the number of intracellular viral genomes for control and 9N-methylharmine treated cultures was detected, significantly lower amounts of extracellular viral genomes were measured in 9N-methylharmine treated cultures with respect to control ( $p < 0.05$ ). Therefore, the ratio between intracellular and extracellular viral genomes was 4 fold higher in treated cells in comparison to control cells (Fig. 3C), indicating that the compound impairs the release of viral particles. On the other hand, the ratio between viral genomes and infectious viral particles in the extracellular medium of 9N-methylharmine treated cells was 3.6 fold higher than the ratio calculated from control supernatants (Fig. 3D), suggesting that besides the impairment in viral release, a higher proportion of non-infective released particles was present in the extracellular medium of treated cultures. Altogether these results suggest that treatment with 9N-methylharmine does not affect macromolecular synthesis but impairs the maturation and release of infectious virus to the extracellular space.

### 3.4. Effect of 9N-methylharmine on the spreading of DENV-2 infection

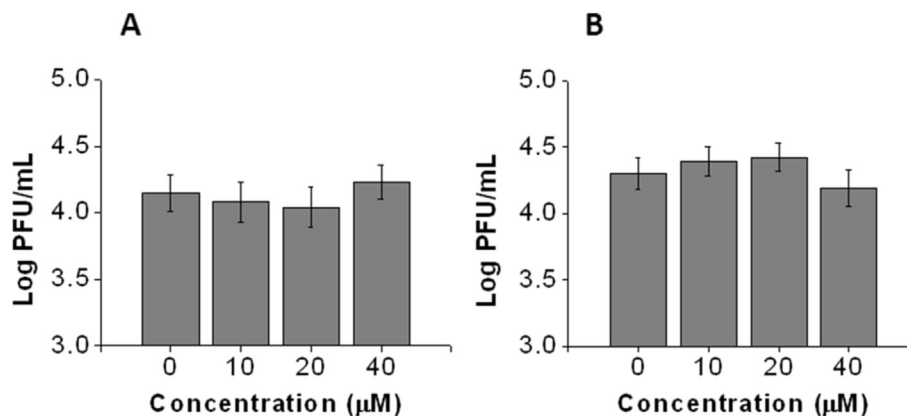
To further explore the effect of 9N-methylharmine on virus infection, we achieved an indirect immunofluorescence assay for the detection of viral E glycoprotein in DENV-2 infected cells treated with 9N-methylharmine from 1 h p.i. As Fig. 3E shows, the number of cells expressing viral glycoprotein at 48 h p.i. was reduced by 80.5% by 9N-methylharmine treatment confirming that the inhibition of virus release affects the spreading of infection.

### 3.5. Effect of 9N-methylharmine on DENV-induced activation of p38 mitogen activated protein kinase (MAPK) pathway

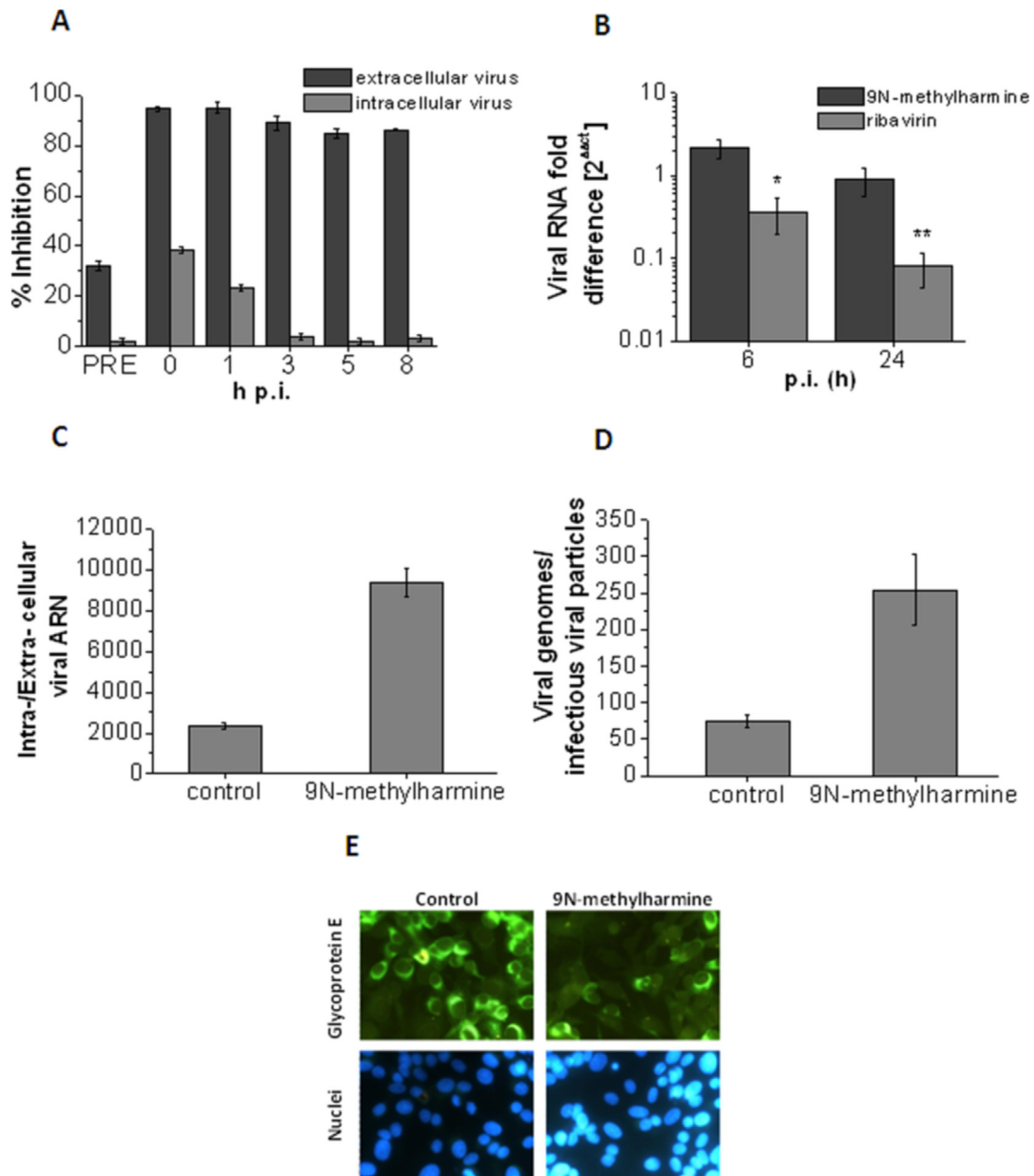
It has been recently reported that the  $\beta$ -carboline harmine blocks HSV infection by downregulation of p38 and c-Jun N-terminal kinase (JNK) MAPK cellular pathways (Chen et al., 2015). The activation of MAPK pathways during DENV infection has been described in different cell types (Albarnaz et al., 2014; Ceballos-Olvera et al., 2010; Fu et al., 2014; Huerta-Zepeda et al., 2008; Smith et al., 2014) and the requirement of p38 and JNK cascades for DENV multiplication has also been proposed (Ceballos-Olvera et al., 2010). Therefore, we decided to investigate whether 9N-methylharmine exerts its antiviral action through the inhibition of DENV-induced p38 phosphorylation. For this purpose, uninfected or DENV-2 infected cells were treated with different concentrations of 9N-methylharmine and after 24 h cell lysates were obtained and the level of p38 phosphorylation was assessed by western-blot. In the absence of the  $\beta$ -carboline derivative we showed that DENV-2 infection of Vero cells induces p38 activation since the level of p38 phosphorylation increased in untreated infected cells in comparison to untreated uninfected cultures (Fig. 4). Treatment with 9N-methylharmine (40  $\mu$ M) seems to affect basal levels of p38 phosphorylation in uninfected cells; by contrast p38 phosphorylation in DENV-2 infected cells is not affected by the compound either at a concentration that does not inhibit viral multiplication (1  $\mu$ M) or at inhibitory concentrations of 9N-methylharmine (10 and 40  $\mu$ M) (Fig. 4), indicating that the antiviral activity of 9N-methylharmine is not related to its ability to downregulate p38 activation.

## 4. Discussion

Despite the increasing risk of DENV infection in tropical and subtropical regions of the world there is no available antiviral



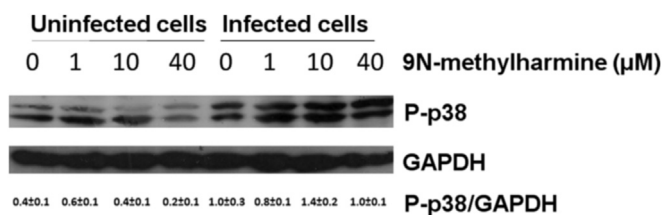
**Fig. 2. Virucidal activity of the active  $\beta$ -carbolines.** To assay virucidal activity of the compounds DENV-2 was incubated with harmol (A) or 9N-methylharmine (B) at different concentrations for 2 h at 37 °C. Remaining infectivity was determined by plaque assay in Vero cells. Viral titers are mean values from three replicates of one representative experiment  $\pm$ 95% CI.



**Fig. 3. Mode of action of 9N-methylharmine on DENV-2 infection.** (A) Vero cells were infected and 9N-methylharmine (40  $\mu$ M) was added at different times p.i. At 48 h p.i. intracellular and extracellular virus yields were determined by plaque assay. Data are mean values from three independent experiments  $\pm$  SD. (B) Vero cells were infected with DENV-2 (MOI = 1) and after 1 h at 37  $^{\circ}$ C cells were covered with MM containing 9N-methylharmine (40  $\mu$ M), or ribavirin (80  $\mu$ M). At 6 and 24 h p.i. total RNA was extracted and real-time RT-PCR was performed to determine the relative amount of viral RNA with respect to untreated infected cultures, using DENV-2 3' UTR specific primers and cellular actin amplification for normalization. Data are mean values from three independent experiments  $\pm$  SD. \* $p$  < 0.05, \*\* $p$  < 0.01. (C) Vero cells were infected with DENV-2 (MOI = 1) and after 1 h at 37  $^{\circ}$ C cells were covered with MM (control) or MM containing 9N-methylharmine (40  $\mu$ M). At 24 h p.i. intracellular and extracellular viral genome copies were quantified by absolute quantitative RT-PCR. Data represent the ratio between intra- and extra-cellular viral RNA from three independent determinations  $\pm$  SD (D) Viral RNA and infectivity from supernatants of untreated cultures (control) or cultures treated with 9N-methylharmine (40  $\mu$ M) were measured by real time RT-PCR and plaque assay, respectively. Data represent the ratio between viral genomes and viral infectious particles from three independent determinations  $\pm$  SD (E) Vero cells were infected with DENV-2 (MOI = 1) and after 1 h of infection MM or MM containing 9N-methylharmine (40  $\mu$ M) was added. At 48 h p.i. supernatants were removed, cultures were fixed with methanol and cells expressing viral E glycoprotein were detected by an indirect immunofluorescence assay. Cell nuclei were stained with Hoechst. Magnification 400 $\times$ .

chemotherapy to face dengue (Guzman and Harris, 2015; John et al., 2015). Even though the inhibitory effect of  $\beta$ -carbolines on the multiplication of several viruses has been reported, there has been no research into the potential of these compounds as antiviral agents against DENV. In the present study we evaluated the antiviral activity of 4 natural  $\beta$ -carbolines and 8 synthetic derivatives against DENV-2 in Vero cell cultures. We found that harmol and 9N-methylharmine inhibit DENV-2 multiplication and lack direct inactivating effect on DENV-2 particles.

Up to now, the mechanism of antiviral action of natural or synthetic  $\beta$ -carbolines has been scarcely investigated. A synthetic  $\beta$ -carboline analogue bearing a substituted carbohydrate group at C-3 and a phenyl-substituted group at C-1 inhibits PV replication when added after virus adsorption and displays no virucidal activity. On the contrary, another structurally related derivative prevents HSV multiplication when it is present during viral adsorption and exhibits virucidal effect (Nazari Fromagio et al., 2009). On the other hand, the natural  $\beta$ -carbolines harmine and harmaline exert



**Fig. 4. Effect of 9N-methylharmine on DENV-2 induced p38 phosphorylation.** Uninfected or DENV-2 infected Vero cells were treated with different concentrations of 9N-methylharmine during 24 h and p38 activation was analyzed based on the levels of phosphorylated p38 (P-p38) assessed by western blot using GAPDH as loading control. The relative intensity of P-p38 bands with respect to GAPDH band for each sample is shown. Each value represents the mean  $\pm$  SD of three independent experiments.

their anti-HSV activity by downregulation of immediate-early transcriptional events (Bag et al., 2014; Chen et al., 2015). Although there are no reports about the antiviral activity of this kind of alkaloids against DENV, structural related compounds such as synthetic  $\gamma$ -carbolines were proved to inhibit the replication of the flavivirus BVDV. The fact that  $\gamma$ -carbolines were inactive against BVDV mutants resistant to viral RNA polymerase (NS5B) inhibitors and the probable interaction between active  $\gamma$ -carbolines and NS5B revealed by docking studies suggest that the compounds may target viral RNA synthesis (Salim et al., 2010).

Our study showed a clearly different mode of action of 9N-methylharmine against DENV-2. This compound does not interfere either with initial events of viral multiplication cycle or with viral RNA synthesis. Furthermore, time course studies revealed that intracellular infectivity was only slightly affected by treatment with the derivative. On the contrary, extracellular virus production was reduced even when the compound was added at 8 h p.i. Lower amounts of viral genomes and a higher proportion of non-infective DENV particles were quantified in the supernatants of 9N-methylharmine treated cultures in comparison to untreated ones, indicating that not only viral particle release but also viral particle maturation is affected by the compound.

Viruses take advantage of cellular machinery to promote their assembly and release.

DENV assembly takes place in the endoplasmic reticulum and nascent viral particles need to be transported to the Golgi apparatus. Cellular trafficking machinery is involved in virus assembly followed by transportation of viral particles through the exocytic pathway to the extracellular space. During the secretion process, prM and E viral proteins interact with cellular factors and the cleavage of prM by furin is necessary for virus maturation and infectivity (Wang et al., 2014). The ADP-ribosylation factor (Arf) family of proteins and Rab 11, which belong to the Ras superfamily of small GTPases, were proved to be important for efficient secretion of DENV particles (Kudelko et al., 2012; Wang et al., 2009). A possible explanation for our results is that 9N-methylharmine would exert its antiviral action by interfering with these or other still unknown virus-cellular factor interactions that promote viral maturation and the formation of transport vesicle containing mature DENV particles.

Although 9-N-methylharmine and harmol were inhibitory of the four DENV serotypes, a dissimilar serotype susceptibility was observed, with increased EC<sub>50</sub> values for DENV-1, DENV-3 and DENV-4 viruses in comparison to DENV-2, the most susceptible one (Tables 1 and 3). Similarly, a variation of antiviral effect according to virus serotype was also reported for antibiotic derivatives (Kaptein et al., 2010; Rothan et al., 2014) and sulfated polysaccharides (Hidari et al., 2008; Talarico et al., 2005; Talarico and Damonte, 2007) affecting cell entry as well as for compounds targeted to

viral or cellular components that interfere with DENV RNA synthesis such as NS4B inhibitors (Wang et al., 2015) and inosine monophosphate dehydrogenase (IMPDH) inhibitors (Mazzucco et al., 2015; Sepúlveda et al., 2008), respectively. The inhibitory effect of celgosivir, an  $\alpha$ -1 glucosidase 1 inhibitor, was also found to be differently modulated by diverse DENV-2 virus strains in HuH-7, Vero and THP-1 cells whereas the efficacy is comparable among all virus strains in BHK-21 cells (Watanabe et al., 2016). Although it has been suggested that all these variable antiviral responses may be related to a serotype/strain-specific differential interaction with viral or cellular factors during infection, the mechanistic basis for these differences is not clear at this moment and further studies are required to elucidate it.

On the other hand, in contrast to the results obtained with harmine in HSV infection (Chen et al., 2015) no reduction in p38 levels of phosphorylation was observed in DENV-infected cells treated with inhibitory concentrations of 9N-methylharmine. Therefore, the antiviral action of the  $\beta$ -carboline derivative would not be due to the modulation of this signaling cascade.

In conclusion we identified two  $\beta$ -carbolines, harmol and the synthetic derivative 9N-methylharmine, that exhibit inhibitory action against DENV. Both compounds are not virucidal agents and our results indicate that 9N-methylharmine exerts its antiviral action by interfering with virus maturation and egress. Further investigation of  $\beta$ -carbolines by performing a structure-activity analysis of new derivatives obtained from the active compounds and additional study of their mechanism of action would represent a promising approach for the development of novel antiviral agents to deal with DENV infections.

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## References

- Albarnaz, J.D., De Oliveira, L.C., Torres, A.A., Palhares, R.M., Casteluber, M.C., Rodrigues, C.M., Cardozo, P.L., De Souza, A.M., Pacca, C.C., Ferreira, P.C., Kroon, E.G., Nogueira, M.L., Bonjardim, C.A., 2014. MEK/ERK activation plays a decisive role in yellow fever virus replication: implication as an antiviral therapeutic target. *Antivir. Res.* 111, 82–92.
- Alvarez, D.E., De Lella Ezcurra, A.L., Fucito, S., Gamarnik, A.V., 2005. Role of RNA structures present at the 3'UTR of dengue virus on translation, RNA synthesis, and viral replication. *Virology* 339, 200–212.
- Ashok, P., Chander, S., Balzarini, J., Pannecouque, C., Murugesan, S., 2015. Design, synthesis of new  $\beta$ -carboline derivatives and their selective anti-HIV-2 activity. *Bioorg. Med. Chem. Lett.* 25, 1232–1235.
- Bag, P., Ojha, D., Mukherjee, H., Halder, U.C., Mondal, S., Biswas, A., Sharon, A., Van Kaer, L., Chakrabarty, S., Das, G., Mitra, D., Chattopadhyay, D., 2014. A dihydro-pyrido-indole potently inhibits HSV-1 infection by interfering the viral immediate early transcriptional events. *Antivir. Res.* 105, 126–134.
- Begum, S., Hassan, S.I., Siddiqui, B.S., 2004. Synthesis and antimycobacterial activity of some beta-carboline alkaloids. *Nat. Prod. Res.* 18, 341–347.
- Brahmbhatt, K.G., Ahmed, N., Sabde, S., Mitra, D., Singh, I.P., Bhutani, K.K., 2010. Synthesis and evaluation of  $\beta$ -carboline derivatives as inhibitors of human immunodeficiency virus. *Bioorg. Med. Chem. Lett.* 20, 4416–4419.
- Cao, R., Chen, Q., Hou, X., Chen, H., Guan, H., Ma, Y., Peng, W., Xu, A., 2004. Synthesis, acute toxicities, and antitumor effects of novel 9-substituted beta-carboline derivatives. *Bioorg. Med. Chem.* 12, 4613–4623.
- Cao, R., Chen, H., Peng, W., Ma, Y., Hou, X., Guan, H., Liu, X., Xu, A., 2005. Design, synthesis and in vitro and in vivo antitumor activities of novel beta-carboline derivatives. *Eur. J. Med. Chem.* 40, 991–1001.
- Cao, R., Peng, W., Wang, Z., Xu, A., 2007.  $\beta$ -carboline alkaloids: biochemical and

- pharmaceutical functions. *A. Curr. Med. Chem.* 14, 479–500.
- Castilla, V., Piccini, L.E., Damonte, E.B., 2015. Dengue virus entry and trafficking: perspectives as antiviral target for prevention and therapy. *Future Virol.* 10, 625–645.
- Ceballos-Olvera, I., Chávez-Salinas, S., Medina, F., Ludert, J.E., del Angel, R.M., 2010. JNK phosphorylation, induced during dengue virus infection, is important for viral infection and requires the presence of cholesterol. *Virology* 396, 30–36.
- Chen, D., Su, A., Fu, Y., Wang, X., Lv, X., Xu, W., Xu, S., Wang, H., Wu, Z., 2015. Harmine blocks herpes simplex virus infection through downregulating cellular NF- $\kappa$ B and MAPK pathways induced by oxidative stress. *Antivir. Res.* 123, 27–38.
- Fu, Y., Yip, A., Seah, P.G., Blasco, F., Shi, P.Y., Hervé, M., 2014. Modulation of inflammation and pathology during dengue virus infection by p38 MAPK inhibitor SB203580. *Antivir. Res.* 110, 151–157.
- Guzman, M.G., Harris, E., 2015. Dengue. *Lancet* 385, 453–465.
- Hidari, K.I.P.J., Takahashi, N., Arihara, M., Nagaoka, M., Morita, K., Suzuki, T., 2008. Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga. *Biochem. Biophys. Res. Comm.* 376, 91–95.
- Huerta-Zepeda, A., Cabello-Gutiérrez, C., Cime-Castillo, J., Monroy-Martínez, V., Manjarrez-Zavala, M.E., Gutiérrez-Rodríguez, M., Izaguirre, R., Ruiz-Ordaz, B.H., 2008. Crosstalk between coagulation and inflammation during Dengue virus infection. *Thromb. Haemost.* 99, 936–943.
- John, D.V., Lin, Y.S., Perng, G.C., 2015. Biomarkers of severe dengue disease - a review. *J. Biomed. Sci.* 22, 83.
- Kaptein, S.J.F., De Burghgraave, T., Froeyen, M., Pastorino, B., Alen, M.M.F., Mondotte, J.A., Herdewijn, P., Jacobs, M., de Lammallerie, X., Schols, D., Gamarnik, A.V., Sztaricskai, F., Neyts, J., 2010. A derivative of the antibiotic doxorubicin is a selective inhibitor of dengue and yellow fever virus replication in vitro. *Antimicrob. Agents Chemother.* 54, 5269–5280.
- Khan, F.A., Maalik, A., Iqbal, Z., Malik, I., 2013. Recent pharmacological developments in  $\beta$ -carboline alkaloid "harmaline". *Eur. J. Pharmacol.* 721, 391–394.
- Kudelko, M., Brault, J.B., Kwok, K., Li, M.Y., Pardigon, N., Peiris, J.S., Bruzzone, R., Després, P., Nal, B., Wang, P.G., 2012. Class II ADP-ribosylation factors are required for efficient secretion of dengue viruses. *J. Biol. Chem.* 287, 767–777.
- Liu, Y., Song, H., Huang, Y., Li, J., Zhao, S., Song, Y., Yang, P., Xiao, Z., Liu, Y., Li, Y., Shang, H., Wang, Q., 2014. Design, synthesis, and antiviral, fungicidal, and insecticidal activities of tetrahydro- $\beta$ -carboline-3-carbohydrazide derivatives. *J. Agric. Food Chem.* 62, 9987–9999.
- Maeto, C.A., Knott, M.E., Linero, F.N., Ellenberg, P.C., Scolaro, L.A., Castilla, V., 2011. Differential effect of acute and persistent Junin virus infections on the nucleocytoplasmic trafficking and expression of heterogeneous nuclear ribonucleoproteins type A and B. *J. Gen. Virol.* 92, 2181–2190.
- Mazzucco, M.B., Talarico, L.B., Vatansever, S., Carro, A.C., Fascio, M.L., D'Accorso, N.B., García, C.C., Damonte, E.B., 2015. Antiviral activity of an N-allyl acridone against dengue virus. *J. Biomed. Sci.* 22, 29.
- Moloudizargari, M., Mikaili, P., Aghajanshakeri, S., Asghari, M.H., Shayegh, J., 2013. Pharmacological and therapeutic effects of Peganum harmala and its main alkaloids. *Pharmacogn. Rev.* 7, 199–212.
- Nazari Formaggio, A.S., Santos, P.R., Zanoli, K., Ueda-Nakamura, T., Düsman Tonin, L.T., Nakamura, C.V., Sarragiotto, M.H., 2009. Synthesis and antiviral activity of  $\beta$ -carboline derivative bearing a substituted carbohydrazide at C-3 against poliovirus and herpes simplex virus (HSV-1). *Eur. J. Med. Chem.* 44, 4695–4702.
- Nenaah, G., 2010. Antibacterial and antifungal activities of (beta)-carboline alkaloids of Peganum harmala (L) seeds and their combination effects. *Fitoterapia* 81, 779–782.
- Patel, K., Gadewar, M., Tripathi, R., Prasad, S.K., Patel, D.K., 2012. A review on medicinal importance, pharmacological activity and bioanalytical aspects of beta-carboline alkaloid 'harmine'. *Asian Pac. J. Trop. Biomed.* 2, 660–664.
- Rothan, H.A., Mohamed, Z., Paydar, M., Rahman, N.A., Yusof, R., 2014. Inhibitory effect of doxycycline against dengue virus replication in vitro. *Arch. Virol.* 159, 711–718.
- Sako, K., Aoyama, H., Sato, S., Hashimoto, Y., Baba, M., 2008. Gamma-carboline derivatives with anti-bovine viral diarrhoea virus (BVDV) activity. *Bioorg. Med. Chem.* 16, 3780–3790.
- Salim, M.T., Goto, Y., Hamasaki, T., Okamoto, M., Aoyama, H., Hashimoto, Y., Musiu, S., Paeshuyse, J., Neyts, J., Froeyen, M., Herdewijn, P., Baba, M., 2010. Highly potent and selective inhibition of bovine viral diarrhoea virus replication by  $\gamma$ -carboline derivatives. *Antivir. Res.* 88, 263–268.
- Sepúlveda, C.S., Fascio, M.L., Mazzucco, M.B., Docampo Palacios, M.L., Pellón, R.F., García, C.C., D'Accorso, N.B., Damonte, E.B., 2008. Synthesis and evaluation of N-substituted acridones as antiviral agents against haemorrhagic fever viruses. *Antivir. Chem. Chemother.* 19, 41–47.
- Simmons, C.P., McPherson, K., Van Vinh Chau, N., Hoai Tam, D.T., Young, P., Mackenzie, J., Wills, B., 2015. Recent advances in dengue pathogenesis and clinical management. *Vaccine* S0264–410X: 01393–01396.
- Smith, J.L., Stein, D.A., Shum, D., Fischer, M.A., Radu, C., Bhinder, B., Djaballah, H., Nelson, J.A., Früh, K., Hirsch, A.J., 2014. Inhibition of dengue virus replication by a class of small-molecule compounds that antagonize dopamine receptor d4 and downstream mitogen-activated protein kinase signaling. *J. Virol.* 88, 5533–5542.
- Song, H.J., Liu, Y.X., Liu, Y.X., Huang, Y.Q., Li, Y.Q., Wang, Q.M., 2014. Design, synthesis, anti-TMV, fungicidal, and insecticidal activity evaluation of 1,2,3,4-tetrahydro- $\beta$ -carboline-3-carboxylic acid derivatives based on virus inhibitors of plant sources. *Bioorg. Med. Chem. Lett.* 24, 5228–5233.
- Talarico, L.B., Pujol, C.A., Zibetti, R.G.M., Faria, P.C.S., Nosedá, M.D., Duarte, M.E.R., Damonte, E.B., 2005. The antiviral activity of sulfated polysaccharides against dengue virus is dependent on virus serotype and host cell. *Antivir. Res.* 66, 103–110.
- Talarico, L.B., Damonte, E.B., 2007. Interference in dengue virus adsorption and coating by carrageenans. *Virology* 363, 473–485.
- van Maarseveen, J.H., Scheeren, H.W., Clercq, E., Balzarini, J., Kruse, C.G., 1999. Antiviral and tumor cell antiproliferative SAR studies on tetracyclic eudistomins-II. *Bioorg. Med. Chem.* 5, 955–970.
- Wang, P.G., Kudelko, M., Lo, J., Siu, L.Y., Kwok, K.T., Sachse, M., Nicholls, J.M., Bruzzone, R., Altmeyer, R.M., Nal, B., 2009. Efficient assembly and secretion of recombinant subviral particles of the four dengue serotypes using native prM and E proteins. *PLoS One* 4, e8325.
- Wang, P.G., Kudelko, M., Kwok, K.T., Bruzzone, R., Nal, B., 2014. Cellular enhancing and restricting factors of dengue virus egress. *Hong Kong Med. J.* 20, 44–46.
- Wang, Q.-Y., Dong, H., Zou, B., Karuna, R., Wan, K.F., Zou, J., Susila, A., Shan, C., Yeo, K.L., Xu, H., Chan, W.L., Gu, F., Seah, P.G., Liu, W., Lakshminarayana, S.B., Kang, C., Lescar, J., Blasco, F., Smith, P.W., Shi, P.-Y., 2015. Discovery of dengue virus NS4B inhibitors. *J. Virol.* 89, 8233–8244.
- Wang, Y.-H., Tang, J.-G., Wang, R.-R., Yang, L.-M., Dong, Z.J., Du, L., Shen, X., Liu, J.-K., Zheng, Y.-T., 2007. Flazinamide, a novel  $\beta$ -carboline compound with anti-HIV actions. *Bioorg. Biophys. Res. Comm.* 355, 1091–1095.
- Watanabe, S., Chan, K.W.-K., Dow, G., Ooi, E.E., Low, J.G., Vasudevan, S.G., 2016. Optimizing celgosivir therapy in mouse models of dengue virus infection of serotypes 1 and 2: the search for a window for potential therapeutic efficacy. *Antivir. Res.* 127, 10–19.
- Whitehorn, J., Yacoub, S., Anders, K.L., Macareo, L.R., Cassetti, M.C., Nguyen Van, V.C., Shi, P.Y., Wills, B., Simmons, C.P., 2014. Dengue therapeutics, chemoprophylaxis, and allied tools: state of the art and future directions. *PLoS. Negl. Trop. Dis.* 8, e3025.