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In Vitro Inhibition of Zika Virus Replication with Amantadine and Rimantadine Hydrochlorides

Jorge L. Arias-Arias ^{1,2*}, Francisco Vega-Aguilar ³, Dihalá Picado-Soto ³, Eugenia Corrales-Aguilar ^{2,3} and Gilbert D. Loria ^{2,3,4}

¹ Sección de Biología Celular y Molecular, Facultad de Microbiología, Universidad de Costa Rica, San José, 11501-2060, Costa Rica

² Centro de Investigación en Enfermedades Tropicales (CIET), Universidad de Costa Rica, San José, 11501-2060, Costa Rica; eugenia.corrales@ucr.ac.cr (E.C.-A.); gilbert.loria@ucr.ac.cr (G.D.L.)

³ Sección de Virología, Facultad de Microbiología, Universidad de Costa Rica, San José, 11501-2060, Costa Rica; francisco.vega@ucr.ac.cr (F.V.-A.); dpicado@inciensa.sa.cr (D.P.-S)

⁴ Centro de Investigación en Hematología y Trastornos Afines (CIHATA), Universidad de Costa Rica, San José, 11501-2060, Costa Rica

* Correspondence: jorgeluis.arias@ucr.ac.cr

Abstract: Zika virus (ZIKV) is a mosquito-born flavivirus which human infection became relevant during recent outbreaks in Latin America, due to its unrecognized association with fetal neurological disorders. Currently there are no approved effective antivirals or vaccines for treatment or prevention of ZIKV infections. Amantadine and rimantadine are approved antivirals used against susceptible influenza A virus infections, that have been shown to have antiviral activity against other viruses, such as dengue virus (DENV). Here, we report the *in vitro* effectiveness of both amantadine and rimantadine hydrochlorides against ZIKV replication, resulting in a dose-dependent reduction in viral titers of a ZIKV clinical isolate and two different ZIKV reference strains. Additionally, we demonstrate similar *in vitro* antiviral activity of these drugs against DENV-1 and yellow fever virus (YFV), although at higher drug concentrations for the later. ZIKV replication was inhibited at drug concentrations well below cytotoxic levels of both compounds, as denoted by the high selectivity indexes obtained with the tested strains. Further work is absolutely needed to determine a potential clinical use of these antivirals against ZIKV infections, but our results suggest the existence of a highly conserved mechanism across flavivirus, susceptible to be blocked by modified more specific adamantane compounds.

Keywords: Zika; Dengue; Yellow fever; Antivirals; Adamantanes; Amantadine; Rimantadine

1. Introduction

Zika virus (ZIKV) is a member of the genus *Flavivirus* within the *Flaviviridae* family, first isolated in 1947 from a rhesus macaque in the Zika Forest, Uganda [1]. It is a mosquito-borne arbovirus transmitted to humans by *Aedes aegypti* and *Aedes albopictus* [2]. The virion comprises an enveloped spherical particle with a positive single-stranded RNA genome, phylogenetically related to other flaviviruses of medical importance such as dengue (DENV), yellow fever (YFV), West Nile (WNV), and Japanese encephalitis (JEV) [3]. The first ZIKV human infection was reported in Nigeria in 1954 [4] and more than 50 years later the virus re-emerged, first in Yap Island in 2007 [5], then in French Polynesia in 2013 [6], and later in South and Central America, particularly in Brazil in 2015 [7]. This last outbreak was declared as a public health emergency of international concern by the World Health Organization (WHO) between February and November 2016.

The clinical spectrum produced by ZIKV range from asymptomatic infections in the majority of cases, to a mild influenza-like illness in 20% of infected people [5]. Severe manifestations such as Guillain-Barré syndrome occurred in 1/6,500 to 1/17,000 individuals in endemic regions [8], and during pregnancy consequences such as microcephaly in 2.3%

of babies from infected mothers [9], congenital ZIKV syndrome (CZS) and fetal demise, especially when infection occur during the first trimester of pregnancy, were observed [10]. Therefore, antivirals against ZIKV are necessary to treat and prevent these virus-induced neurological disorders during fetal development and in adulthood. From an epidemiological perspective, drug therapy is also needed to impair the chain of transmission of the virus [11].

Currently, there are no approved vaccines or antivirals to prevent or treatment against ZIKV infections. Researchers have been working on either the re-purposing of long-used drugs or the development of novel antivirals, especially those based on the use of antibodies as therapeutics [12,13]. To date, tested drugs are directed against viral and cellular targets and include antibiotics, anticancer and anti-inflammatory compounds, anti-parasite compounds, nucleosides analogues and nucleoside synthesis inhibitors, and drugs targeting viral enzymes[14]. However, most of the ongoing ZIKV clinical trials are focused on testing vaccine candidates and none include the evaluation of small molecule-based drug therapies [12].

Amantadine and its structural analogue rimantadine are two Food and Drug Administration (FDA)-approved antiviral therapies with known pharmacokinetics and pharmacodynamics, originally developed against influenza A infections and then applied to other respiratory viruses, i.e. Human parainfluenza viruses (HPIVs) and Respiratory syncytial virus (RSV) [15–20]. Consequently, people have been highly exposed to adamantanes for decades, as these drugs are sold without medical prescription in many countries around the world for the treatment of common colds [21]. Adamantanes have also shown antiviral *in vitro* activity against the severe acute respiratory syndrome coronavirus SARS-CoV-1 [22,23], and have been suggested as a possible therapeutic option for the treatment of the COVID-19 coronavirus SARS-CoV-2 [24].

Amantadine and rimantadine are suitable antiviral agents due to their high selectivity, their effectiveness at low concentrations, and their low toxicity at cellular and systemic levels with negligible side effects [25]. Adamantanes have also shown antiviral activity against flaviviruses, such as hepatitis C virus (HCV) [26], WNV [27], and DENV [28,29]. Nevertheless, to date there are no reports on the use of adamantanes to suppress ZIKV infection. Here we present our work that describes the antiviral *in vitro* activity of both amantadine and rimantadine hydrochlorides against a ZIKV clinical isolate and two different reference strains.

2. Materials and Methods

2.1. Viruses

The clinical isolate ZIKV CIET-01 (ZIKV/CR/CIET-01/2016) was previously isolated from sera of a diagnosed patient in Vero cells from *Cercopithecus aethiops* (ATCC) and confirmed as ZIKV by sequencing [30]. ZIKV MR 766 (ZIKV/UG/MR766/1947) and ZIKV R103451 (ZIKV/US/R103451/2016) strains were purchased from American Type Culture Collection (ATCC). Vaccine strain YFV 17D (YFV/US/17D/1937) was isolated from the commercial vaccine YF-VAX® (Sanofi Pasteur). ZIKV and YFV viruses stocks were produced in Vero cells by inoculating cellular monolayers at a multiplicity of infection (MOI) of 0.1 and incubating for 5 days with Minimum Essential Medium (MEM, Gibco) supplemented with 2% fetal bovine serum (FBS, Gibco) at 37 °C in an atmosphere of 5% CO₂. Culture supernatants were collected, centrifuged at 3000 × g for 10 min, aliquoted, and stored at -80 °C. Culture supernatant from uninfected Vero cells was also collected, stored, and used for mock infections. DENV-1 Angola (D1/AO/XX/1988) strain was supplied by the Instituto de Medicina Tropical Pedro Kourí, Havana, Cuba [31]. DENV-1 stock was produced in C6/36 cells from *Aedes albopictus* (ATCC) by inoculating a cellular monolayer at a MOI of 0.01 and incubating for 3 days with Roswell Park Memorial Institute medium (RPMI-1640, Gibco) supplemented with 2% FBS at 33 °C in 5% CO₂. Then, culture supernatant was collected and centrifuged at 3000 × g for 10 min. Before storage at -80 °C, 23%

newborn calf serum (NBCS, Gibco) was added [32]. All viruses were titrated by plaque assay in Vero cells as previously described [33]. Briefly, 10-fold serial dilutions of viruses were added to Vero confluent monolayers. After 2 hours of adsorption, cells were incubated at 37 °C in 5% CO₂ for 5 days with MEM AutoMod™ (Sigma) supplemented with 2% FBS and 1% carboxymethylcellulose (Sigma). Plaque numbers were counted after staining with crystal violet.

2.2. Antivirals

Amantadine and rimantadine hydrochlorides (Sigma) were dissolved in PBS, pH 7.2 (Gibco) by means of incubation in an ultrasonic bath (JPS) for 30 min at 37 °C, to obtain 1 mg/ml stock solutions that were used immediately upon preparation. Working solutions were prepared by 2-fold serial dilution in MEM 2% FBS.

2.3. Antiviral activity assay

Vero cells were seeded on 48-well tissue culture plates (Greiner Bio-One) at a density of 60 000 cells per well with MEM 10% FBS. After 18 hours of incubation at 37 °C in an atmosphere of 5% CO₂, cells were infected with either ZIKV CIET-01, ZIKV MR 766, ZIKV R103451, YFV 17D or DENV-1 Angola at a MOI of 0.1 and allowed to adsorb for 2 hours at 37 °C. Then, cells were washed once with PBS and incubated for 96 hours at 37 °C 5% CO₂ with MEM 2% FBS containing 0, 6.25, 12.5, 25, 50, and 100 µg/ml of amantadine or rimantadine hydrochlorides. Samples of culture supernatants were taken at 48, 72, and 96 hours post-infection and virions were quantified by plaque assays in Vero cells, as described above. The 50% inhibitory concentration (IC₅₀) and 90% inhibitory concentration (IC₉₀), defined as the concentrations of antiviral required to reduce virion production by 50% and 90%, respectively, were calculated using non-linear regression in the software GraphPad Prism 8.0.

2.4. Cell viability assay

Vero cells were seeded on µClear black 96-well plates (Greiner Bio-One) at a density of 15 000 cells per well with MEM-10% FBS. After 18 hours of incubation at 37 °C - 5% CO₂, cells were mock-infected for 2 hours and labeled with 1 µg/mL Hoechst 33342 (Invitrogen) for 10 min. Then, cells were washed once with PBS and incubated for 48 hours at 37 °C 5% CO₂ with MEM 2% FBS containing 0, 6.25, 12.5, 25, 50, and 100 µg/ml of amantadine or rimantadine hydrochlorides and 500 nM SYTOX green (Invitrogen). After incubation, fluorescence images of cells were acquired at 200X magnification with a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek). Image analysis was performed with the software CellProfiler 2.0 (<http://www.cellprofiler.org>; Broad Institute) to calculate the percentages of cells with condensed chromatin (brighter Hoechst 33342 + nuclei) and dead cells (SYTOX green + nuclei). The 50% cytotoxic concentration (CC₅₀), defined as the concentration of antiviral that reduces cell viability by 50%, was calculated using the percentage of dead cells and non-linear regression in the software GraphPad Prism 8.0.

2.5. Statistics

Data are expressed as mean ± standard deviation (SD) of three independent experiments. Statistical significance of the differences between mean values was determined by using a one-way ANOVA followed by a Tukey's post hoc test with the software SigmaPlot 14 (Systat Software Inc.). The level of significance is denoted in figure legends.

3. Results

3.1. Amantadine and rimantadine hydrochlorides have antiviral activity against a ZIKV clinical isolate at different times post-infection

To test the antiviral activity of amantadine and rimantadine hydrochlorides against ZIKV, we infected Vero cells monolayers with the clinical isolate ZIKV CIET-01 and incubate them with different doses of both drugs in the range 0-100 µg/ml. Then, we titrated by plaque assay at 48, 72, and 96 hours post-infection. We observed a dose-dependent reduction in ZIKV titers upon incubation with both amantadine and rimantadine hydrochlorides at all tested times post-infection, as depicted in the raw data and in plots shown in Figure 1 and Figure 2, respectively. The antiviral activity of both drugs against ZIKV was evident as soon as 48 hours post-infection, therefore we used this time point for subsequent experiments.

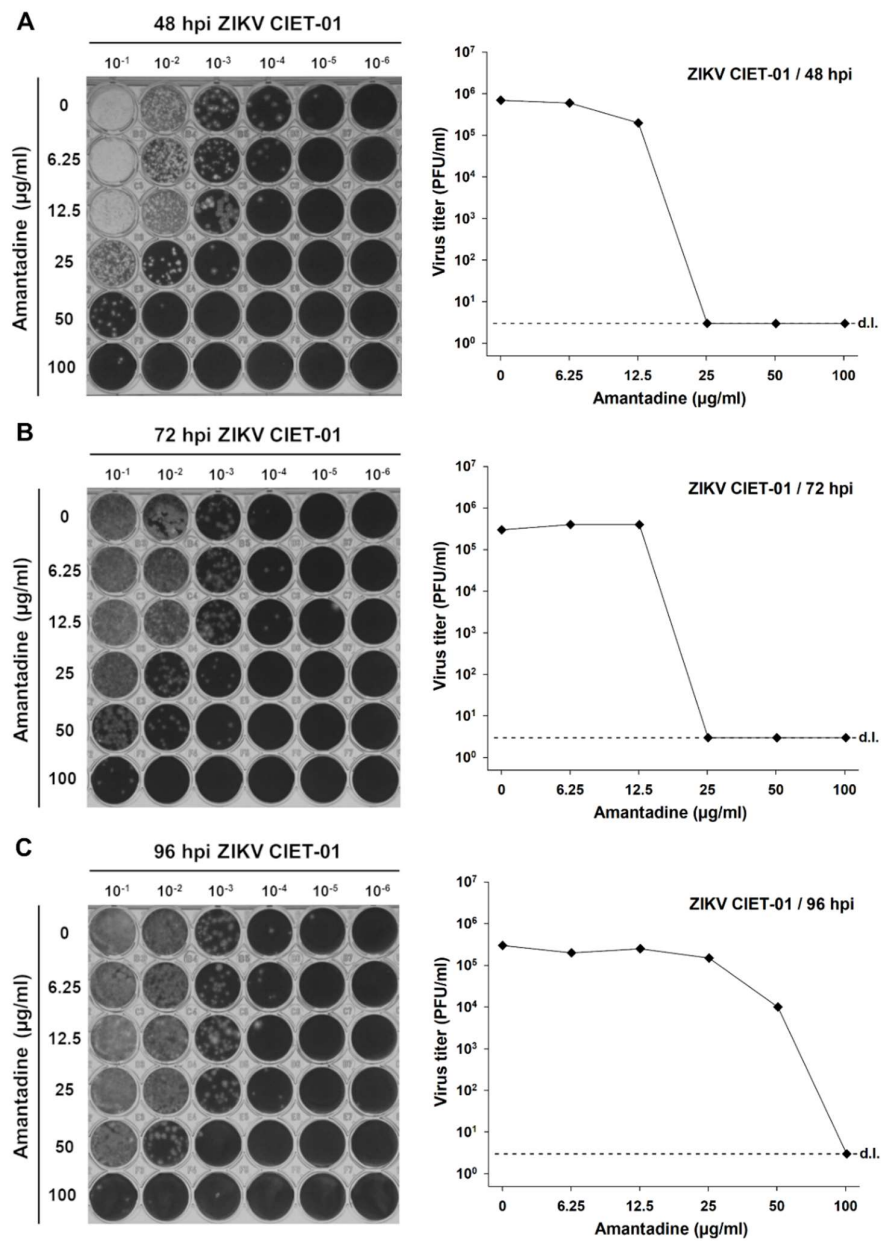


Figure 1. Amantadine hydrochloride suppresses the *in vitro* replication of a ZIKV clinical isolate. Virion production was quantified by plaque assays of culture supernatants of Vero cells treated

with different concentrations of amantadine hydrochloride at 48 (A), 72 (B), and 96 (C) hours post-infection (hpi) with ZIKV clinical isolate CIET-01 at a MOI of 0.1. Data from a single experiment are shown. d.l. = assay detection limit.

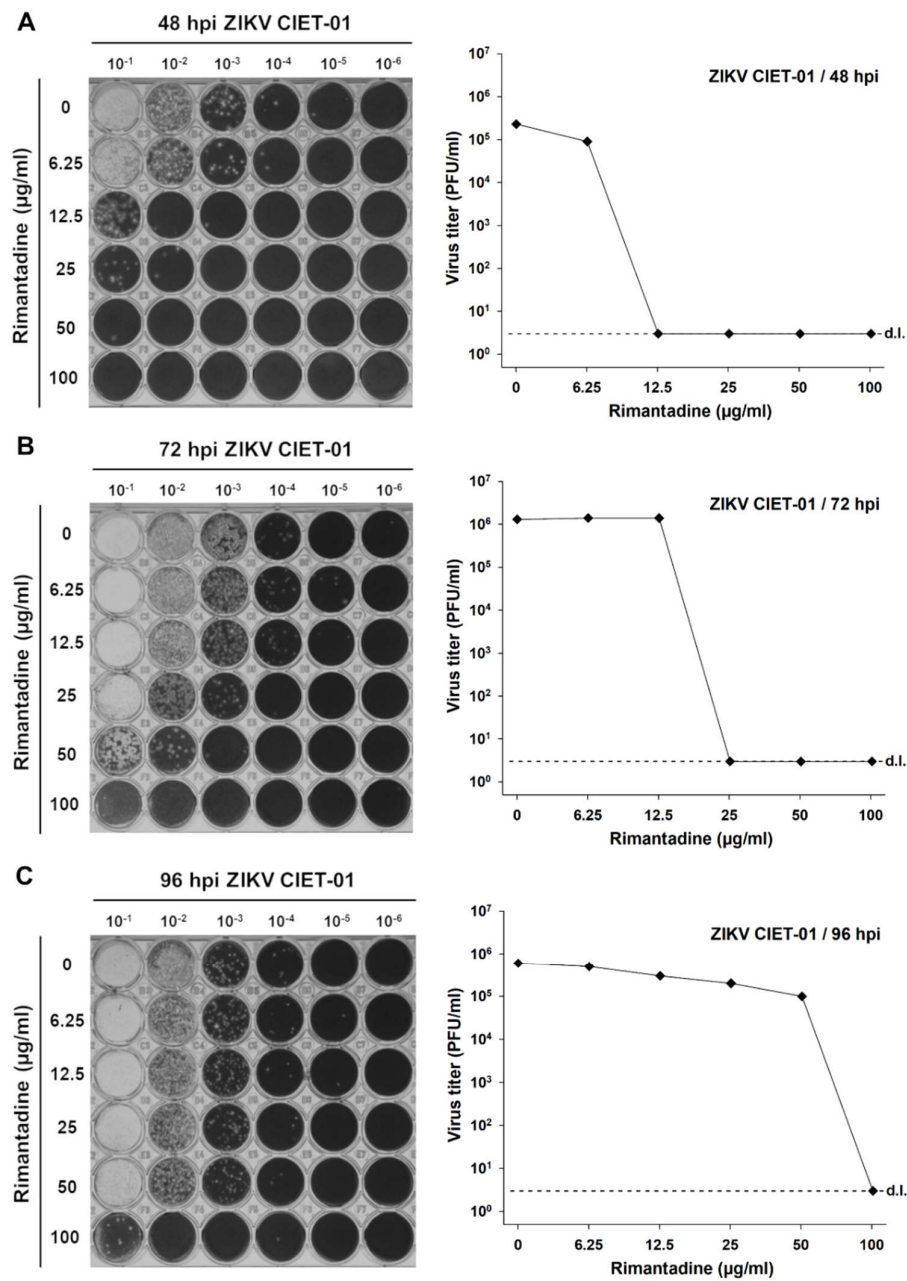


Figure 2. Rimantadine hydrochloride suppresses the *in vitro* replication of a ZIKV clinical isolate. Virion production was quantified by plaque assays of culture supernatants of Vero cells treated with different concentrations of rimantadine hydrochloride at 48 (A), 72 (B), and 96 (C) hours post-infection (hpi) with ZIKV clinical isolate CIET-01 at a MOI of 0.1. Data from a single experiment are shown. d.l. = assay detection limit.

3.2. Antiviral activity against ZIKV is not explained by a cytotoxic effect induced by the tested concentrations of adamantanes

To demonstrate that the observed antiviral activity on ZIKV replication was not produced by a cytotoxic side effect of adamantanes on Vero cells, we performed a cell viability assay at 48 hours post-treatment with the previously tested doses (0-100 µg/ml). The percentages of death cells and cells with condensed chromatin were calculated by SYTOX green and Hoechst 33342 staining, respectively, as previously described [30]. Even though, the percentages of death cells (cytotoxicity) and cells with condensed chromatin (cell damage) were significantly higher ($p < 0.001$) on cells treated with 100 µg/ml of both amantadine and rimantadine hydrochlorides compared to the untreated cells (Figure 3B and 3C, respectively), the calculated 50% cytotoxic concentrations (CC_{50}) were >100 µg/ml for both drugs. These results demonstrate that the tested doses of adamantanes have no considerable cytotoxic effects on Vero cells and are not responsible of the observed antiviral effect against ZIKV (Figure 1 and Figure 2).

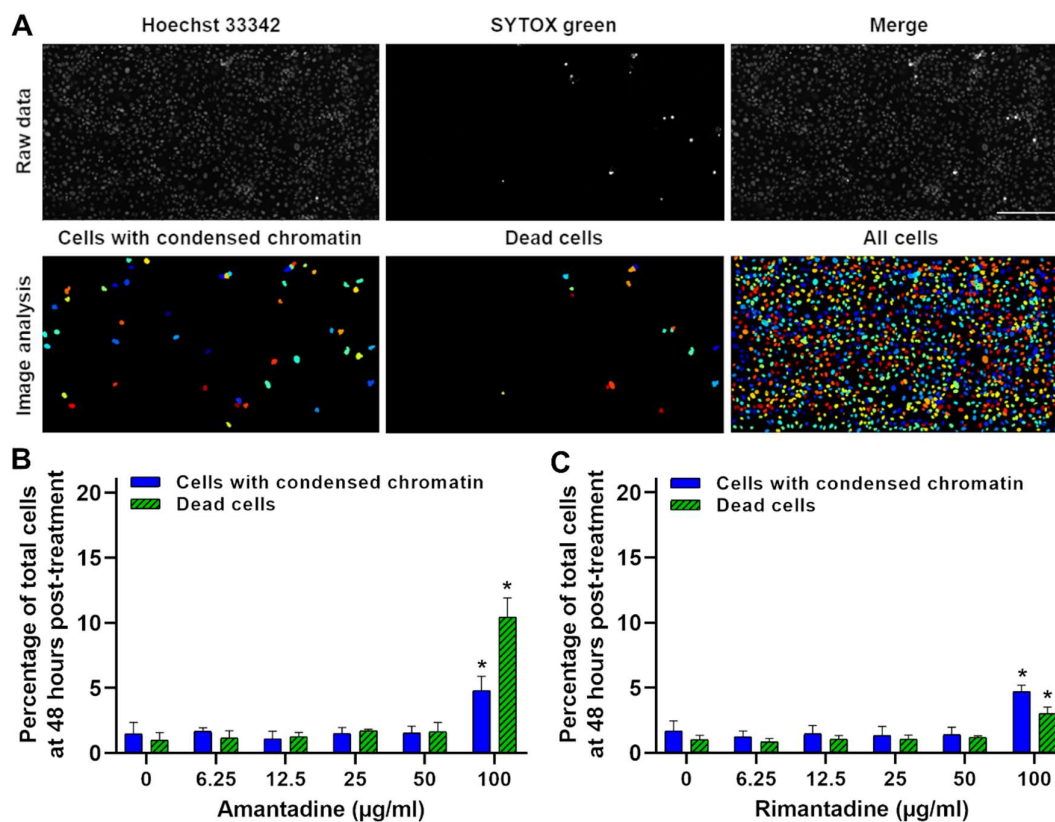
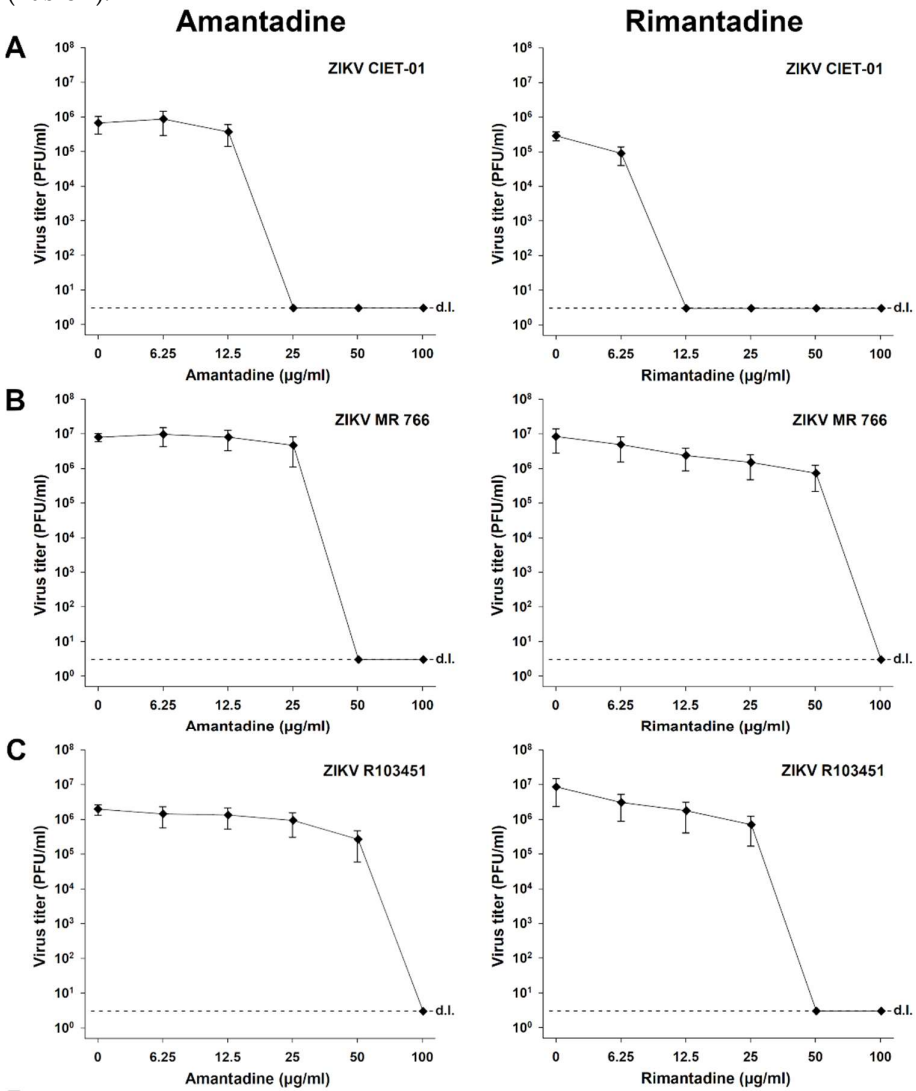


Figure 3. Amantadine and rimantadine hydrochlorides have no significant effect on Vero cells viability at concentrations with the shown antiviral activity against ZIKV. Vero cells monolayers were Hoechst 33342 stained and at 48 hours post-treatment with culture medium containing a combination of SYTOX green and different concentrations of amantadine or rimantadine hydrochlorides, cells were analyzed by live-cell imaging. (A) Image analysis of Hoechst 33342 and SYTOX green stained Vero cells at 48 hours post-treatment with 50 µg/ml of amantadine hydrochloride, to quantify the number of cells with condensed chromatin and dead cells, respectively. A representative experiment is shown (n = three independent experiments, magnification of 200X, scale bar = 200 µm). (B and C) Percentage of total Vero cells with condensed chromatin and dead cells at 48 hours post-treatment with different concentrations of amantadine or rimantadine hydrochlorides, respectively. CC_{50} values were >100 µg/ml for both treatments. Data are expressed as mean \pm SD of three independent experiments. * $p < 0.001$ calculated to cells with the same staining among different antiviral doses.

3.3. The antiviral activity of adamantanes is also observed for other ZIKV strains and DENV-1, though only slightly for YFV

Finally, we tested the antiviral activity of amantadine and rimantadine hydrochlorides in independent experiments for different ZIKV strains (ZIKV CIET-01, ZIKV MR 766, and ZIKV R103451), DENV-1, and YFV. As defined in previous experiments, we applied doses of both antivirals in the range 0-100 µg/ml and virion production was quantified at 48 hours post-infection by plaque assays. A dose-dependent reduction in the viral titers was observed for all tested ZIKV strains upon treatment with both adamantanes (Figure 4A-C). The antiviral activity against ZIKVs was also evidenced by the calculated 50% and 90% low inhibitory concentrations (IC₅₀ and IC₉₀, respectively) and high selectivity index (SI) values, as shown in Table 1. Similar results were obtained for DENV-1 (Figure 4D, Table 1), in accordance to previous reports [28,29], but not for YFV (Figure 4E). Among the tested flaviviruses, YFV showed the higher IC₅₀ and IC₉₀ values and the lower SI values (Table 1). These results demonstrate that the observed antiviral activity of adamantanes is reproducible with different ZIKV strains. Compared with other flaviviruses, this activity is similar to that obtained against DENV-1 and superior than the observed for YFV (Table 1).



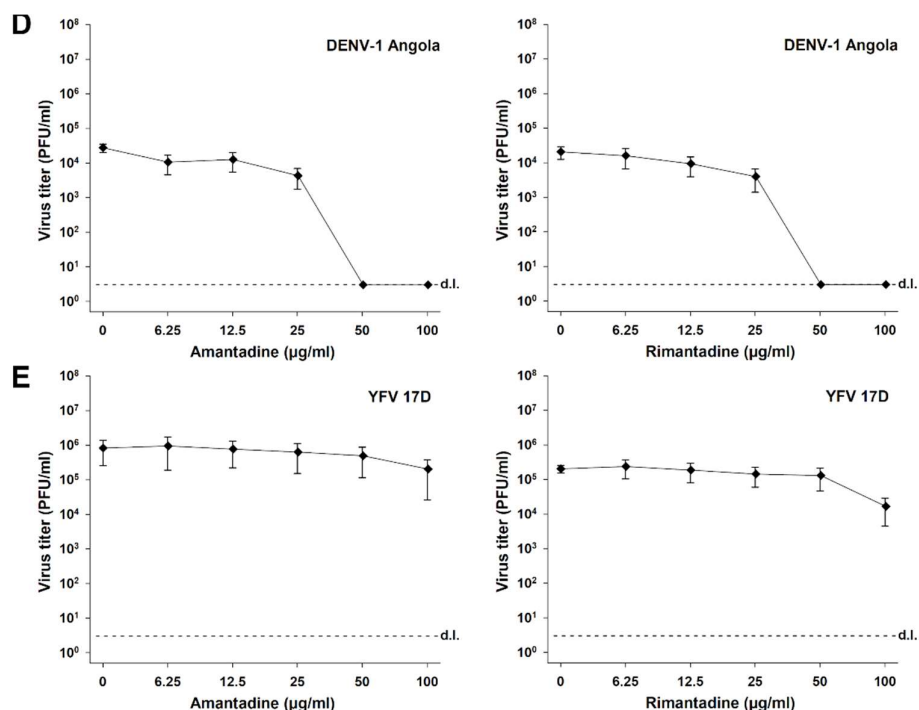


Figure 4. Amantadine and rimantadine hydrochlorides suppress the *in vitro* replication of different ZIKV strains, DENV-1, and YFV. Virion production was quantified by plaque assays of culture supernatants of Vero cells treated with different concentrations of amantadine or rimantadine hydrochlorides at 48 hours post-infection with ZIKV CIET-01 (A), ZIKV MR 766 (B), ZIKV R103451 (C), DENV-1 Angola (D), and YFV 17D (E) at a MOI of 0.1. Data are expressed as mean \pm SD of three independent experiments. d.l. = assay detection limit.

Table 1. Antiviral activity of amantadine and rimantadine hydrochlorides against ZIKV, DENV and YFV *in vitro*^a.

Virus	Amantadine (CC ₅₀ ^b >100 µg/ml)			Rimantadine (CC ₅₀ >100 µg/ml)		
	IC ₅₀ ^c (µg/ml)	IC ₉₀ ^d (µg/ml)	SI ^e	IC ₅₀ (µg/ml)	IC ₉₀ (µg/ml)	SI
ZIKV CIET-01	12.25 \pm 2.28	16.04 \pm 1.94	>8.16	5.89 \pm 0.22	6.96 \pm 0.19	>16.98
ZIKV MR 766	26.52 \pm 12.79	32.62 \pm 9.26	>3.77	8.95 \pm 3.79	36.04 \pm 11.19	>11.17
ZIKV R103451	29.05 \pm 13.79	61.48 \pm 19.00	>3.44	4.91 \pm 2.38	19.60 \pm 4.87	>20.37
DENV-1 Angola	12.81 \pm 3.56	35.11 \pm 5.06	>7.81	12.26 \pm 1.67	37.14 \pm 5.54	>8.17
YFV 17D	50.62 \pm 8.51	>100	>1.98	74.69 \pm 13.09	>100	>1.34

^a Data are expressed as mean \pm SD of three independent experiments

^b Concentration of antiviral that reduces cell viability by 50%

^c Concentration of antiviral that reduces virus titer by 50%

^d Concentration of antiviral that reduces virus titer by 90%

^e Selectivity index (CC₅₀/IC₅₀)

Altogether, these results demonstrate that both amantadine and rimantadine hydrochlorides have an antiviral activity against ZIKV that is not explained by a cytotoxic effect on the cellular model and this antiviral effect is reproducible with different ZIKV strains.

4. Discussion

Currently, no specific antiviral therapies have been approved for the treatment of ZIKV infections. The search for drugs against ZIKV is being conducted through different approaches, such as the screening of existing compound libraries and the repurposing of validated drugs used in the clinic for the treatment of other diseases, including many already known and used antivirals [34]. In the present work, we show that already ap-

proved and widely used antiviral drugs amantadine and rimantadine hydrochlorides inhibit ZIKV replication *in vitro*, as demonstrated by a dose-dependent reduction in viral titers of a ZIKV clinical isolate and two different reference strains upon incubation with these antivirals (Figure 4). Moreover, the replication of ZIKV was reduced at drug concentrations well below cytotoxic levels with both antivirals, as denoted by the high selectivity indexes obtained with all tested strains (Table 1).

These observations mentioned above are promising and constitute a good starting point aiming to the evaluation of the effectiveness of adamantanes to putatively hinder

ZIKV infections *in vivo*. There is extended evidence about the safety and effectiveness of *in vivo* use of amantadine and rimantadine hydrochlorides for the prophylaxis and treatment for influenza A infections [35,36]. Amantadine has also shown therapeutic potential in clinical trials for multiple sclerosis [37], depression [38], post-traumatic disorders of consciousness [39], and Parkinson's disease [40], among others conditions. Furthermore, amantadine has been used *in vivo* as a therapeutic option against other flaviviral diseases, such as hepatitis C and dengue fever, contributing to the clinical improvement of patients in terms of symptoms reduction and shortened recovery time, without notable side-effects at high doses of 200 and 300 mg/day, respectively [41,42].

Taking into account that the majority of ZIKV infections are asymptomatic or produce mild flu-like syndromes, the real target population for a possible antiviral intervention would be high risk patients and pregnant women. This sets an additional challenge on ZIKV antiviral research as the candidate drugs must be active against the virus but harmless to the mothers and fetuses. Despite theoretical concerns, adamantanes have not been demonstrated to be human teratogens and there is no causal reports that link their use with any obstetric complications. Pregnancy abnormalities reported in animal studies occur only at very high doses, 16 times higher than the normal dose concentrations used in humans [43,44]. Even more, amantadine treatment (200 mg/day) throughout pregnancy has already been reported with favorable fetal outcomes and non-associated complications [44,45]. Nevertheless, more studies and careful evaluation are needed on the administration of adamantanes as antiviral therapy in pregnant women.

A hypothetical mechanism for the observed antiviral activity of adamantanes on ZIKV can be proposed by previous findings in the literature. Many clinically important viruses, including HCV and SARS-CoV-1, contain small hydrophobic proteins with ion channel activity, usually less than 100 aminoacids in length with 1 or 2 transmembrane domains (TMDs), collectively known as viroporins [46–50]. By far the best-characterized member of the viroporin family is the M2 protein, a H⁺ channel of influenza A virus, which furthermore is the target of amantadine and rimantadine [16,51,52]. Among flaviviruses, amantadine has shown antiviral activity against HCV by a direct action on its P7 viroporin, as demonstrated by the fact that a L20F mutation in the P7 protein confers resistance to this drug in combined therapy with IFN- α [47,53,54]. This sets a precedent for the use of adamantanes as antivirals to target flaviviral viroporins. Interestingly, several viroporin candidates have been identified in other amantadine-sensitive flaviviruses, such as the 2K peptide, the membrane protein (M), and the non-structural proteins NS2A and NS2B of DENV [46,55–57] and the M protein of WNV [46]. Based on these observations and on our data, we hypothesize the presence and dependence of ZIKV replication on a yet unidentified viroporin. The blockage of this putative viroporin could be the mechanism of action of the herein tested adamantanes against ZIKV (Figure 4, Table 1). We found support to this hypothesis in the preliminary work from Brown and collaborators [58]. This research group demonstrated a reduction in ZIKV envelope protein (E) expression in Vero cells upon treatment with the viroporin inhibitor, rimantadine, in a dose dependent manner. Like us, this observation made them envisage the potential role of viroporin activity in ZIKV infection. Indeed, they combined molecular dynamic simulations with biochemical approaches to provide evidence that ZIKV M protein functions as a rimantadine-sensitive viroporin within virion membranes, with a potential role during

the entry and uncoating of infectious viral particles. They also used *in vivo* ZIKV preclinical models to demonstrate that rimantadine reduces viraemia, supporting that M protein channel activity is a relevant physiological target to block ZIKV infection [58].

Here we report the *in vitro* ZIKV susceptibility to both amantadine and rimantadine hydrochlorides. More work is needed to test the effectiveness of these antivirals against ZIKV *in vivo*, both in animal models and in clinical studies, as this may represent a new approach in the treatment of ZIKV infections by broadening the spectrum of use of these well validated and already approved antivirals.

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