International Journal of Peptide Research and Therapeutics (2020) 26:811–821
https://doi.org/10.1007/s10989-019-09888-2

Smp76, a Scorpine-Like Peptide Isolated from the Venom of the Scorpion *Scorpio maurus palmatus*, with a Potent Antiviral Activity Against Hepatitis C Virus and Dengue Virus

Alaa M. H. El-Bitar[1,2] · Moustafa Sarhan[1,2] · Mohamed A. Abdel-Rahman[4] · Veronica Quintero-Hernandez[5,6] · Chie Aoki-Utsubo[3] · Mohsen A. Moustafa[1] · Lourival D. Possani[5] · Hak Hotta[2,3]

Accepted: 2 July 2019 / Published online: 6 July 2019
© Springer Nature B.V. 2019

Abstract

Growing global viral infections have been a serious public health problem in recent years. This current situation emphasizes the importance of developing more therapeutic antiviral compounds. Hepatitis C virus (HCV) and dengue virus (DENV) belong to the *Flaviviridae* family and are an increasing global health threat. Our previous study reported that the crude venom of *Scorpio maurus palmatus* possessed anti-HCV and anti-DENV activities in vitro. We report here the characterization of a natural antiviral peptide (scorpion-like peptide Smp76) that prevents HCV and DENV infection. Smp76 was purified from *S. m. palmatus* venom and contains 76 amino acids with six residues of cysteine. Smp76 antiviral activity was evaluated using a cell culture technique utilizing Huh7it-1, Vero/SLAM, HCV (JFH1, genotype 2a) and DENV (Trinidad 1751, type 2). A potential antiviral activity of Smp76 was detected in culture cells with an approximate IC_{50} of 0.01 µg/ml. Moreover, Smp76 prevents HCV infection and suppresses secondary infection, by inactivating extra-cellular infectious particles without affecting viral replication. Interestingly, Smp76 is neither toxic nor hemolytic in vitro at a concentration 1000-fold higher than that required for antiviral activity. Conclusively, this report highlights novel anti-HCV and anti-DENV activities of Smp76, which may lay the foundation for developing a new therapeutic intervention against these flaviviruses.

Keywords Animal venom · Antiviral peptide · DENV · HCV · *Scorpio maurus palmatus* · Smp76

✉ Moustafa Sarhan
msarhan@azhar.edu.eg

✉ Mohamed A. Abdel-Rahman
mohamed_hassanain@science.suez.edu.eg

✉ Hak Hotta
hotta@kobe-u.ac.jp

Alaa M. H. El-Bitar
elbitar@azhar.edu.eg

Veronica Quintero-Hernandez
vquinterohe@conacyt.mx

Chie Aoki-Utsubo
nu_chie@people.kobe-u.ac.jp

Mohsen A. Moustafa
mohsenamoustafa@azhar.edu.eg

Lourival D. Possani
possani@ibt.unam.mx

[1] Zoology Department, Faculty of Science, Al-Azhar University, Assiut, Egypt

[2] Department of Microbiology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

[3] Department of International Health, Kobe University Graduate School of Health Sciences, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan

[4] Zoology Department, Faculty of Science, Suez Canal University, Ismailia 41522, Egypt

[5] Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad, 2001, Colonia Chamilpa, Apartado Postal 510-3, 62210 Cuernavaca, Morelos, Mexico

[6] CONACYT-Laboratorio de Ecología Molecular Microbiana, Centro de Investigaciones en Ciencias Microbiológicas-Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, Ciudad Universitaria, C.P. 72570 Puebla, Mexico

Introduction

Hepatitis C virus (HCV) is a single-stranded RNA viruses that belongs to family Flaviviridae (Mohammed et al. 2013; Supanee et al. 2014). Around 150 million people worldwide are chronically infected with HCV and the annual mortality from HCV-related liver diseases reach up to 700,000 individual (Ministry of Health and 2015; World Health Organization 2016; Jefferies et al. 2018). In the past decade, interferon-based therapy was the gold standard for HCV treatment with a sustained virological response (SVR) rate hovering around 50%. The recent approval of oral direct-acting antivirals (DAAs), like HCV NS3 protease inhibitors, NS5A inhibitors and NS5B RNA-dependent RNA polymerase inhibitors, for clinical use improved the SVR rates to more than 90% (Pawlotsky 2016; Falade-Nwulia et al. 2017). Nevertheless, cirrhosis patients remain at risk for severe complications. In addition, treatment with DAAs is not affordable for many patients and they are still not readily available around the globe. Therefore, uncovering novel HCV inhibitors is still a clinical priority.

Dengue virus (DENV) is another single-stranded RNA Flaviviridae virus that is transmitted by mosquitoes causing dengue fever (Rodenhuis-Zybert et al. 2010). DENV is currently endemic in more than 100 countries with the highest prevalence in South-East Asia, Africa and the Americas (Mackenzie et al. 2004; Malavige et al. 2004; Deen et al. 2006; Bhatt et al. 2013). Each year, there are around 390 million DENV infections are recorded worldwide and among them 50 to 100 million patients are presented with the clinical manifestations of dengue fever (Bhatt et al. 2013). Dengue fever leads to 20,000 annual deaths, mainly in young children (Rui-feng et al. 2008). To date, four DENV serotypes, DENV-1, DENV-2, DENV-3 and DENV-4, have been identified and infections with one serotype does not offer protection from infection with the remaining three serotypes (Weaver and Vasilakis 2009; Messina et al. 2014; Mustafa et al. 2015). A major impediment to the development of vaccines is that vaccines should have a tetravalent effect, i.e. sufficient protective immune responses against all four DENV serotypes. Owing to the absence of specific treatments against DENV and the limitations of the available vaccine (Dengvaxia® or CYD-TDV), the global burden of DENV infection is becoming enormous (Behnam et al. 2016). Therefore, the development of new antiviral compounds against DENV infections is urgently needed.

Scorpion venom is a rich source for drug discovery and prototyping (Ortiz et al. 2015; Ghosh et al. 2019). Scorpion venoms are highly complex mixture of nucleotides, enzymes, mucoproteins, biogenic amines, nucleotides, salts, as well as peptides and proteins (Omran 2003; Rodriguez de la Vega and Possani 2005; Ozkan et al. 2006a, b, c; Feng et al. 2008; Kanoo and Deshpande 2008; Ortiz et al. 2015). Antimicrobial peptides (AMPs), isolated from several venomous animals, exhibit a wide range of antibacterial and antiviral activity with direct or indirect microbicide activity (Hv et al. 2006; Ortiz et al. 2015). Several studies demonstrated an antiviral effect for certain scorpion venom peptides (Carballar-Lejarazu et al. 2008; El-Bitar et al. 2015; Ortiz et al. 2015). In this study, we report the molecular and functional characterization of a new antiviral peptide (Smp76), a scorpion-like peptide derived from an Egyptian scorpion's venom, *S. m. palmatus*. Our findings will broaden the currently known antiviral peptides and open a new avenue for the development of novel HCV and DENV therapies.

Materials and Methods

Collection of Scorpions and Venom Preparation

Adult *S. m. palmatus* scorpions were collected from the Western Coastal Mediterranean Desert (Alexandria Governorate, Egypt) and were housed individually in clear plastic containers. Scorpions were fed small insects and were given water. Crude venom was extracted using electrical stimulation (20 V) and the milked venom was collected and centrifuged for 20 min at 13,000 rpm/4 °C as detailed previously (Abdel-Rahman et al. 2013). Clear supernatants were pooled, freeze-dried and stored at − 20 °C until use. Venom samples were dissolved in bi-distilled water and the total protein concentration was determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) according to the standard protocols.

Cell Culture and Virus Production

The human hepatoma-derived cell line, Huh7it-1, was cultured in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with non-essential amino acids (Invitrogen, Carlsbad, CA, USA), fetal bovine serum (Biowest, Nuaille, France), streptomycin (100 µg/ml) and penicillin (100 IU/ml) (Invitrogen) in a 5% CO_2 incubator at 37 °C (Aoki et al. 2014). Huh7it-1 cells were infected with cell culture-adapted HCV (JFH1 strain of genotype 2a) and supernatants were collected at day 3 post-infection (Wakita et al. 2005; Yu et al. 2010). Next, supernatants were, concentrated by 100 K Amicon centrifugal filters and used for antiviral screening.

DENV type 2 (Trinidad 1751 strain) (Hotta et al. 1983; Hotta and Homma 1994) was infected into Vero/SLAM cells (Ono et al. 2001). Following an hour of virus adsorption, the

virus-infected cells were cultured with DMEM medium containing 10% fetal bovine serum at 37 °C in 5% CO_2. Supernatants were collected at 3 to 5 days post-infection and stored at − 80 °C. Measles virus (K52 strain) was inoculated to Vero/SLAM cells and the culture supernatants was collected from the virus-infected cells as described previously (Otaki et al. 2006).

Cytotoxicity Assay

The cytotoxicity of Smp76 was estimated using WST-1 assay as described previously with a some modification (Deng et al. 2008). Briefly, Huh7it-1 cells seeded in 96-well plate (2.5×10^4 cells/well) were treated with serial dilutions of Smp76 (0.1 to 10 µg/ml) or medium (control) for 48 h at 37 °C in 5% CO_2. Then, supernatants were discarded and replaced with fresh DMEM medium containing 10 µl of WST-1 reagent (Roche, Mannheim, Germany) and incubated for 4 h. The number of viable cells was quantified by using a microplate reader at 450 and 630 nm. For each dilution, the percentage of viable cells were compared to the control sample and used to calculate the 50% cytotoxic concentrations (CC_{50}) values according to the following formula:

Absorbance of sample/Absorbance of control × 100.

Hemolysis Assay

Hemolytic activity of Smp76 was performed as previously described (Evans et al. 2013). Briefly, a total of 10 µl of Smp76 peptide was mixed with 190 µl of diluted human red blood cells (RBCs) to achieve a final dilution 1/20 of the original venom peptide per well. Alternatively, the RBCs were incubated with 200 µl of 0.5% Triton X-100 or PBS to serve as both positive and negative controls, respectively. After an hour incubation period at 37 °C, the plate was centrifuged for 5 min at 500×*g* and 100 µl of supernatant was transferred to a clear 96-well plate. The released hemoglobin was measured on a microplate reader at 400:541 nm. The percentage of hemolysis was calculated relative to the positive control (0.5% Triton X100). The hemolysis concentration (HC_{50}) value was defined as the peptide concentration that can lyse 50% of the RBCs.

Antiviral Activities of the Venom Fractions

Huh7it-1 cells were grown on coverslips (13-mm in diameter; 1.9×10^5 Cells/well) 1 day before viral infection. Different concentrations of the venom fractions were mixed with HCV at multiplicity of infection (MOI: 1) for 2 h at 37 °C. Then, the virus/venom fraction mixture was inoculated in Huh7it-1 cells for 2 h at 37 °C. Medium-treated virus and cells were used as controls. The percentage of inhibition for virus infectivity was compared to the control samples and the 50% inhibitory concentrations (IC_{50}) were calculated.

Virus Titration (Immunofluorescence Staining)

HCV infectivity was determined as described previously (Deng et al. 2008). In brief, Huh7it-1 cells, grown on glass coverslips, were incubated with tenfold serially diluted virus samples for 2 h; then, the cells were washed with free medium and cultured for another 24 h. Following fixation and permeabilization, Huh7it-1 cells were incubated for 1 h with the serum of HCV-infected patients, followed by FITC-conjugated goat anti-human IgG (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Finally, the cells were counterstained by Hoechst 33342 (Molecular Probes, Eugene, OR, USA) and mounted using Vectashield H-1000 reagent (Vector Laboratories, Inc. Burlingame, CA, USA). HCV antigen positive cells were counted under a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

For dengue virus infectivity, serially diluted venom fractions and Smp76 were mixed with fixed amount of DENV and incubated for 2 h at 37 °C. The virus/venom fractions mixture was inoculated for 2 h at 37 °C on Vero/SLAM cells. The cells were washed twice after the virus inoculation and incubated with a fresh medium for 24 h. The infected cells were incubated with mouse monoclonal antibody against dengue virus followed\Alexa Fluor A488 goat anti-mouse IgG (Life Technologies).

To determine the infectivity of measles virus, serially diluted Smp76 was mixed separately with fixed amount of measles virus and incubated for 2 h at 37 °C. Virus/venom fraction mixture was inoculated to Vero/SLAM cells for 2 h at 37 °C and the cells were washed twice then, incubated with fresh medium for 24 h. The plaques (virus-induced syncytia) forming on the infected monolayer cells were counted.

Virocidal Activity Assay

The Smp76 venom peptide was mixed with a fixed amount of HCV JFH1 for 2 h at 37 °C. Next, the virus/Smp76 mixture was inoculated to Huh7it-1 cells and incubated for 2 h at 37 °C. The cells were washed and cultured without Smp76 for 24 h. Finally, the cells were subjected to an indirect immunofluorescence assay as previously described (El-Bitar et al. 2015).

Immunoblot Analysis

Huh7it-1 cells were lysed in SDS sample buffer and equal amounts of protein were separated on a SDS–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (PVDF) (Millipore, Bedford, MA, USA). The PVDF membrane was blocked by 5% skim milk

and probed with anti-HCV NS3 antibody and anti-GAPDH antibody (Millipore). Followed by horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Invitrogen) as a secondary antibody and visualized using the enhanced chemiluminescence detection system (ECL; GE Healthcare, Buckinghamshire, UK).

Real-Time Quantitative RT-PCR

The amounts of HCV RNA in the infected cells were determines as described previously (El-Bitar et al. 2015). RNA was extracted by RNA cell miniprep system ReliaPrep (Promega, Madison, WI, USA). The cDNA was transcribed from one µg total RNA using a GoScript Reverse Transcription system (Promega) with oligo(dT) primers. Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara, Kyoto, Japan) in a MicroAmp 96-well reaction plate. PCR was conducted on a ABI PRISM 7500 fast system (Applied Biosystems, Foster City, CA, USA) with specific primers used to amplify the NS5A region of the HCV genome 5′-AGACGT ATTGAGGTCCATGC-3′ (sense) and 5′-CCGCAGCGA CGGTGCTGATAG-3′ (antisense). the expression of GAPDH mRNA was also measured as a housekeeping gene using the 5′- GCCATCAATGACCCCTTCATT-3′ (sense) and 5′ TCT CGCTCCTGGAAGATGG-3′primers.

RP-HPLC Fractionation of *S. m. palmatus* Venom

Chromatographic separation of *S. m. palmatus* venom was conducted using reverse phase high performance liquid chromatography (RP-HPLC; Waters, Milford, Massachusetts, United States) (Abdel-Rahman et al. 2013).A total of 4 mg scorpion venom was reconstituted in 200 ml 0.05% trifluoroacetic acid (TFA) and fractionated by a C18 RP-HPLC column (250 × 10 mm, 5 µm; Vydac, California, United States). A gradient of buffer A (0.12% TFA in MilliQ water) and sixty percent buffer B (0.10% TFA in acetonitrile) were used to separate scorpion venom in 1 h (1 ml/min flow rate). Individual venom fractions were collected manually according to the peak's absorbance (at 230 nm). All collected fractions were dried using a rotary evaporator (Savant Speed Vac SC210A, Minnesota, United States). The active fraction eluted at retention time 36.4 min was further characterized using mass spectrometry and amino acids sequencing. In addition, recombinant and synthetic Smp76 derivatives (N-terminal 32 aa and C-terminal 44 aa) were prepared as described below.

Determination of Molecular Mass and N-Terminal Sequencing of Native and Recombinant Smp76 Peptides

The average molecular mass of native Smp76 peptide (8398 Da), the recombinant fusion protein Thioredoxine-Smp76 (22123 Da) and a recombinant C-terminal of Smp76 (4775.57 Da) were determined using ESI–MS, ESI LCQ FLEET spectrometer (Thermo Scientific, CA, USA). The sequence of native Smp76 (approximately 250 pmol) was determined using Edman degradation (Protein Sequencer PPSQ-31A, Shimadzu Scientific Biotech, Maryland, United States). Synthetic N, C-terminal and full-length Smp76 peptides were manufactured by GenScript Japan Inc.

Construction of Recombinant Smp76C-Terminal Peptide (44 aa)

Six oligonucleotides were designed to cover the C-terminal region of Smp76 (44 aa) (Supplementary Table 1). The oligonucleotides BHEK-Dir1 and scSMP-LW5 included the *Bam*HI and *Xho*I restriction sites, respectively. Subsequently, this enabled the cloning into the pET22b-Thio-EK expression vector as detailed previously (Jiménez-Vargas et al. 2017; Vargas-Jaimes et al. 2017). PCR assembly of the C-terminal peptides was carried out using Vent DNA Polymerase (New England Biolabs, MA, United States). The final concentration of external primers BHEK-Dir1 and scSMP-LW5 was 0.2 pmol/µl while the concentration of internal oligonucleotides was 0.02 pmol/µl.

Expression and Purification of Recombinant C-Terminal (44 aa) Peptide of Smp76

In order to express the fusion protein Thioredoxine-C-terminal, pET22b-Thio-C-terminal plasmid was transformed into *E. coli* BL21 (DE3) using electroporation. The pellet was harvested and the fusion protein was purified using the Ni–NTA agarose resin columns (QIAGEN) as previously described (Vargas-Jaimes et al. 2017). HPLC purification was further performed using a C18 RP-HPLC column (250 × 10 mm, 5 µm; Vydac, California, United States). The purified fusion protein Thioredoxine-C-terminal was digested with enterokinase (New England Biolabs) in 200 mM Tris HCl (pH 8.0), 500 mM NaCl, 20 mM $CaCl_2$ for 16 h at 25 °C. Then, the pure recombinant C-terminal of Smp76 was finally isolated and purified by HPLC as described above.

Statistical Analysis

Data are presented as mean ± standard error of mean (SEM). The difference between data sets was determined by Student's two-tailed *t* test. A *P* value of < 0.05 was considered to be statistically significant.

Table 1 Anti-HCV and anti-DENV activities (IC_{50}) of selected fractions from the venom of the scorpion *S. m. palmatus*

No.	RT (min)	Protein conc. (mg/ml)	HCV IC_{50} (μg/ml)	DENV IC_{50} (μg/ml)	No.	RT (min)	Protein conc. mg/ml	HCV IC_{50} (μg/ml)	DENV IC_{50} (μg/ml)
1	3.3	0.496	>10.0	>10.0	38	23.2	ND	NT	NT
2	3.9	ND	NT	NT	39	24.1	1.819	>10.0	>10.0
3	4.5	ND	NT	NT	40	24.9	ND	NT	NT
4	5.2	ND	NT	NT	41	26.9	0.683	>10.0	>10.0
5	5.7	ND	NT	NT	42	27.1	1.886	>10.0	>10.0
6	6.9	ND	NT	NT	43	28.0	ND	NT	NT
7	8.4	1.037	>10.0	>10.0	44	28.4	1.926	>10.0	>10.0
8	9.0	ND	NT	NT	45	29.0	4.491	>10.0	>10.0
9	9.2	ND	NT	NT	46	29.8	ND	NT	NT
10	9.5	ND	NT	NT	47	30.7	0.804	>10.0	>10.0
11	9.7	ND	NT	NT	48	31.4	0.891	>10.0	>10.0
12	10.5	ND	NT	NT	49	31.6	0.533	>10.0	>10.0
13	11.4	ND	NT	NT	50	32.1	0.554	>10.0	>10.0
14	11.7	ND	NT	NT	51	32.2	ND	NT	NT
15	12.3	ND	NT	NT	52	33.0	ND	NT	NT
16	12.5	ND	NT	NT	53	33.5	ND	NT	NT
17	13.0	ND	NT	NT	54	33.7	ND	NT	NT
18	13.2	1.273	>10.0	>10.0	55	35.5	0.797	0.10	≤10
19	13.4	ND	NT	NT	56	36.2	3.773	0.05	NT
20	13.7	ND	NT	NT	57[a]	**36.4**	**4.462**	**0.01**	**0.01**
21	13.9	ND	NT	NT	58	37.9	0.619	0.10	NT
22	14.7	ND	NT	NT	59	38.7	0.971	1.0	1.0
23	15.0	ND	NT	NT	60	39.2	1.214	1.0	1.0
24	15.5	ND	NT	NT	61	40.8	0.648	1.0	1.0
25	15.7	4.446	>10.0	>10.0	62	41.2	ND	NT	NT
26	17.0	5.034	>10.0	>10.0	63	41.6	ND	NT	NT
27	17.4	2.132	>10.0	>10.0	64	42.2	0.226	1.0	3.0
28	18.0	3.209	>10.0	>10.0	65	42.9	ND	NT	NT
29	18.9	2.371	>10.0	>10.0	66	43.8	2.279	6.0	>10.0
30	19.5	ND	NT	NT	67	45.5	ND	NT	NT
31	20.0	ND	NT	NT	68	46.1	0.099	NT	NT
32	20.2	0.209	>10.0	>10.0	69	46.7	ND	NT	NT
33	20.5	1.692	>10.0	>10.0	70	48.2	ND	NT	NT
34	21.1	ND	NT	NT	71	48.7	0.501	NT	NT
35	21.5	ND	NT	NT	72	50.9	0.126	NT	NT
36	21.9	0.687	>10.0	>10.0	73	56.6	0.082	NT	NT
38	22.6	1.821	>10.0	>10.0	74	59.9	0.001	NT	NT

ND not detected, *NT* not tested

[a]Venom fraction containing Smp76

Results

Fractionation of Crude Venom Extract of *S. m. palmatus*

Since a whole *S. m. palmatus* soluble venom strongly inhibited HCV infectivity in vitro and displayed anti-HCV activity in cell culture (El-Bitar et al. 2015), the crude venom was fractionated to identify active molecule(s) with anti-HCV activity. Accordingly, 74 fractions from four milligrams of the venom were separated by HPLC analytical method (Fig. 1a; Table 1).

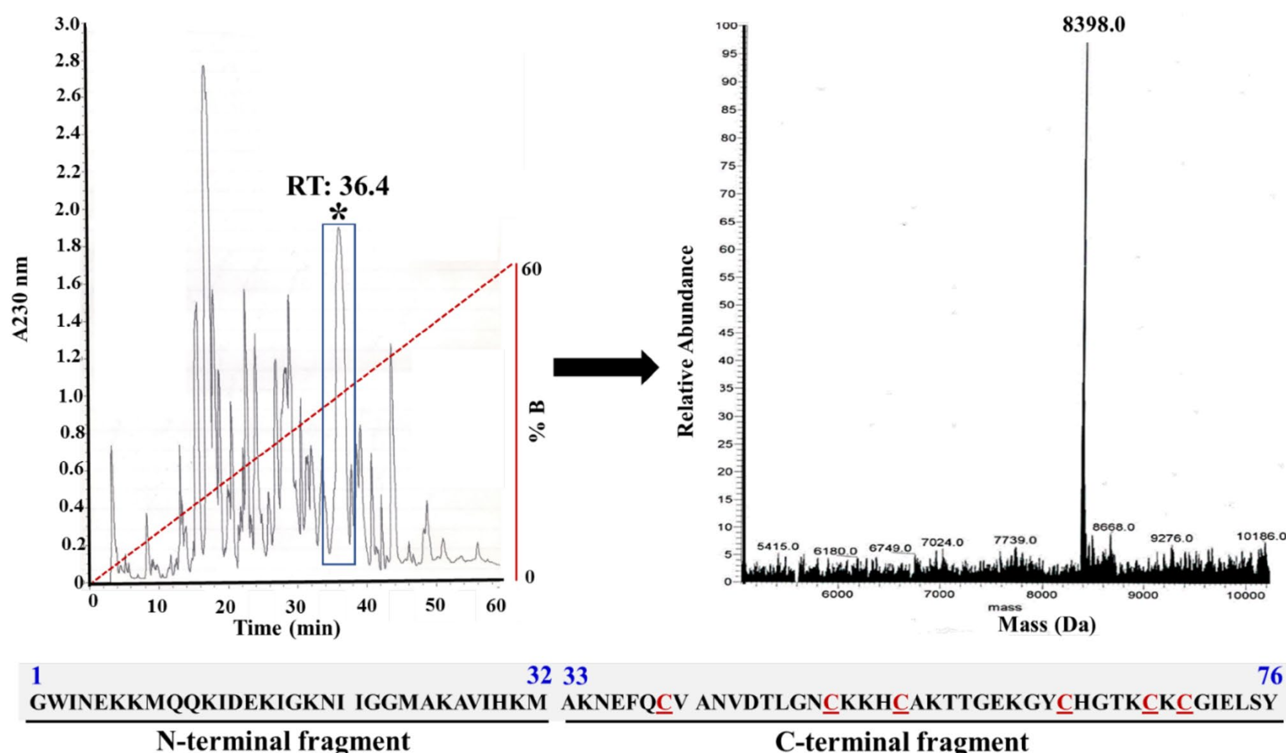

Fig. 1 Scorpion venom separation and characterization of Smp76. **a** Purification of Smp76 from the venom of *S. m. palmatus* using RP-HPLC. 4.0 mg crude venom was separated in a C18 analytical column with a gradient from buffer A (0.12% TFA) and 60% buffer B (ACN in 0.10% TFA) for an hour (Abdel-Rahman et al. 2013)74 fractions have been obtained and only the fraction eluted at RT 36.4 min (*) revealed strong antiviral activity against both HCV and DENV (IC_{50} 0.01 µg/ml). **b** Determination of the molecular mass of Smp76 (8398.0 Da) using LC–MS–ESI. **c** Automatic amino acids sequencing of Smp76 determined by Automated Edman Degradation. Cysteine residues are marked red and underlined (Color figure online)

Anti-HCV Activities of *S. m. palmatus* Venom Fractions

Subsequently, the anti-HCV activity of fractions obtained from the venom of *S. m. palmatus* were tested against JFH1 strain of genotype 2a. Based on protein concentrations, 30 fractions were tested for anti-HCV activity. The other fractions showed very low protein concentrations and, therefore, it was not possible to check their antiviral activity (Table 1). Each fraction was incubated separately with fixed amount of HCV for 2 h at 37 °C. Then, Huh7it-1 cells were infected with the virus/venom-fraction mixture (Fig. 2a) and virus infectivity was measured by infectious center assay. The anti-HCV activity started to appear from the retention time (RT) 35.5 until 43.8 min. The fraction at RT 36.4 min showed the most potent anti-HCV activity with IC_{50} being 0.01 µg/ml (Fig. 2b; Table 1).

Identification of Smp76 From the Active Fraction RT 36.4 min

In order to identify the bioactive compound(s) present in RT 36.4, LC–MS–ESI analysis was performed. Interestingly, the data of mass spectrometry showed that the active fraction contains a unique peptide with molecular mass of 8398 Da (Fig. 1b). To go further into the characterization of the active peptide, the amino acid sequence was determined (Fig. 1c). The sequence of this peptide contains 76 amino acids (GWINEKKMQQKIDEKIGKNIIGGMAKAVIHKMAKNEFQCVANVDTLGNCKKHCAKTTGEKGYCHGTKCKCGIELSY). The obtained sequence belongs to the scorpion venom antimicrobial peptides and matched with the scorpine-like peptide Smp76, which was identified in the scorpion venom gland of *S. m. palmatus* using transcriptomic analysis (Abdel-Rahman et al. 2013). The amino acid sequence of Smp76 was confirmed until the amino acid number 40 by Edman degradation method and the molecular mass was confirmed by mass spectrometry resulting in 8398 Da (see "Materials and Methods").

Cytotoxicity, Hemolytic Activity and Selectivity Index of Smp76

The cytotoxic activity of Smp76 against Huh7it-1 cells was tested using the WST-1 assay, and hemolytic activity was examined on human red blood cells. The CC_{50} and the HC_{50}

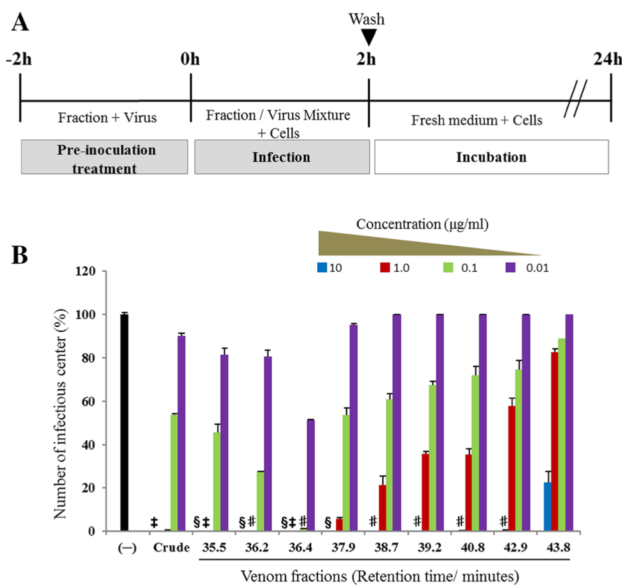

Fig. 2 Screening of anti-HCV activities of the venom fractions of *S. m. palmatus*. HCV (JFH1a strain) was treated with decreasing concentrations of the crude venom of *S. m. palmatus* and collected fractions (10, 1.0, 0.1 and 0.01 μg/ml) for 2 h or left untreated as a control (−) and then inoculated to Huh7it-1 cells and cultivated for 24 h. **a** Schematic of infection assay. **b** Amounts of HCV infectious particles. The data represents Mean ± SEM of two independent experiments. [§]Below the detection limit; [‡]≤ 0.07%; [#]< 0.5%

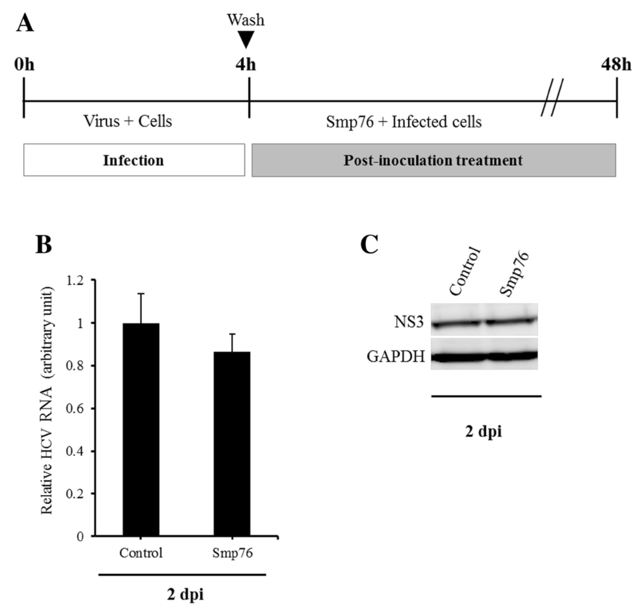

Fig. 3 Analysis of HCV RNA expression and NS3 protein accumulation. Huh7it-1 cells were infected with HCV and treated with Smp76 (0.1 μg/ml) to check its post-treatment effect or left untreated as a control (−) and both groups were incubated for 44 h post-infection. **a** Schematic of infection assay. **b** Amounts of HCV RNA in the cells. HCV RNA amounts were normalized to GAPDH mRNA expression. **c** HCV NS3 protein accumulation in the cells. GAPDH used as an internal control to verify equal amounts of sample loading. Data represents Mean ± SEM of two independent experiments

were calculated. As shown in Table 2, CC_{50} of Smp76 against Huh7it-1 cells and HC_{50} were > 10 μg/ml. These results indicate that this peptide has no cytotoxic or hemolytic effects up to 10 μg/ml with selectivity index (SI) > 1000.

Smp76 did not Inhibit the HCV Replication in Culture Cells

Since Smp76 displayed a significant inhibitory effect at the early stage of HCV infection, we examined whether the Smp76 peptide can also inhibit HCV NS3 protein production and HCV RNA replication in the cells. Virus at multiplicity of infection of 2 pfu/cell was inoculated to the Huh7it-1 cells for 3 ~ 4 h at 37 °C. After virus adsorption, the cells were cultured with media supplemented with 0.1 μg/ml of Smp76 for 44 h at 37 °C (Fig. 3a). The cells were harvested and subjected to immunoblot and RT-qPCR analyses. The results showed that the post-treatment of HCV RNA replication was not significantly inhibited (Fig. 3b) or HCV NS3 protein synthesis in the cells (Fig. 3c). The above results suggest that the Smp76 directly affects HCV particles and/or host cells in the culture medium to inhibit the viral infection and does not have an antiviral effect in the cells.

Table 2 Cytotoxicity (CC_{50}), hemolytic activity (HC_{50}) and selectivity index (SI) of Smp76

Venom peptide	CC_{50} (μg/ml)	HC_{50} (μg/ml)	SI
Smp76	>10	>10	>1000

Anti-DENV Activity of Smp76

We previously showed that the crude venom of *S. m. palmatus* inhibits DENV (El-Bitar et al. 2015). Therefore, anti-DENV activity of the selected 30-fractions obtained from the crude venom of *S. m. palmatus* was tested. Each fraction was incubated separately with fixed amount of DENV for 2 h at 37 °C. After that, the virus/venom fractions mixtures were used to infect Vero/SLAM cells and virus infectivity was measured by infectious center assay. Interestingly, the results were consistent with the data of anti-HCV activity obtained in this study (Table 1). Also, the fraction identified at 36.4 min which contains Smp76 showed the potent anti-DENV activity with IC_{50} being 0.01 μg/ml (Table 1 and Fig. 4b).

Specificity of Antiviral Activity of Smp76

To determine whether the antiviral activity of Smp76 peptide (previously described) was specific to HCV and DENV,

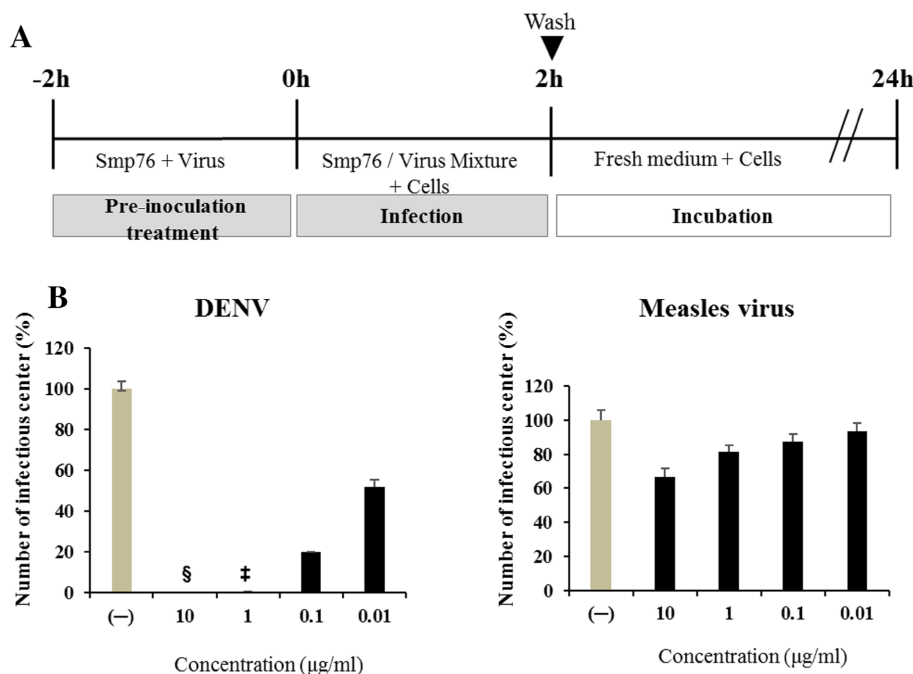

Fig. 4 Antiviral activity of Smp76 against dengue and measles viruses. Dengue and measles viruses were treated with a tenfold serial dilution of Smp76 (10, 1.0, 0.1 and 0.01 μg/ml) for 2 h or left untreated as a control (−) and then inoculated to Vero/SLAM cells. **a** Schematic of infection assay. **b** Amounts of DENV and measles virus infectious particles. The data represents Mean ± SEM of two independent experiments. §< 0.01%; ‡≤ 0.07% of the control

we tested its possible effects on another enveloped virus such as measles virus (Otaki et al. 2006). In this investigation, the virus was incubated with Smp76 (10–0.01 μg/ml) for 2 h. Then, Vero/SLAM cells were infected with the virus/Smp76 mixture (Fig. 4a) and virus infectivity was measured using an infectious center or plaque assay. The results revealed that while Smp76 peptide showed strong activity against DENV with IC_{50} 10 ng/ml, it induced weak inhibition on measles virus at 10 μg/ml (Fig. 4B).

Lack of Antiviral Activity of Smp76 Synthetic and Recombinant Derivatives (N- and C-Terminals)

In an attempt to identify the active domain of Smp76, the antiviral activity (anti-HCV and anti-DENV) of synthetic N-terminal (32 aa) and C-terminal (44aa without disulfide bonds) were tested. Although, the purified native Smp76 showed strong antiviral activity with IC_{50} of 10 ng/ml, there was no antiviral activity for both synthetic terminals (Table 3). Moreover, antiviral activity of recombinant C-terminal (44 aa) was examined. Also, no antiviral activity for recombinant C-terminal was detected (Table 3). These results indicate that the full-length of Smp76 may be required for its activity against HCV and DENV.

Lack of Antiviral Activity of Full-length Synthetic Smp76 Without Disulfide Bonds

The above mentioned results imply that the full-length of Smp76 may be required for its activity against HCV and DENV. The full-length Smp76 peptide (76 aa) was synthesized but without disulfide bonds. The antiviral activity of the synthesized Smp76 peptide was examined against HCV and DENV. These results showed no antiviral activity for the synthetic full-length peptide without disulfide bonds against HCV and DENV (Table 3).

Table 3 Antiviral activity of Smp76 and its derivatives

Source	Smp76/derivatives	IC_{50} (ng/ml)	
		Anti-HCV	Anti-DENV
Synthetic	N-terminal 32 aa	> 10,000	> 10,000
	C-terminal 44 aa (without disulfide bonds)	> 10,000	> 10,000
	Full-length 76 aa (without disulfide bonds)	> 10,000	> 10,000
Recombinant	C-terminal 44 aa	> 10,000	> 10,000
Native or purified Smp76	Full-length 76 aa	10	10

Discussion

Initial fingerprint analysis of the soluble venom of *S. m. palmatus* allowed the identification of at least 65 different components with molecular masses ranging between 413 and 14,009 Da (Abdel-Rahman et al. 2013). Most peptides showed a molecular weight ranging from 3 to 5 kDa. In the present study 74 fractions were obtained from 4 mg of the crude venom of *S. m. palmatus*. According to HPLC fraction peaks profile and the protein concentrations, we examined antiviral activity of 30 fractions against HCV and DENV to identify the active fraction. The fraction eluted at RT 36.4 min showed the most potent anti-HCV and anti-DENV activity with IC_{50} being ~ 0.01 µg/ml. According to the data of mass spectrometry and amino acids sequencing, this active fraction contains only one peptide with molecular mass of 8398 Da and consists of 76 amino acids. The obtained sequence matches with scorpine-like peptide Smp76 (Abdel-Rahman et al. 2013).

Scorpine is firstly isolated from the venom of *Pandinus imperator*. The structure of scorpine is a hybrid between a cecropin and a defensin. The sequence of scorpine carboxyl terminal region is similar to that of β-KTx family, with cysteine-stabilized α/β fold, and three disulfide bridges. On the other hand, its amino-terminal region is identical to the cecropin family peptides (Conde et al. 2000). Scorpine has also amino acid sequences similar to AMPs and K^+ channel blocking peptides (Luna-Ramirez et al. 2015). Scorpine homologs were thereafter identified from the venom of various scorpions such as *Opistophthalmus carinatus* (Zhu and Tytgat 2004), *Heterometrus laoticus* (Uawonggul et al. 2007), *H. gertschi* (Schwartz et al. 2007), *S. m. palmatus* (Abdel-Rahman et al. 2013), genus Vaejovis (Quintero-Hernandez et al. 2015) and *Urodacus yaschenkoi* (Luna-Ramirez et al. 2015). Importantly, all these peptides possess anti-malaria as well as antimicrobial activities (Conde et al. 2000; Carballar-Lejarazu et al. 2008) and act also as potassium channel blockers (Diego-Garcia et al. 2007). The present data clearly showed that smp76 inhibits the ability of HCV virus to infect the host cells. Indeed, our previous study demonstrated that the crude venom of *S. m. palmatus* venom prevents HCV infection with direct virocidal activity (El-Bitar et al. 2015). On the other hand, we cannot rule out the possibility that, smp76 peptide might has an independent effect on the receptor complexes of host cell components or interacts with components that inactivate viral entry. This possibility will be further investigated by the incubation of smp76 with cells in a free-virus condition prior to HCV infection. However, it worth to mention that the incubation of *S. m. palmatus* crude venom with cells prior to the HCV infection of cells did not impair the viral infectivity (El-Bitar et al. 2015). Thus, the possible effect of smp76 on the host cell to abrogate HCV infection is unlikely. In the present study, Smp76 prevents the early stages of life cycle of HCV and DENV most probably through interacting with viral particles. The viral particle can be neutralized by targeting the envelope of the HCV or host factors related to the mature viral particle (Zeisel et al. 2013). Notably, it has been reported in various studies that the structure of biological membranes could be altered by AMPs (Zasloff 2002; Harrison et al. 2016).

Currently, the new approach for HCV infection treatment probably based on the combination of several drugs (Pereira and Jacobson 2009; Sarrazin and Zeuzem 2010; Zeisel et al. 2011; Qian et al. 2016). Therefore, the use of Smp76 with anti-HCV drugs for treatment of HCV infection may have synergistic effect. However, further experiments are needed to check this possibility.

The present study reported distinctive data on the ability of Smp76 peptide to protect cellular systems from attack of DENV and neutralize viral infection. Currently dengue virus considered as one of the most important arthropod born viral disease worldwide (Botta et al. 2018). Despite the global efforts, there is no antiviral therapy against DENV infections clinically approved and only symptomatic treatment and hospital supportive care setting are available for infected people (Behnam et al. 2016). It was shown that recombinantly expressed scorpine (RScrp) inhibited DENV-2 replication in C6/36 mosquito cells. Also, it was suggested that the development of transgenic mosquitoes that overexpress and correctly secrete RScrp and could eventually break the dengue fever transmission (Carballar-Lejarazu et al. 2008). On the other hand, Smp76, as an infection inhibitor, has some advantages compared to antiviral drugs that target the viral replication stages inside the target cell. Smp76 can inhibit DENV infection before viral entry and is, therefore, helpful for treatment of DENV viraemia.

The prospective pharmaceutical potential of Smp76 cannot be neglected, especially considering its potent antiviral activity. However, Smp76 isolation from natural sources is ineffectual and time-consuming. Synthetic Smp76 without disulfide bonds showed no antiviral activities against HCV and DENV. One possibility is that the synthetic Smp76 peptide without disulfide bonds did not have the properly folded structure necessary for activity. Recently, recombinant scorpine with antimalarial and antibacterial activities was produced by different fusion technology using small ubiquitin-related modifier (SUMO) (Zhang et al. 2014) and maltose binding protein (MBP) (Zhang et al. 2016). These methods have improved efficiency and reduced the cost of producing scorpine and can contribute to the future production of active recombinant Smp76. Interestingly, the recombinant Smp76 was shown to inhibit DENV and ZIKV infections in cultured cell lines and primary mouse

macrophages. However, rSmp76 did not inactivate the viral particles directly but suppressed the established viral infection by upregulating the expression of IFN-β (Ji et al. 2018). This mechanism is significantly different from the virucidal effect of native Smp76 peptides. The exact mechanism by which Smp76 exerts its antiviral activity against HCV and DENV to inhibit infecting their target cells need further studies. In vivo studies should also assess the future role of Smp76 in managing HCV and DENV infections.

Acknowledgements The authors are grateful to Dr. Lin Deng and Dr. Ming Chen, Division of Microbiology, Kobe University Graduate School of Medicine, Japan, for their assistance in virological analyses. The authors also acknowledge Dr. Fernando Zamudio, M. Sc. Leonel Vargas Jaimes and M. Sc. María Teresa Romero Gutiérrez for their assistance in the proteomics work done in this work. We also grateful to Dr. Ahmed El-Shamy, California Northstate University, CA, USA for revising the manuscript. This study was conducted under collaboration between Zoology Department, Faculty of Science, Al Azhar University (Assiut, Egypt) and Division of Microbiology, Graduate School of Medicine, Kobe University (Japan), and was supported in part by a scholarship from the Egyptian Ministry of Higher Education (Cultural Affairs and Missions Sector) according to Channel System between Egypt and Japan. This study has also been supported in part by grants-in-aid for Research on Viral Hepatitis from the Ministry of Health, Labour and Welfare (Japan), and for Special Research on Dengue Vaccine Development from Tokyo Metropolitan Government (Japan).

Compliance with Ethical Standards

Conflict of interest Drs. Hak Hotta, Moustafa M Sarhan, Alaa MH El-Bitar, Lourival D. Possani and Mohamed A. Abdel-Rahman have a patent (anti-virus drug: PCT/JP2017/286) pending containing some of the information described in this work.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animal performed by any of the authors.

References

Abdel-Rahman MA, Quintero-Hernandez V, Possani LD (2013) Venom proteomic and venomous glands transcriptomic analysis of the Egyptian scorpion *Scorpio maurus palmatus* (Arachnida: Scorpionidae). Toxicon 74:193–207

Aoki C, Hartati S, Santi MR et al (2014) Isolation and identification of substances with anti-hepatitis c virus activities from *kalanchoe pinnata*. Int J Pharm Pharm Sci 6(2):211–215

Behnam MA, Nitsche C, Boldescu V, Klein CD (2016) The medicinal chemistry of dengue virus. Med Chem 59:5622–5649

Bhatt S, Gething PW, Brady OJ et al (2013) The global distribution and burden of dengue. Nature 496:504–507

Botta L, Rivara M, Zuliani V, Radi M (2018) Drug repurposing approaches to fight Dengue virus infection and related diseases. Front Biosci 23:997–1019

Carballar-Lejarazu R, Rodriguez MH, de la Cruz Hernandez-Hernandez F et al (2008) Recombinant scorpine: a multifunctional antimicrobial peptide with activity against different pathogens. Cell Mol Life Sci 65:3081–3092

Conde R, Zamudio FZ, Rodriguez MH, Possani LD (2000) Scorpine, an anti-malaria and anti-bacterial agent purified from scorpion venom. FEBS Lett 471:165–168

Deen JL, Harris E, Wills B et al (2006) The WHO dengue classification and case definitions: time for a reassessment. Lancet 368:170–173

Deng L, Adachi T, Kitayama K et al (2008) Hepatitis C virus infection induces apoptosis through a bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. J Virol 82:10375–10385. https://doi.org/10.1128/jvi.00395-08

Diego-Garcia E, Schwartz EF, D'Suze G et al (2007) Wide phylogenetic distribution of Scorpine and long-chain beta-KTx-like peptides in scorpion venoms: identification of "orphan" components. Peptides 28:31–37

El-Bitar AM, Sarhan HM, Aoki C et al (2015) Virocidal activity of Egyptian scorpion venoms against hepatitis C virus. Virology 12:47

Evans BC, Nelson CE, Yu SS et al (2013) Ex vivo red blood cell hemolysis assay for the evaluation of ph-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. J Vis Exp 73:50166

Falade-Nwulia O, Suarez-Cuervo C, Nelson DR, Fried MW, Segal JB, Sulkowski MS (2017) Oral direct-acting agent therapy for hepatitis c virus infection: a systematic review. Ann Intern Med 166:637–648

Feng L, Gao R, Gopalakrishnakone P (2008) Isolation and characterization of a hyaluronidase from the venom of Chinese red scorpion *Buthus martensi*. Comp Biochem Physiol C: Pharmacol Toxicol Endocrinol 148:250–257

Ghosh A, Roy R, Nandi M, Mukhopadhyay A (2019) Scorpion venom–toxins that aid in drug development: a review. Int J Pept Res Ther 25:27–37. https://doi.org/10.1007/s10989-018-9721-x

Harrison PL, George RH, Benjamin RGJ, Mohamed AA-R, Peter NS, Stephen DE, Keith M (2016) Phospholipid dependent mechanism of smp24, an α-helical antimicrobial peptide from scorpion venom. Biochim et Biophys Acta 1858:2737–2744

Hotta H, Homma M (1994) Lectin-mediated enhancement of dengue virus infection in a mouse macrophage cell line Mk1. Arch Virol 134:51–59

Hotta H, Hotta S, Takada H, Kotani S, Tanaka S, Ohki M (1983) Enhancement of dengue virus type 2 replication in mouse macrophage cultures by bacterial cell walls, peptidoglycans, and a polymer of peptidoglycan subunits. Infect Immun 41:462–469

Hv J, Hamill P, Hancock REW (2006) Peptide antimicrobial agents. Clin Microbiol Rev 19:491–511

Jefferies M, Rauff B, Rashid H, Lam T, Rafiq S (2018) Update on global epidemiology of viral hepatitis and preventive strategies World J Clin Cases 6:589–599

Ji Z, Li F, Xia Z et al (2018) The scorpion venom peptide Smp76 inhibits viral infection by regulating type-I interferon response. Virol Sin 33:545–556

Jiménez-Vargas JM, Quintero-Hernández V, González-Morales L, Ortiz E, Possani LD (2017) Design and expression of recombinant toxins from Mexican scorpions of the genus Centruroides for production of antivenoms. Toxicon 128:5–14

Kanoo S, Deshpande SB (2008) Involvement of phospholipase A2 pathway for the Indian red scorpion venom-induced augmentation of cardiopulmonary reflexes elicited by phenyldiguanide. Neurosci Lett 440:242–245

Luna-Ramirez K, Quintero-Hernandez V, Jarez-Gonzalez VR, Possani LD (2015) Whole transcriptome of the venom gland from *Urodacus yaschenkoi* Scorpion. PLoS ONE 10:e0127883

Mackenzie JS, Gubler DJ, Petersen LR (2004) Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10:S98–S109

Malavige GN, Fernando S, Fernando DJ, Seneviratne SL (2004) Dengue viral infections. Postgrad Med J 80:588–601

Messina JP, Brady O, Scott T et al (2014) Global spread of dengue virus types: mapping the 70 year history. Trends Microbiol 22:138–146

Ministry of Health and PE, El-Zanaty and Associates Egypt, I. C. F. International (2015) Egypt Health Issues Survey 2015. Ministry of Health and Population/Egypt and ICF International, Cairo

Mohammed AA, Alwin KR, Seok MW, Rishya M, Shamala DS (2013) Inhibition of dengue virus entry into target cells using synthetic antiviral peptides. Int J Med Sci 10:719–729

Mustafa MS, Rasotgi V, Jain S, Gupta V (2015) Discovery of fifth serotype of dengue virus (DENV-5): a new public health dilemma in dengue control. Med J Armed Forces India 1:70–97

Omran MAA (2003) Cytotoxic and apoptotic effects of scorpion *Leiurus quinquestriatus* venom on 293T and C2C12 eukaryotic cell lines. J Venom Toxins Incl Trop Dis 9:255–276

Ono N, Tatsuo H, Hidaka Y, Aoki T, Minagawa H, Yanagi Y (2001) Measles viruses on throat swabs from measles patients use signaling lymphocytic activation molecule (CDw150) but not CD46 as a cellular receptor. J Virol 75:4399–4401

Ortiz E, Gurrola GB, Schwartz EF, Possani LD (2015) Scorpion venom components as potential candidates for drug development. Toxicon 93:125–135

Otaki M, Sada K, Kadoya H, Nagano-Fujii M, Hotta H (2006) Inhibition of measles virus and subacute sclerosing panencephalitis virus by RNA interference. Antiviral Res 70:105–111

Ozkan O, Adiguzel S, Ates C, Bozyigit I, Filazi A (2006a) Optimization of antiscorpion venom production. J Venom AnimToxins Incl Trop Dis 12:390–399

Ozkan O, Adiguzel S, Yakistiran S, Cesaretli Y, Orman M, Karaer Z (2006b) (Oliver, 1807) scorpionism in the Saniliurfa region in Turkey. Acta Parasitol Turcica 30:239–245

Ozkan O, Adiguzel S, Yakistiran S, Filazi A (2006c) Study of the relationship between *Androctonus crassicauda* (Oliver, 1807; Scorpiones, Buthidae) venom toxicity and telson size, weight and storing condition. J Venom Anim Toxins Incl Trop Dis 12:297–309

Pawlotsky J-M (2016) Hepatitis C Virus resistance to direct-acting antiviral drugs in interferon-free regimens. Gastroenterology 151:70–86

Pereira AA, Jacobson IM (2009) New and experimental therapies for HCV. Nat Rev Gastroenterol Hepatol 6:403–411

Qian X-J, Zhu Y-Z, Zhao P, Qi Z-T (2016) Entry inhibitors: new advances in HCV treatment Emerg. Microbes Infect 5:e3

Quintero-Hernandez V, Ramirez-Carreto S, Romero-Gutierrez MT, Valdez-Velazquez LL, Becerril B, Possani LD, Ortiz E (2015) Transcriptome analysis of scorpion species belonging to the *Vaejovis* genus. PLoS ONE 10:e0117188

Rodenhuis-Zybert IA, Wilschut J, Smit JM (2010) Dengue virus life cycle: viral and host factors modulating infectivity. Cell Mol Life Sci 67:2773–2786

Rodriguez de la Vega RC, Possani LD (2005) Overview of scorpion toxins specific for Na+ channels and related peptides: biodiversity, structure function relationships and evolution. Toxicon 46:831–844

Sarrazin C, Zeuzem SR (2010) Esistance to direct antiviral agents in patients with hepatitis C virus infection. Gastroenterology 138:447–462

Schwartz EF, Diego-Garcia E, de la Vega RCR, Possani LD (2007) Transcriptome analysis of the venom gland of the Mexican scorpion *Hadrurus gertschi* (Arachnida: Scorpiones). BMC Genom 8:119

Supanee P, Stéphane P, Barbara S, Bruno C (2014) Comparison of dengue virus and HCV: from impact on global health to their RNA-dependent RNA polymerases. Fut Virol 9:53–67

Uawonggul N, Thammasirirak S, Chaveerach A et al (2007) Purification and characterization of Heteroscorpine-1 (HS-1) toxin from *Heterometrus laoticus* scorpion venom. Toxicon 49:19e29

Vargas-Jaimes L, Xiao L, Zhang J, Possani LD, Valdivia HH, Quintero-Hernández V (2017) Recombinant expression of Intrepicalcin from the scorpion Vaejovis intrepidus and its effect on skeletal ryanodine receptors. Biochim Biophys Acta - Gen Subj 1861:936–946

Wakita T, Pietschmann T, Kato T et al (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11:791–796

Weaver SC, Vasilakis N (2009) Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. Infect Genet Evol 9:523–540

World Health Organization (2016) Guidelines for the screening, care and treatment of persons with chronic hepatitis, C infection. World Health Organization, Geneva

Yu L, Aoki C, Shimizu Y et al (2010) Development of a simple system for screening anti-hepatitis C virus drugs utilizing mutants capable of vigorous replication. J Virol Methods 169:380–384

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415:389–395

Zeisel MB, Fofana I, Fafi-Kremer S, Baumert TF (2011) Hepatitis C virus entry into hepatocytes: molecular mechanisms and targets for antiviral therapies. J Hepatol 54:566–576

Zeisel MB, Lupberger J, Fofana I, Baumert TF (2013) Host-targeting agents for prevention and treatment of chronic hepatitis C—Perspectives and challenges. Journal of Hepatology 58:375–384

Zhang X, Xie J, Sun Y et al (2014) High-level expression, purification, and characterization of bifunctional ScFv-9R fusion protein. Appl Microbiol Biotechnol 98:5499–5506

Zhang C, Zhou H, Gu Y, He X, Cao J, Gao Q (2016) Production and characterization of scorpine by MBP fusion technology in *Escherichia coli*. Indian J Biotechnol 15:69–74

Zhu S, Tytgat J (2004) The scorpine family of defensins: gene structure, alternative polyadenylation and fold recognition. CMLS 61:1751–1763

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.