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The narrow range of anti-dengue activity of ivermectin *in vitro*

Surat Punyahathaikul[1], Visanu Thamlikitkul[2] and Nuananong Jirakanjanakit[3,*]

[1]Center for Vaccine Development, Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand
[2]Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand
[3]Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand
[*]Corresponding author: nuananong.jir@mahidol.ac.th

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Abstract

To verify the anti-viral mechanisms of Ivermectin (IVM) for its potential application as a dengue (DEN) anti-viral drug. Inhibition of virus binding/entry, virucidal activity, anti-virus plaque formation, and the inhibition of viral growth were determined based on a comparison with Ribavirin (RBV). No direct effects of IVM on the viral attachment and entry step were observed. However, the inhibitory effects of IVM on plaque formation and viral growth were demonstrated in monkey kidney epithelial cells (LLC-MK2) infected cells. The inhibition concentration of 50 of IVM was 8.8 times lower than that of RBV (9.16 µg/mL vs. 80.82 µg/mL) after 24 h. of exposure in DEN infected cells. Meanwhile, the virucidal activity of IVM was not observed to be similar to that of RBV. However, toxicity in the HepG2 cells, which could be human target cells for drug metabolism, provided a narrow range of IVM applications. Considering the nature of DEN in which all 4 serotypes could develop into severe forms of the disease, IVM remains a valuable anti-viral drug, even with the narrow range of applications. Furthermore, IVM could reduce burden of DEN infections during an outbreak.

Keywords: Antiviral drug, Dengue, Ivermectin, Toxicity, Antiviral mechanism

1. Introduction

Dengue (DEN) is among the most rapidly spread vector-borne viral diseases with a 4-fold increase in incidence rates during the past 20 years [1]. Nonetheless, it is improbable to predict which patients with DEN infection will be at risk for severe or even fatal infections given that an effective antiviral drug remains unavailable. The prevention approach via vaccination and vector control is considered to be the best solution. However, incomplete vaccinations could impair disease protection and possibly heighten the risk of a severe form of DEN disease. Up until the present, the available dengue vaccine on the market has shown incomplete protection for the vaccinees [2,3] whereas the vaccine under Phase III of the clinical trials has also revealed an incomplete efficacy in preventing all 4 dengue serotypes [4]. Currently, various candidate vaccines remain under development [5].

Meanwhile, since dengue fever (DF) and dengue hemorrhagic fever (DHF) are considered to be the most fatal fevers, therapeutic approaches, such as antiviral drugs, could become a valuable treatment. Naturally, the duration of viremia in DEN infection is rather short lasting for approximately 3-5 days, thus the administration of an antiviral drug would be crucial. Consequently, reduction of the viral load during viremic peak could possibly reduce the disease severity. It has been recommended that during epidemics, antiviral treatments could be valuable for early administration in DEN patients with febrile illnesses. Additionally, decreasing the viral load of the infected patient could also lower the number of *Aedes* mosquitoes to get infection [6], especially during DEN epidemic. Several classes of agents have been under investigation as potential anti-dengue drugs, including direct antivirals, host modulators, and RNAi therapeutics.

Ivermectin (IVM), an old drug used for treatment of several parasitic infestations in humans and animals, [7-9] has been reported as an effective inhibitor of Flavivirus replication [10]. It could inhibit yellow fever virus induced cytopathic formation with effective concentration 50 (EC_{50}) at nano molar level. Additionally, IVM has

been found to interrupt virus replication of all DEN serotypes, giving its potential as a dengue antiviral agent [11]. Inhibitory effect of IVM on Newcastle virus was also reported with cytotoxicity effect to the tested cells at 100 µg/mL [12]. Moreover, it was also active against Chikungunya, Semliki Forest virus and Sindbis virus, suggesting a broad antiviral activity of IVM [13]. *In vitro* and *in vivo* testing of IVM activity on corona virus, SARS-CoV-2 have also been reported [14-16]. However, the use of IVM for the treatment of coronavirus disease starting in 2019 (COVID-19) cases requires careful risk-benefit considerations, especially in critical cases [15].

Mechanisms of action of IVM have also been approved for its antiviral activity against several ribonucleic acid (RNA) viruses by blocking the nuclear trafficking of viral proteins [10], and by disrupting integrase protein and polymerase enzyme [17]. It could be observed that most of the study had focused on the activity of the inhibitor on the viral replication process, while the precise mechanisms have not been defined. Thus, the antiviral mode of action tested under the appropriate *in vitro* systems could be a qualitative indicator of potential efficacy. The mechanisms of DEN antiviral drugs of IVM were then determined, such as the inhibition of virus binding/entry, virucidal activity, antivirus plaque formation and the inhibition of virus growth.

2. Materials and methods

2.1 Cells and virus

Monkey kidney epithelial cells (LLC-MK_2) and $HepG_2$ cells (ATCC: CCL-7, CCL-81 and HB-8065) were grown in modified Eagle's medium (MEM; Life Technologies) supplemented with 10% fetal calf serum (HyClone; Thermo Scientific). Dengue-2 strain 16681 (DEN-2), derived from a dengue hemorrhagic fever patient, was used. The stock seed virus was amplified in Vero cell culture and was kept at -80°C for all tests.

2.2 Tested drugs

IVM, a white crystalline powder (Batch No. 1270456) was obtained from the Huashu Pharmaceutical Corporation (China). Ribavirin (RBV, R9644), a known anti-dengue compound was purchased from Sigma-Aldrich to be used as control. The tested compound was dissolved in 95% ethanol as stock. They were used at the final concentration which contained ethanol at less than 1% in the tested solution.

2.3 Cytotoxicity test

Briefly, confluent culture cells grown in 96-well plates were exposed to different concentrations of the tested drug with 5 wells for each concentration and 5 wells for cell control. After incubation for 24 h, 1 mg/mL of MTT (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was replaced to each well and the tested cells were further incubated for 4 h. After that, Dimethyl Sulfoxide (DMSO) was added to each well to solubilize the formazan crystals and then the absorbance was measured using a microplate reader at 570 nm. The cytotoxicity of the tested drug was computed from 3 experiments as the percentage of cell viability in the tested wells compared to the control wells.

2.4 Virucidal activity

DEN-2 virus suspension was incubated with an equal volume of eagle's minimum essential medium (MEM) with or without different concentrations of IVM or RBV at the non-toxic dose for 60 min at 37°C. After that, the virus-drug mixtures were inoculated onto LLC-MK_2 cell monolayer grown in 6 well-plates. After 90 min adsorption, the carboxy-methyl cellulose overlayer medium was replaced and the culture plates were further incubated for 7 days. The virucidal concentration 50 (VC_{50}) was calculated as the concentration required to inactivate the virions by 50%.

2.5 Antiviral activity

2.5.1 Inhibition of the viral plaque

Antiviral activity was evaluated by a viral plaque reduction assay. LLC-MK_2 cell monolayers, which had been grown on 6-well plates, were infected with approximately 50-200 plaque-forming unit (PFU)/well of DEN-2 virus (plaque forming unit). After 90 min of absorption, varying non-toxic concentrations of IVM (0.1, 1, 10, 20, and 30 µg/mL or RBV (0.1, 1, 10, 100, 200, 300, and 400 µg/mL) were added in triplicate. The control wells of the tested drug at the same concentration without viral infection were also verified. After incubation for 24 h. at 37°C, the tested materials were discarded, and the overlayer medium was added. The numbers of plaques were counted

after 7 days of incubation at 37°C. The inhibitory concentration 50 (IC_{50}) was calculated as the drug concentration, which had reduced the number of viral plaques by 50% of the virus control from the dose response curve.

2.5.2 The reduction of viral replication

LLC-MK_2 cells were infected with DEN virus at 0.1 MOI (multiplicity of infection). The drug effects on virus replication were examined in the infected cells incubated with the non-toxic concentrations of IVM at 2.5, 5, 10, and 20 µg/mL. RBV at 12.5, 25, 50, and 100 µg/mL were used as control Virus growth under the presence or absence of either IVM or RBV was quantified for the number of DEN viruses by plaque assay during 1-5 days of incubation. The IC_{50} was calculated as the drug concentration which reduced the number of viral plaques by 50 % of the viral control from the dose response curve.

2.6 The effects on virus adsorption and internalization

2.6.1 Virus adsorption

LLC-MK2 cells grown on 6-well plate were exposed to DEN virus in the presence or absence of varying concentrations of the tested drug in triplicate. The non-toxic concentrations of IVM at 2.5, 5, 10 and 20 µg/mL and RBV at 12.5, 25, 50, and 100 µg/mL were used. After 90 min of incubation at 4°C, the compounds and unabsorbed virus were removed. The tested cells were washed with cold PBS and over-laid with plaquing medium. Numbers of plaques were counted after 7 days of incubation.

2.6.2 Internalization

LLC-MK2 cells grown on 6-well plate were exposed to DEN virus in the presence of varying concentrations of the tested drugs in triplicate. The non-toxic concentrations of IVM at 2.5, 5, 10 and 20 µg/mL and RBV at 12.5, 25, 50, and 100 µg/mL were used. After incubation for 90 min at 37°C, the compounds and virus mixture were removed. The tested cells were washed with PBS and treated with citrate buffer for 1 min to inactivate the adsorbed but not internalized virus. After re-washing with PBS, overlay medium was added. Numbers of plaques were counted after 7 days of incubation.

2.7 Statistical analysis

The results were expressed as means standard deviation (SD). Linear regression was used to calculate the IC_{50} of the tested drugs from the dose response curve. Statistical comparisons were carried out using the Student's t-test and the significant difference was set at $p \leq 0.05$.

3. Results

3.1 Cytotoxicity

Ethanol, which was used as drug solvent, had no effect on viability of both LLC-MK_2 and $HepG_2$ cells at the highest concentration tested at 2%. It was observed that IVM was toxic to LLC-MK_2 and $HepG_2$ cells at the concentration of 10 µg/mL after 24 h. exposure. Cell viability was reduced to 78.96% and 45.79%, respectively as compared to the control cells (Figure 1). On the other hand, RBV showed no cytotoxic to either LLC-MK_2 or $HepG_2$ cells after 24 h. of exposure to the highest concentration at 1000 µg/mL as determined by MTT assay.

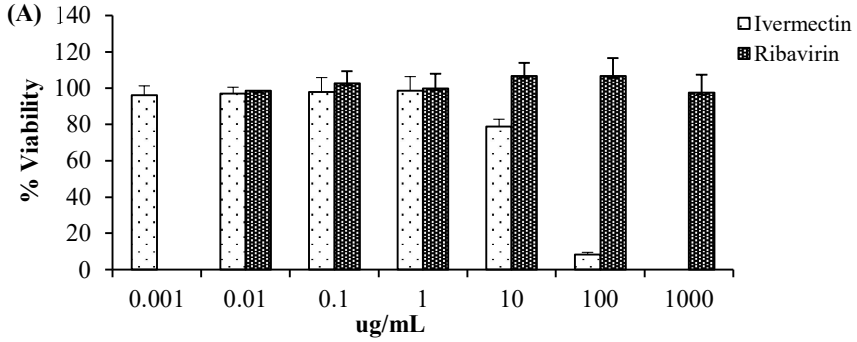

Figure 1 Cytotoxicity of Ivermectin and Ribavirin on (A) LLC-MK_2 cells as determined by MTT assay after 24 h. exposure (Values are expressed as means ±SD).

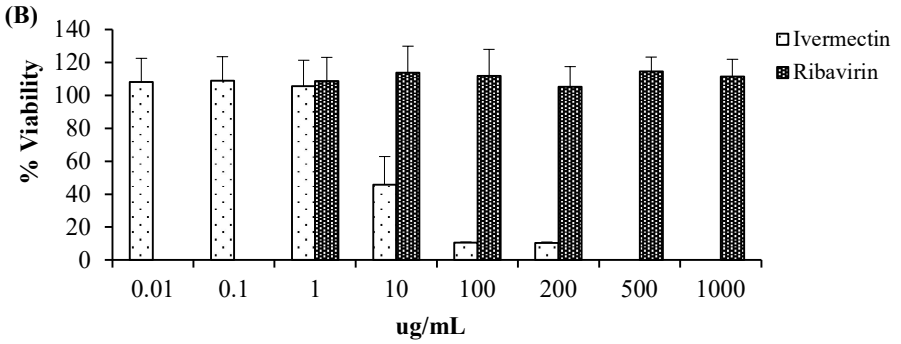

Figure 1 (continued) Cytotoxicity of Ivermectin and Ribavirin on (B) $HepG_2$ cells as determined by MTT assay after 24 hrs. exposure (Values are expressed as means ±SD).

Cytotoxicity of IVM to LLC-MK_2 cells at varying days of incubation was also verified to ensure the antiviral activity to dengue virus in the tested cells. As presented in Figure 2, IVM was toxic to the cells at 1 µg/mL during Day 4 and Day 5, while those at ≥ 2.5 µg/mL were toxic to the tested cells from Day 2 onwards. IVM at 7.5 and 10 µg/mL were also found to be slightly toxic to the cells after 1 day of incubation. In contrast, RBV at varying concentrations (10, 100, 200, 300 and 400 µg/mL) showed no toxicity to LLC-MK_2 cells after 5 days of incubation (data not shown).

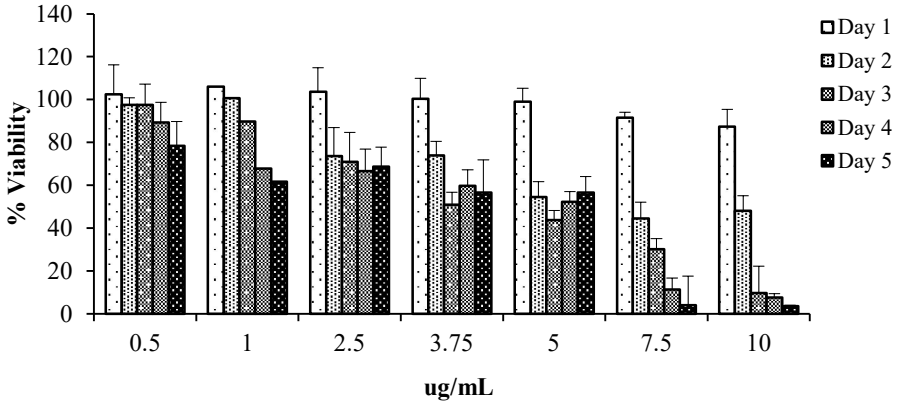

Figure 2 Cytotoxicity of Ivermectin on LLC-MK_2 cells at 1, 2, 3, 4 and 5 days of incubation as determined by MTT assay (Values are expressed as means ±SD).

3.2 Virucidal activity

At the non-toxic concentrations (0.01-30 µg/mL and 0.1-400 µg/mL, respectively), IVM, as well as RBV, showed no virucidal activity to the DEN-2 virus given that the number of viral plaques in the tested wells had shown no significant differences from those in the control wells. Thus, the VC_{50} could not be determined.

3.3 Antiviral activity

3.3.1 Inhibition of viral plaques

The tested drugs exhibited different inhibitory effects on viral plaque forming depending on the number of viruses used (Table 1). At 200 PFU, RBV provided 50% plaque reduction at 297 µg/mL, while the highest concentration of IVM used at 20 µg/mL showed a slightly inhibitory effect after 24 h. of incubation. Increasing in IVM concentration (≥30 µg/mL) caused cell damage due to its toxicity to the tested cells. However, significantly lower IC50 of IVM than that of RBV (p<0.01) was presented when tested with DENV at 100 PFU and 70 PFU. It was noted that IVM exhibited 3-5 times higher inhibitory effect on DENV plaque forming than RBV at the tested conditions.

Table 1 Dengue virus plaque inhibition concentration 50 of Ivermectin and Ribavirin at 200, 100 and 70 virus particles after 24 h exposure.

Tested drugs	Inhibition concentration 50 (μg/mL)		
	200 PFU	100 PFU	70 PFU
Ivermectin	0	15.94* ± 2.55	14.25# ± 1.86
Ribavirin	297.66 ± 25.66	84.76* ± 11.87	39.52# ± 11.95

PFU = plaque forming unit. Values are expressed as means ± SD.
*A significant difference was found between Ivermectin and Ribavirin tested at 100 PFU (p<0.01).
A significant difference was found between Ivermectin and Ribavirin tested at 70 PFU (p<0.01).

3.3.2 Reduction of virus replication

The inhibitory effects of IVM and the RBV control on DENV growth are presented in Table 1. Since IVM at 2.5, 5, and 10 μg/mL had also been toxic to the cells from Day 2 onward (Table 2), the reduction in virus particles at these concentrations between Day 2-Day 5 had been due to the mortality of the host cells rather than any antiviral effects. As a result, IC50 could only be computed from the results obtained on Day 1. On the other hand, RBV exhibited inhibitory effect on DEN virus growth relatively in corresponding to concentration used. However, IC50 of IVM on day 1 was approximately 8.8 times lower than that of RBV (p<0.001). Although, IVM seemed to be more effective than RBV, a narrow range of IVM concentration (5-10 μg/mL) was effective during 24 h. due to toxicity in the tested conditions. Alternatively, antiviral activity of RBV remained effective on DEN virus growth relatively at the same IC50 during 5 days of treatment.

Table 2 Inhibition concentration 50 of Ivermectin and Ribavirin on dengue virus growth during 5 days of incubation.

Incubation period	Inhibition concentration 50 (μg/mL)	
	Ivermectin	Ribavirin
1 Day	9.16[a] ± 1.15	80.82[b] ± 1.44
2 Days	-	113.96 ± 11.09
3 Days	-	94.68 ± 10.03
4 Days	-	104.73 ±4.01
5 Days	-	104.76 ±10.85

- Not applicable. Value are expressed as mean ±SD.
[a, b] Significant difference of IC50 between Ivermectin and Ribavirin after 1 day of incubation (p<0.001).

3.4 The effects on viral adsorption and internalization

Under the tested condition, the number of virus plaque in the presence of different concentrations of either RBV or IVM showed no significant differences to that in the control wells from both experiment on virus adsorption and internalization. Thus, the 2 tested drugs showed no inhibitory effect at the virus entry step on LLC-MK_2 cells.

4. Discussion

IVM has been tested on its antiviral activity against several RNA viruses by disrupting viral proteins in the replication process [17]. Time of addition experiments also revealed that IVM acted on the replication phase of the viral infection cycle. Therefore, it was determined to be a viral inhibitory compound. These findings had led to the prove of its efficacy as the antiviral drug for DEN. The placebo-controlled trial phase II and III had been conducted and a significant difference of NS1 antigen clearance in IVM treated was presented as compared with the placebo. [18,19]. However, no significant differences in the viremic levels was presented as measured by qPCR [18], thus clinical efficacy of IVM could not be determined. In addition, this outcome of ivermectin-accelerated NS1 clearance was diverse from the previously reported clinical associations between elevated plasma NS1 levels and DEN disease severity [20-22].

In the present study, the effectiveness of IVM was determined by testing on possible mechanism(s) of DEN antiviral drug. Nonetheless, it was observed that IVM did not express inhibitory effect on virus entry step in LLC-MK_2 infected cells. The number of virus plaque showed no significant difference to that in the control wells from both experiments on virus adsorption and internalization. It has also been reported that IVM showed no inhibitory effect on the virus entry process of the porcine reproductive and respiratory syndrome virus [23]. In addition, it was discovered that IVM and RBV at the tested concentration (0.01- 30 μg/mL and 0.1- 400 μg/mL) exhibited no virucidal activity to DEN-2 virus. Similarly, IVM also did not demonstrate any virucidal activity to either SFV or CHIKV virus [13].

On the other hand, treatment of DEN infected cells with varying concentration of IVM revealed that IVM could inhibit DEN virus plaque formation. Antiviral activity of IVM could be determined in the tested well with 70 and 100 virus plaques and provided the IC50 at 14.25 and 15.94 µg/mL, respectively. Whereas significantly lower activity of RBV was presented with the IC50 at 39.5 and 84.76 µg/mL, respectively. Surprisingly, IVM at the non-toxic level of 20 µg/mL showed no remarkable inhibition on virus plaque formation when tested with 200 plaques, while RBV remained active (IC50 at 297 µg/mL). Therefore, IVM might have some limitations as a viral inhibitor at high viral loads due to the concentration used in the *in vitro* testing. However, IVM at the non-toxic level provided 3-5 times more effective as compared to RBV.

In another experiment, incubation of DEN infected cells with varying concentrations of the tested compounds revealed that IVM has 8.8 times higher antiviral activity compared to RBV by 24 h. of incubation. However, RBV showed robust antiviral potential at all tested concentrations, while IVM at 2.5, 5 and 10 µg/mL were rather toxic to the tested cell after Day 2 of incubation. Therefore, the reduction in viral particles at these concentrations during day 2-5 was due to the mortality of the host cells, rather than the antiviral effect of IVM. Cytotoxicity of IVM was then depending on the concentration used, as well as the time of exposure. Caution must be taken in the experiment using cell culture since misinterpretation was possible. IVM were also found to be more toxic to the liver cell (HepG2) than to kidney cell (LLC-MK2 cell). Thus, IVM might have been metabolized to a more toxic substance by the enzyme in HepG2 cells. On the other hand, toxicity of IVM to chicken primary fibroblast cell line was reported at 100 µg/mL [11]. Therefore, its toxicity may vary depending on different cell lines as well as different tested conditions. Nonetheless, elimination half-life of IVM in human was approximately by 1 day [24], thus toxicity could be insignificant with a single daily dose regimen. In the combined phase 2/3 randomized double-blinded placebo-controlled trial, a 3-day 1 daily dose of 400 µg/kg oral ivermectin had confirmed safety use [18]. Nevertheless, the clinical efficacy was not observed at the tested dose regimen. Limitation of IVM application could partially due to its toxicity at high dose, thus the use of Ivermectin as the antiviral drug was restricted. Interestingly, high dose IVM at 1200 µg/kg for 5 days of treatment was tested in Phase II with dose-finding and proof of concept clinical trial without any serious adverse drug reactions [25]. However, no significant reduction in viral load between IVM and placebo was presented. Meta-analysis of IVM for treatment of COVID-19 suggested that ivermectin does not reduce mortality risk, and thus it was lack of clinical benefit [26]. These consequences supported the recommendation by WHO that it is not suggested to use IVM in COVID-19 patient except in research purpose [27].

Although, it was not recommended to use in breastfeeding mothers, since IVM at 14.1 mg/mL was detected in the milk [28]. Concern on adverse reaction of neurotoxicity due to IVM passing through the blood-brain barrier in the breast-fed child was likely. In addition, IVM is not recommended for use in children under 5 years of age [28] due to neurotoxicity since the drug possibly being able to cross the incompletely developed blood-brain barrier. While the majority of dengue cases were mainly in children under 15 years of age [29], application of IVM in infected patient was possible. Although toxicity of IVM could limit its potential use, conclusive data from the present work provided a benefit for the application of IVM as an antiviral compound for DENV treatment in adult. In addition, IVM was effective on various type of virus, thus it could be favorable in the area where numerous diseases with similar symptoms could complicate disease diagnosis for early treatment. Moreover, a broad-spectrum antiviral of IVM made it become a good choice of DEN antiviral drug in the case of co-infection. Antiviral drugs are also essential in the short therapeutic time period since the effective tools to predict the high-risk patients did not exist.

5. Conclusion

The effectiveness of IVM as DEN antiviral drug has been verified. The IC_{50} of IVM was 8.8 times lower than that of RBV after 24 h. exposure under the tested condition. This data supports the possible usage of IVM as the antiviral drug for DEN cases. However, the limitation of IVM due to its toxicity provided a narrow range for antiviral application. Since, all DEN serotypes could develop severe forms of disease, IVM could be valuable antiviral drug for infected patients, especially during the outbreak.

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7. References

[1] Yacoub S, Mongkolsapaya J, Screaton G. Recent advances in understanding dengue [version 1; referees: 3 approved] F1000Research 2016;5(F1000 Faculty Rev):78.

[2] Aguiar M, Stollenwerk N, Halstead SB. The risks behind Dengvaxia recommendation. Lancet Infect Dis. 2016;16:882-883.

[3] Sridhar S, Luedtke A, Langevin E, Zhu M, Bonaparte M, Machabert T, et al. Effect of Dengue Serostatus on Dengue Vaccine Safety and Efficacy. N Engl J Med. 2018;379:327-40.

[4] Biswal S, Borja-Tabora C, Martinez Vargas L, et al. TIDES Study Group. Efficacy of a tetravalent dengue vaccine in healthy children aged 4-16 years: a randomized, placebo-controlled, phase 3 trial. Lancet. 2020; 395:1423-1433.

[5] Deng SQ, Yang X, Wei Y, Chen JT, Wang XJ, Peng HJ. A review on dengue vaccine development. Vaccine. 2020;8:63.

[6] Novelo M, Hall MD, Pak D, Young PR, Holmes EC, McGraw EA. Intra-host growth kinetics of dengue virus in the mosquito Aedes aegypti. PLoS Pathog 2019;15(12):e1008218.

[7] Dourmishev AL, Dourmishev LA, Schwartz RA, Bennet DG. Ivermectin: pharmacology and application in dermatology. Int J Dermatol. 2005;44:981-988.

[8] Fox LM. Ivermectin: uses and impact 20 years. Curr Opin Infect Dis. 2006;19:588-593.

[9] Ottesen EA, Cambell WC. Ivermectin in human medicine. J Antimicrob Chemother. 1994;34:195-203.

[10] Mastrangelo E, Pezzullo M, De Burghgraeve T, Kaptein S, Pastorino B, Dallmeier K, et al. Ivermectin is a potent inhibitor of flavivirus replication specifically targeting NS3 helicase activity: new prospects for an old drug. J Antimicrob Chemother. 2012;67:1884-1894.

[11] Tay MYF, Fraser JE, Chan WKK, Moreland NJ, Rathore AP, Wang C, et al. Nuclear localization of dengue virus (DENV) 1-4 nonstructural protein 5: protection against all 4 DENV serotypes by the inhibitor ivermectin. Antivir Res. 2013;99:301-306.

[12] Azeem S, Ashraf1 M, Rasheed MA, Anjum AA, Pak RH. Evaluation of cytotoxicity and antiviral activity of ivermectin against Newcastle disease virus. J Pharm Sci. 2015;28(2):597-602.

[13] Varghese FS, Kaukinen P, Gl€asker S, Bespalov M, Hanski L, Wennerberg K, et al. Discovery of berberine, abamectin and ivermectin as antivirals against chikungunya and other alphaviruses. Antivir Res. 2016;126:117e124.

[14] Calya L, Drucea JD, Cattona MG, Jansb DA, Wagstaff KM. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 *in vitro*. Antivir Res. 2020;178:104787.

[15] Chaccour C, Hammann F, Ramo´ n-Garc´ S, Rabinovich NR. Ivermectin and COVID-19: Keeping Rigor in Times of Urgency. Am J Trop Med Hyg. 2020;102(6):11561157.

[16] Manomaipiboon A, Pholtawornkulchai K, Poopipatpab S, Suraamornkul S, Maneerit J, et al. Efficacy and safety of ivermectin in the treatment of mild to moderate COVID-19 infection: a randomized, double-blind, placebo-controlled trial Trials. Random Controll Trial. 2022;23(1):714.

[17] Wagstaff KM, Sivakumaran H, Heaton SM, Harrich D, Jans DA. Ivermectin is a specific inhibitor of import in α/β-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus. Biochem J. 2012;443:851-856.

[18] Suputtamongkol Y, Avirutnan P, Mairiang D, Angkasekwinai N, Niwattayakul K, Yamasmith E, et al. Ivermectin accelerates circulating nonstructural protein 1 (NS1) clearance in adult dengue patients: a combined phase 2/3 randomized double-blinded placebo-controlled trial. Clin Inf dis. 2021;72:e586-e593.

[19] Avirutnan P. Ivermectin: a promising anti-dengue replication treatment [abstract S634]. Presented at: 26th European Congress of Clinical Microbiology and Infectious Diseases; 2016 April 9-12; Amsterdam: Netherlands; Los Angeles: PMC Disclaimer; 2017.

[20] Libraty DH, Young PR, Pickering D, et al. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J Infect Dis. 2002;186:1165-1168.

[21] Avirutnan P, Punyadee N, Noisakran S, et al. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J Infect Dis 2006;193:1078-1088.

[22] Paranavitane SA, Gomes L, Kamaladasa A, Adikari TN, Wickramasinghe N, et al. Dengue NS1 antigen as a marker of severe clinical disease. BMC Infectious Diseases 2014;14:570.

[23] Lee YJ, Lee C. Ivermectin inhibits porcine reproductive and respiratory syndrome virus in cultured porcine alveolar macrophages. Arch Virol. 2016;161:257–268.

[24] Baraka OZ, Mahmoud BM, Marschke CK, Geary TG, Homeida A, Williams JF. Ivermectin distribution in the plasma and tissues of patients infected with Onchocerca volvulus. Eur J Clin Pharmacol. 1996;50:407-410.

[25] Buonfrate D, Chesini F, Martini D,Roncaglioni MC, Ojeda Fernandez ML, et al. High-dose ivermectin for early treatment of COVID-19 (COVER study): a randomised, double-blind, multicentre, phase II, dose-finding, proof-of-concept clinical trial. Int J Antimicrob Agents. 2022;59(2):106516.
[26] Marcolino MS, Meira KC, Guimaraes NS, Motta PP, Chagas VS, et al. Systematic review and meta-analysis of ivermectin for treatment of COVID-19: evidence beyond the hype. BMC Infect Dis. 2022; 22:639.
[27] Agarwal A, Rochwerg B, Siemieniuk RA, Agoritsas T, Lamontagne F, Askie L, et al. A living WHO guideline on drugs for COVID-19. BMJ. 2020;370:m3379.
[28] Ogbuokiri JE, Ozumba BC, Okonkwo OP. Ivermectin levels in human breast milk. Eur J Clin Pharmacol. 1993;46:389-390.
[29] Tantawichien T. Dengue fever and dengue haemorrhagic fever in adolescents and adults. Paediatr Int Child Health. 2012;32:22-27.