

1 **Title: Chloroquine inhibits Zika Virus infection in different cellular models**

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12 **Summary**

13 Zika virus (ZIKV) infection *in utero* might lead to microcephaly and other  
14 congenital defects. In adults, cases of Guillain-Barré syndrome and meningoencephalitis  
15 associated with ZIKV infection have been reported, and no specific therapy is available so  
16 far. There is urgency for the discovery of antiviral agents capable of inhibiting viral  
17 replication and its deleterious effects. Chloroquine is widely administered as an antimalarial  
18 drug, anti-inflammatory agent, and it also shows antiviral activity against several viruses.  
19 Here we show that chloroquine exhibits antiviral activity against ZIKV in VERO, human  
20 brain microvascular endothelial, and neural stem cells. We demonstrated *in vitro* that  
21 chloroquine reduces the number of ZIKV-infected cells, virus production and cell death  
22 promoted by ZIKV infection without cytotoxic effects. Our results suggest that chloroquine  
23 is a promising candidate for ZIKV clinical trials, since it is already approved for clinical  
24 use and can be safely administered to pregnant woman.

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29           **INTRODUCTION**

30           Zika virus (ZIKV) is an arthropod-borne virus, transmitted by *Aedes* mosquitoes,  
31 that belongs to the *Flavivirus* genus, which also includes other pathogens such as West  
32 Nile Virus (WNV), Yellow Fever Virus (YFV), Japanese Encephalitis Virus (JEV) and  
33 Dengue Virus (DENV). Phylogenetic analysis of the nonstructural protein 5 encoding  
34 region identified three ZIKV lineages: East African, West African and Asian (Lanciotti et  
35 al., 2008).

36           *In utero* exposure to ZIKV might lead to microcephaly and other developmental  
37 malformations including calcifications, arthrogryposis, ventriculomegaly, lissencephaly,  
38 cerebellar atrophy and ocular abnormalities, which altogether are referred as congenital  
39 Zika syndrome (Brasil et al., 2016; Martines et al., 2016; Mlakar et al., 2016; Oliveira Melo  
40 et al., 2016). Although all ZIKV lineages can infect humans, severe manifestations of the  
41 infection have only been associated to Asian lineages, including Brazilian isolates (Calvet  
42 et al., 2016; Mlakar et al., 2016).

43           An unprecedented increase in microcephaly cases associated with ZIKV infection  
44 prompted the World Health Organization (WHO) to declare it a Public Health Emergency  
45 of International Concern. Brazil is currently the most affected country with 1,168 cases  
46 confirmed of ZIKV-related microcephaly (<http://portalsaude.saude.gov.br>).

47           ZIKV was detected in the brain and amniotic fluid of newborns and stillborns with  
48 microcephaly (Brasil et al., 2016; Calvet et al., 2016; Martines et al., 2016; Mlakar et al.,  
49 2016) and it was shown to kill human neuroprogenitor cells *in vitro* as well as decrease the  
50 brain organoid growth rate (Garcez et al., 2016; Tang et al., 2016). After reviewing several  
51 evidences, the Centers for Disease Control and Prevention (CDC) concluded that Zika virus  
52 infection causes microcephaly and other congenital defects (Rasmussen et al., 2016).

53 Symptoms of ZIKV infections include low-grade fever, headache, rash,  
54 conjunctivitis, arthritis and myalgia (Brasil et al., 2016; Duffy et al., 2009). However, in a  
55 minor number of cases, infection is associated with cases of Guillain-Barré syndrome (Cao-  
56 Lormeau et al., 2016) and meningoencephalitis (Carteaux et al., 2016). Currently, there is  
57 no vaccine or specific therapeutic approaches to prevent or treat ZIKV infections. With the  
58 alarming increase in the number of countries affected and the potential for viral spread  
59 through global travel and sexual transmission (D'Ortenzio et al., 2016; Deckard et al.,  
60 2016), there is an urgency to find a treatment capable of lessen the effects of the disease  
61 and inhibit further transmission.

62 Chloroquine, a 4-aminoquinoline, is a weak base that is rapidly imported into acidic  
63 vesicles increasing their pH (Browning, 2014). It is approved by the Food and Drug  
64 Administration (FDA) to treat malaria and has long been prophylactically prescribed to  
65 pregnant women at risk of exposure (Levy et al., 1991). Chloroquine, through inhibition of  
66 pH-dependent steps of viral replication, restricts HIV (Tsai et al., 1990), Influenza virus  
67 (Ooi et al., 2006), DENV (Juvenal et al., 2013), JEV (Zhu et al., 2012) and WNV infection  
68 (Boonyasuppayakorn et al., 2014). Here, we sought to investigate the antiviral effects of  
69 chloroquine on ZIKV infection in different cell types.

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## 73 **RESULTS**

### 74 **ZIKV infection is inhibited by chloroquine in Vero cells**

75 We have initially characterized the antiviral properties of chloroquine in Vero cells,  
76 a model widely used to study viral infections. Vero cells were infected with ZIKV MR766

77 at MOI 2 and then treated with chloroquine in concentrations ranging from 6.25 to 50  $\mu$ M  
78 for 5 days. Viral infectivity was assessed using 4G2 antibody, which detects flavivirus  
79 envelope protein. We observed that chloroquine treatment decreases the number of ZIKV-  
80 infected cells in a dose dependent manner. Flow cytometry analysis showed a reduction of  
81 65% and 95% in ZIKV-infected cells treated with 25  $\mu$ M and 50  $\mu$ M chloroquine,  
82 respectively, compared to untreated infected cells (Fig. 1A). These results were  
83 corroborated by immunofluorescence staining (Fig. 1B). Additionally, chloroquine  
84 decreases the production of infectious virus particles by ZIKV-infected cells (Fig. 1C). To  
85 confirm that viral inhibition is independent of chloroquine cytotoxicity, cell viability was  
86 analyzed in uninfected cells treated with chloroquine (1.56 to 200  $\mu$ M) for 5 days.  
87 Chloroquine does not impact cell viability at 50  $\mu$ M or lower concentrations (Fig. 1D). We  
88 further analyzed whether chloroquine treatment could protect Vero cells from ZIKV-  
89 induced cell death. Chloroquine, ranging from 12.5 to 50  $\mu$ M, rescued cell viability to 55-  
90 100% (Fig. 1E).

### 91 **Chloroquine reduces ZIKV infection in hBMEC, an *in vitro* model of blood-** 92 **brain barrier**

93 Considering that ZIKV infects human brain microvascular endothelial cells  
94 (hBMECs) (Bayer et al., 2016), we investigated whether chloroquine could inhibit viral  
95 infection of these cells. Chloroquine reduces the number of ZIKV-infected hBMECs to  
96 20% and 8% at 25 and 50  $\mu$ M, respectively (Fig. 2A and D). These concentrations are non-  
97 cytotoxic (Fig. 2B) and protected around 80% of hBMECs from ZIKV-induced death (Fig.  
98 2C).

### 99 **Chloroquine inhibits ZIKV infection in human neural progenitor cells**

100 Neural stem cells (NSCs) are key cells in the process of corticogenesis, giving rise  
101 to the three main cell types of central nervous system: neurons, astrocytes and  
102 oligodendrocytes. Primary microcephaly occurs mainly as a result of the depletion of the  
103 NSCs pool (Gilmore and Walsh, 2013). In order to evaluate if chloroquine could protect  
104 these cells from ZIKV infection, they were exposed to up to 50 $\mu$ M chloroquine for 4 days.  
105 Chloroquine treatment decreases 60% of the number of ZIKV-infected hNSC cells and  
106 protects 70% of these cells from ZIKV cytopathic effects, reducing death without  
107 cytotoxicity (Fig. 3A-D).

#### 108 **Chloroquine inhibits ZIKV infection in mouse neurospheres**

109 Neuroprogenitor cells when submitted to differentiation culture conditions generates  
110 neurospheres and induced to differentiate into neurons. Our group showed that ZIKV  
111 infection affects neurospheres size, neurite extension and neuronal differentiation  
112 (Campanati et al., 2016). As we previously observed, neurospheres infected with MR766  
113 ZIKV showed convoluted and misshappen neurites. Neurite extension was evaluated in  
114 chloroquine treated cultures by Map2 staining and phase contrast and although many  
115 neurospheres were severely impacted by the infection, many other showed the same aspect  
116 of mock-infected spheres indicating that chloroquine treatment rescued neurite extension  
117 phenotype (Fig. 4A-C). ZIKV infection decreased when 12.5  $\mu$ M chloroquine was added to  
118 the medium, as evaluated by 4G2 staining (Fig. 4D-F).

#### 119 **Chloroquine inhibits Asian and African ZIKV strains infection.**

120 Microcephaly cases and neurological disorders have only been associated to the  
121 Asian strains of ZIKV, detected in the French Polynesia and in the Americas (Calvet et al.,  
122 2016; Cao-Lormeau, 2014; Mlakar et al., 2016). To determine the inhibition spectrum of  
123 chloroquine against ZIKV infection, Vero cells were infected with the African lineage

124 MR766 and two Brazillian isolates of the Asian lineage (ZIKV BR1 and ZIKV BR2). The  
125 levels of viral RNA in the supernatant of Vero cells were determined as a direct  
126 measurement of ZIKV infection. Treatment with 25 or 50  $\mu$ M chloroquine led to a 30 to  
127 40-fold reduction in ZIKV particle production, regardless the viral lineage used (FIG. S1).

### 128 **Chloroquine inhibits early stages of ZIKV infection**

129 Inhibition of viral infection mediated by chloroquine can occur in both early and  
130 later stages of ZIKV replication cycle (Savarino et al., 2003). To evaluate which step of the  
131 viral cycle was susceptible to inhibition, chloroquine was added to Vero cells at different  
132 time points post-infection. Supernatant was collected 30 hours post-infection and virus  
133 production was evaluated by relative quantification of viral RNA over untreated control by  
134 qPCR. Virus titers were also determined by plaque assay in Vero cells. Incubation of Vero  
135 cells with chloroquine at 0 hpi had a greater impact on production of ZIKV particles,  
136 decreasing 70 times viral RNA over control. Addition of chloroquine from 30 minutes to 12  
137 hours post-infection was able to reduce virus release 9-20 times over untreated, infected-  
138 cells. However, chloroquine added at 24 hpi had no effect on viral production (FIG. 5A).  
139 These results were confirmed by quantification of ZIKV infectious particles release after  
140 chloroquine treatment (FIG. 5B). These data confirm that chloroquine targets mainly early  
141 stages of viral infection.

### 142 **DISCUSSION**

143 From January 2007 to April 2016, ZIKV transmission has been reported in 64  
144 countries and territories (WHO, 2016). Although Zika virus disease is in general mild, the  
145 recent correlation between infection and congenital malformations and neurological  
146 damages in adults has intensified the need for therapeutical approaches. Prophylactic  
147 treatment for women that intend to get pregnant in epidemic areas and travelers going to

148 affected countries would represent relevant tools to reduce ZIKV transmission and avoid  
149 the spread of the disease by travelers. Moreover, a drug that blocks placental transfer of the  
150 virus could decrease the chance of vertical transmission in viremic pregnant women as was  
151 shown for HIV-infected pregnant women treated with anti-retroviral therapy (Connor et al.,  
152 1994).

153 Here we demonstrate that chloroquine decreases the number of ZIKV-infected cells  
154 and protected them from ZIKV-induced cell death at non-cytotoxic concentrations. The half  
155 maximal effective concentration (EC50) of chloroquine, concentration that protected 50%  
156 of cells from ZIKV-induced death, was 9.82-14.2  $\mu\text{M}$  depending on cell model and 50%  
157 cytotoxicity concentration (CC50) ranged from 94.95-134.54  $\mu\text{M}$  (Table 1). These values  
158 of EC50 are lower than those obtained for DENV inhibition (around 25  $\mu\text{M}$ ) and HIV  
159 inhibition (100  $\mu\text{M}$ ) (Juvenal et al., 2013; Tsai et al., 1990).

160 A clinical trial of chloroquine administration to DENV-infected patients during  
161 three days showed that 60% of the patients in the chloroquine treated group reported feeling  
162 less pain and showed improvement in the performance of daily chores during treatment.  
163 Symptoms returned after medication withdrawal and chloroquine treatment did not reduce  
164 the duration and intensity of the fever or duration of disease (Borges et al., 2013).

165 Chloroquine is widely distributed to body tissues as well as its analogue  
166 hydroxychloroquine. Concentration of hydroxychloroquine in the brain is 4-30 times higher  
167 than in the plasma (Titus, 1989). The concentration of chloroquine in the plasma reached 10  
168  $\mu\text{M}$  when a daily intake of 500 mg was prescribed to arthritis patients (Mackenzie, 1983).  
169 Chloroquine is able to cross the placental barrier and is supposed to reach similar  
170 concentrations on maternal and fetal plasma (Law et al., 2008). Concentrations needed to  
171 inhibit ZIKV infection *in vitro*, as shown here, are achieved in the plasma in current

172 chloroquine administration protocols and might even reach the brain. Since it is a molecule  
173 already approved for clinical use by the FDA and other agencies around the world, its  
174 approval as a therapeutic agent against ZIKV should be faster than new compounds.

175         The use of chloroquine during pregnancy was evaluated and when prophylactic  
176 doses of chloroquine were administered for malaria (400 mg/week), no increment in birth  
177 defects was observed (Wolfe and Cordero, 1985). Higher concentrations (250 mg to 500  
178 mg/day) are administered to pregnant women who have severe diseases, such as lupus or  
179 rheumatoid arthritis. Few cases of abortion and fetal toxicity were observed. However, fetal  
180 toxicity or death could not be discarded as consequence of disease itself since, in most  
181 cases, disease was active during pregnancy. In addition, all term deliveries resulted in  
182 healthy newborns (Levy et al., 1991; Parke, 1988).

183         Different mechanisms for chloroquine inhibition of viral infection have been  
184 described (Savarino et al., 2003). We observed a higher reduction of ZIKV particles release  
185 when the drug was added at 0 hours post-infection, suggesting a higher impact on early  
186 stages of infection, possibly during fusion of the envelope protein to the endosome  
187 membrane. Chloroquine inhibits acidification of the endosome, consequently inhibiting the  
188 low pH-induced conformational changes required for the fusion of the envelope protein of  
189 flaviviruses with the endosomal membrane (Smit et al., 2011). However, even when  
190 chloroquine was added after early stages of virus infection, except for addition of  
191 chloroquine 24 hours post-infection, we noticed a decrease in virus release, suggesting later  
192 stages of ZIKV cycle might also be affected, although we cannot eliminate a potential  
193 impact on a new cycle of infection.

194         The proposed mechanism for chloroquine action on later-stages of viral replication  
195 relates to the alteration of post-translational modifications in the trans-golgi network.



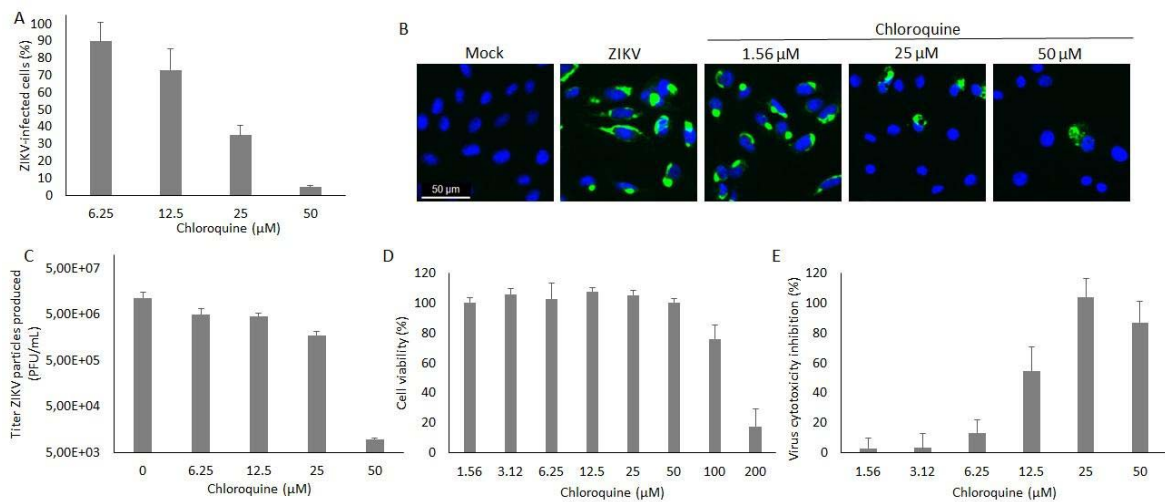
196 Chloroquine treatment impairs *Flavivirus* prM cleavage, which prevents virus maturation  
197 and, consequently, infectivity (Randolph et al., 1990; Zybert et al., 2008). For HIV-1,  
198 chloroquine inhibits the glycosylation of the gp120 protein, responsible for cell attachment  
199 (Tsai et al., 1990). The effect of chloroquine on the glycosylation of ZIKV or other  
200 flavivirus envelope protein has yet to be addressed.

201 ZIKV was detected in the cerebrospinal fluid of ZIKV-infected adult patients that  
202 manifested meningoencephalitis, indicating that ZIKV penetrates the central nervous  
203 system through yet unknown mechanisms. Transcytosis through the endothelial cells of the  
204 blood brain barrier is a known mechanism of viral access to the central nervous system  
205 (Dohgu et al., 2012; Suen et al., 2014). Here we demonstrated that chloroquine protects  
206 hBMEC, an *in vitro* model of the blood-brain barrier, from ZIKV infection and ZIKV-  
207 induced death.

208 Recent studies showed that neural stem cells are highly permissive for ZIKV  
209 infection and one of mechanisms proposed for the cause of microcephaly would be the  
210 depletion of the stem cell pool induced by ZIKV (Garcez et al., 2016; Qian et al., 2016;  
211 Tang et al., 2016). Our data showed that chloroquine inhibits infection; decreasing the  
212 number of induced pluripotent stem cells-derived neural stem cells infected with ZIKV and  
213 partially protecting them against ZIKV-induced death. Using the mouse neurospheres  
214 model to study neural stem cell differentiation into neurons, another process that might be  
215 disturbed in microcephaly, we observed that chloroquine inhibited the infection of neuronal  
216 progenitors and partially protected the ability of these cells to extend neurites. The  
217 protective effect of chloroquine on stem cells and committed progenitors is potentially a  
218 groundbreaking feature of this compound, as it would be prescribed to women at  
219 childbearing age traveling to affected countries and women planning pregnancy in endemic

220 areas. This would decrease the chances of infection and thus fetal damage, especially to the  
221 developing brain.

222 Altogether, our results suggest that chloroquine activity against ZIKV should  
223 immediately be evaluated *in vivo* and hopefully it will mitigate the devastating brain  
224 damage associated with congenital Zika syndrome and neurological damage in affected  
225 adults.

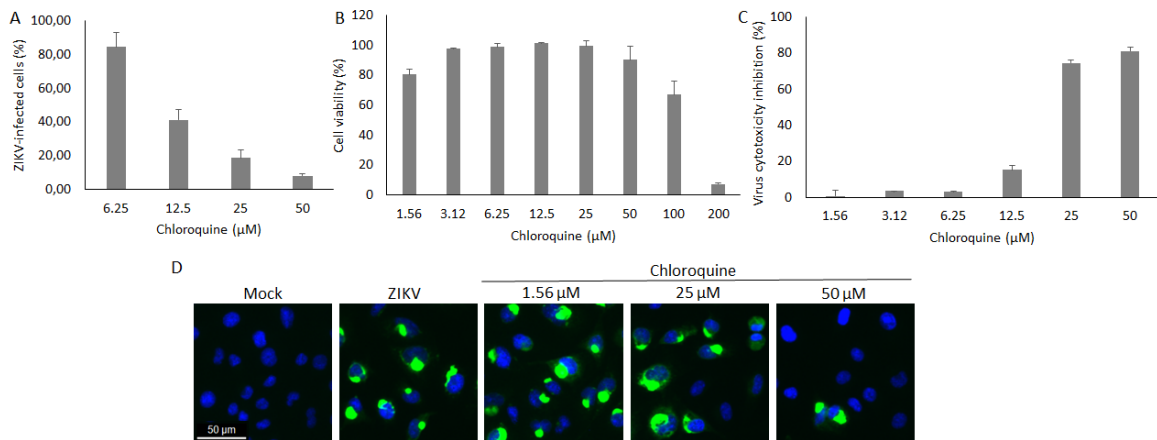


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227 **Figure 1. Inhibition of ZIKV infection by chloroquine in Vero cells.** Vero cells were  
228 infected with ZIKV MR766 at MOI 2 and treated with chloroquine for 5 days when cells  
229 were stained for the viral envelope protein and analyzed by flow cytometry (A). (B)  
230 Chloroquine treatment of ZIKV-infected cells was evaluated by immunofluorescence  
231 staining of 4G2 antibody (green) and DAPI (blue). (C) Infectious virus particles were  
232 quantified on supernatant. (D) Chloroquine cytotoxicity was evaluated by cell viability of  
233 uninfected cells treated with chloroquine. (E) Protection against ZIKV-induced cell death  
234 was evaluated in ZIKV-infected Vero cells treated with chloroquine for 5 days. Data were  
235 normalized by each experiment control. Data are represented as mean  $\pm$  SD.

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240 **Figure 2. Chloroquine reduces the number of ZIKV-infected hBMEC and protects**

241 **hBMEC from ZIKV-induced cell death. (A)** hBMEC were infected with ZIKV MR 766

242 at MOI 2 followed by chloroquine treatment for 5 days. Cells were stained with 4G2

243 antibody and analyzed by flow cytometry. (B) Uninfected hBMECs were incubated with

244 chloroquine for 5 days and cell viability was analyzed. (C) Protection against ZIKV-

245 induced cell death was measured in chloroquine-treated, ZIKV-infected cells. (D)

246 Immunofluorescence with 4G2 antibody (green) and DAPI (blue) of ZIKV-infected cells

247 treated with chloroquine for 5 days. Data are represented as mean  $\pm$  SD.

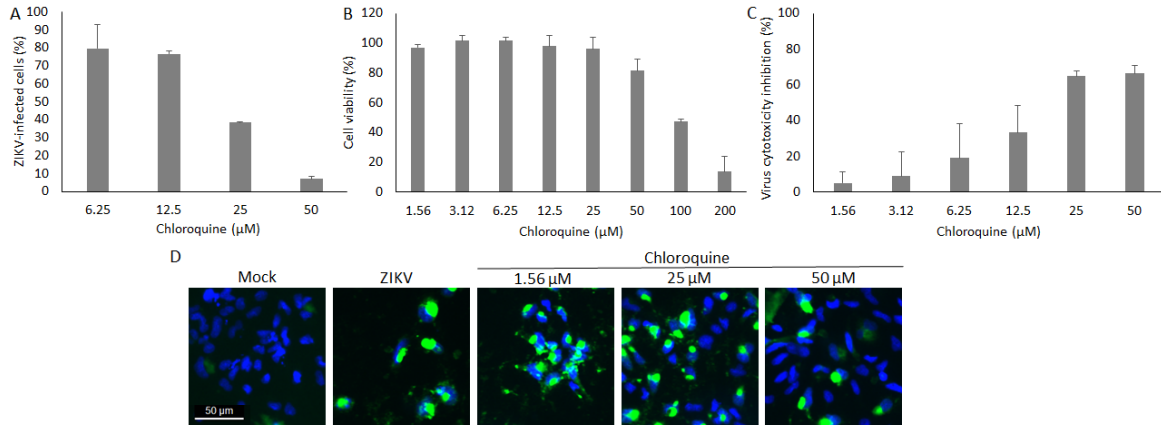
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**Figure 3. Chloroquine inhibits ZIKV infection in human NSCs.** NSCs were infected

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with ZIKV MR 766 at MOI 2 and incubated with increasing concentrations of chloroquine

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for 5 days. (A) The number of ZIKV-infected cells was analyzed by 4G2 staining and flow

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cytometry. (B) Chloroquine cytotoxicity was assessed by the viability of uninfected NSC

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treated with chloroquine. (C) Chloroquine treatment protection from deleterious effects of

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infection was evaluated after 5 days of infection. (D) Immunofluorescence of infected and

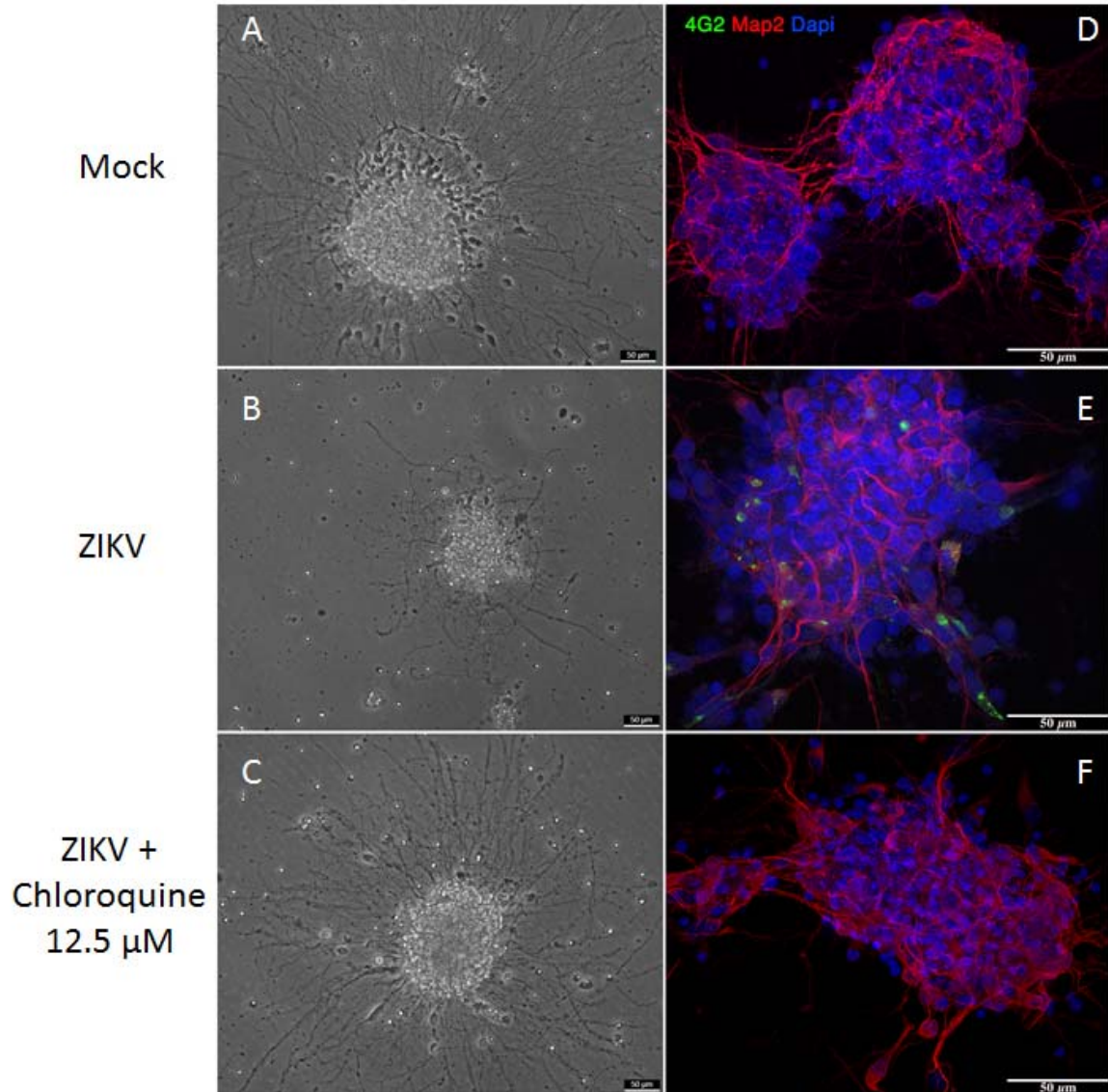
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treated cells with 4G2 antibody (green) and DAPI (blue). Data are represented as mean  $\pm$

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SD.

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**Figure 4. Chloroquine inhibits ZIKV infection in mouse neurospheres.** Mouse

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neurospheres were infected with ZIKV MR766 and treated with chloroquine for 3 days.

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Neurospheres were analyzed by phase contrast microscopy (A-C) and triple stained for

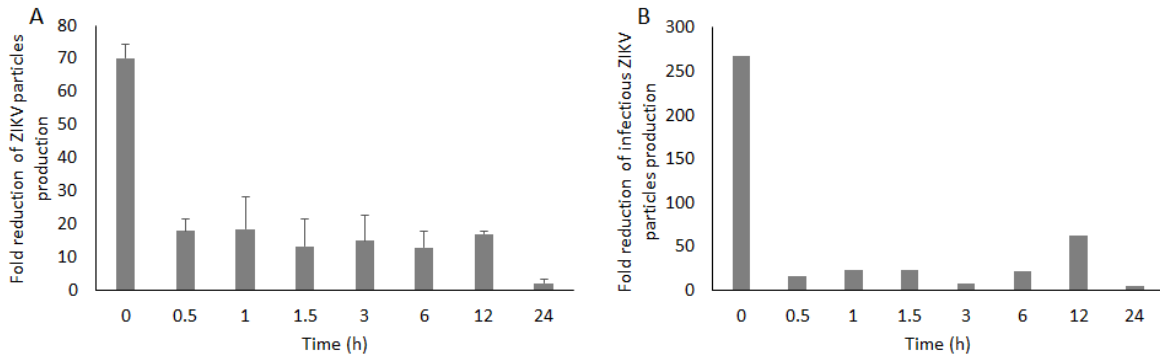
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envelope viral protein (green), Map-2 (red), a neuron-specific protein, and DAPI (blue) (D-

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F).

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271 **Figure 5. Early stages of infection are inhibited by chloroquine.** Vero cells were  
272 infected with ZIKV MR766 at MOI 10 and chloroquine added at different times post-  
273 infection when the supernatant was collected and viral RNA (A) or infectious particles (B)  
274 were quantified.

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276 **Table 1. Pharmacological parameters of chloroquine in each cell type.**

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Cell type	CC50	EC50	TI
Vero	134.54 ± 16.76 µM	9.82 ± 2.79 µM	13.70
hBMEC	116.61 ± 9.70 µM	14.20 ± 0.18 µM	8.21
hNSC	94.95 ± 9.38 µM	12.36 ± 2.76 µM	7.68

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279 CC50 - 50% cytotoxicity concentration; EC50 - half maximal effective concentration; TI - therapeutic index  
280 (CC50/EC50). Data are represented as mean ± SD.

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282 **Author contribution:** **RD** designed and performed experiments, prepared figures and/or

283 tables, analyzed the data and wrote the manuscript, **LH** designed and performed

284 experiments, prepared figures and/or tables, analyzed the data and wrote the manuscript;

285 **PP** designed and performed experiments, prepared figures and/or tables, analyzed the data

286 and wrote the manuscript; **AV** designed and performed experiments, prepared figures and/

287 or tables, analyzed the data and wrote the manuscript; **PG** designed and performed

288 experiments, analyzed the data and wrote the manuscript; **FM** designed and performed  
289 experiments, prepared figures and/ or tables and analyzed the data; **EL** performed  
290 experiments; **SR** designed and performed experiments, contributed with  
291 reagents/materials/analysis tools and wrote the manuscript; **LC** designed and performed  
292 experiments, prepared figures and/or tables, analyzed the data, contributed with  
293 reagents/materials/analysis tools and wrote the manuscript. **RS** designed and performed  
294 experiments, contributed with reagents/materials/analysis tools and wrote the manuscript;  
295 **AT** designed and performed experiments, prepared figures and/or tables, analyzed the data,  
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301 **The authors declare no competing financial interests.**

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