

1 **Title:**

2 The clinically approved antiviral drug sofosbuvir impairs Brazilian zika virus
3 replication

4 **Running title:** sofosbuvir impairs zika virus replication

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Summary

Zika virus (ZIKV) is a member of *Flaviviridae* family, as other agents of clinical significance, such as dengue (DENV) and hepatitis C (HCV) viruses. ZIKV spread from Africa to Pacific and South American territories, emerging as an etiological pathogen of neurological disorders, during fetal development and in adulthood. Therefore, antiviral drugs able to inhibit ZIKV replication are necessary. Broad spectrum antivirals, such as interferon, ribavirin and favipiravir, are harmful for pregnant animal models and women. The clinically approved uridine nucleotide analog anti-HCV drug, sofosbuvir, has not been affiliated to teratogenicity. Sofosbuvir target the most conserved protein over the members of the *Flaviviridae* family, the viral RNA polymerase. We thus studied ZIKV susceptibility to sofosbuvir. We initially characterized a Brazilian ZIKV strain for use in experimental assays. Sofosbuvir inhibits the Brazilian ZIKV replication in a dose-dependent manner, both in BHK-21 cells and SH-Sy5y, by targeting ZIKV RNA polymerase activity, with the involvement of conserved amino acid residues over the members of *Flaviviridae* family. The identification of clinically approved antiviral drugs endowed with anti-ZIKV could reduce the time frame in pre-clinical development. Altogether, our data indicates that sofosbuvir chemical structure is endowed with anti-ZIKV activity.

Key-words: Zika virus, antiviral, sofosbuvir, nucleoside/nucleotide analogs, microcephaly and Guillain-Barré syndrome, inhibitors.

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84 INTRODUCTION

85 Zika virus (ZIKV) is a member of the *Flaviviridae* family, which includes
86 several agents of clinical significance, such as dengue (DENV), hepatitis C (HCV), west
87 Nile (WNV) and Japanese encephalitis (JEV) viruses, among others. This emerging
88 pathogen is an enveloped, positive-sense single stranded RNA virus. Although ZIKV is
89 an arthropod-borne virus (arbovirus) transmitted by mosquitos of the genus *Aedes* (1),
90 transmission through sexual contact have been described (2).

91 In 1947, in the Zika forest of Uganda, ZIKV was originally identified in sentinel
92 monkeys (3). After occasional episodes of infection in humans in the 50's, outbreaks
93 have been registered in 2007 (Federated States of Micronesia) and 2013 (French
94 Polynesia) (1). Computational analyses suggest that ZIKV may have been introduced in
95 Brazil already in 2013 (4). In 2015, ZIKV explosively spread across the Brazilian
96 territory and to neighbor countries. Although the epidemiological numbers of zika
97 infection in Brazil may be underestimated – due to limited resources for patient
98 assessments in area where people live below the poverty line – it was predicted that
99 more than 4 million persons were infected (5). ZIKV provokes mild and self-limited
100 exanthematic disease with no or low-grade fever for many patients (6). Remarkably,
101 however, ZIKV infection has been associated to congenital malformations, including
102 microcephaly, and Guillain-Barré syndrome (GBS), based on clinical and laboratorial
103 data (7, 8). Consequently, the World Health Organization (WHO) declared ZIKV
104 infection as a public health emergency of international concern. Antiviral treatments
105 against ZIKV are therefore necessary, because they could not only mitigate ZIKV
106 morbidities but also impair chain of transmission and possess prophylactic activity.

107 Several broad-spectrum antiviral agents are harmful over pregnant animal
108 models and women. Interferons (IFNs) are abortive, ribavirin and favipiravir are
109 teratogenic (9, 10). Currently, at least three studies have been published in the field of
110 small molecules inhibitors of anti-ZIKV replication (11-13). In these studies, the
111 authors used in their experimental infections the African ZIKV (ZIKV^{AFR}) as a
112 prototype (11-13). Importantly, it has been shown that ZIKV^{AFR} is more virulent than
113 the ZIKV strain circulating in Brazil (ZIKV^{BRA}) (14), meaning that one might neglected

114 a promising clinically approved compound by screening libraries of compounds over
115 ZIKV^{AFR}.

116 Delvecchio et al. evaluated the pharmacological activity of chloroquine against
117 ZIKV replication (11). Whether chloroquine main mechanism of action relates to the
118 blockage of viral life cycle or promotion of cellular defenses needs to be detailed (11).
119 The studies from Zmurko et al. and Eyer et al. show the anti-ZIKV activity novel
120 nucleoside analogs (12, 13). As these last two works have focused on novel molecules,
121 extensive pre-clinical studies must be carried out before the translation of their data into
122 clinical trials.

123 Among the *Flaviviridae* family, the gene encoding the RNA polymerase shows
124 the highest degree of conservation (15). Therefore, new therapeutic options against
125 HCV, specially targeting the viral RNA polymerase, could have a broader spectrum
126 over other members of the *Flaviviridae* family. In this regard, sofosbuvir was clinically
127 approved in the last years for therapeutic intervention against HCV infection.
128 Sofosbuvir is a phosphoramidate uridine nucleotide prodrug, which has to be
129 triphosphorylated within the cells to aim the viral RNA polymerase (16). The Food and
130 Drug Administration (FDA) categorizes sofosbuvir as a class B substance: “Animal
131 reproduction studies have failed to demonstrate a risk to the fetus and there are no
132 adequate and well-controlled studies in pregnant women”. The Australian’s equivalent
133 agency, Therapeutic Goods Administration (TGA), suggests a safer use of sofosbuvir,
134 by categorizing this substance as B1: “Drugs which have been taken by only a limited
135 number of pregnant women and women of childbearing age, without an increase in the
136 frequency of malformation or other direct or indirect harmful effects on the human fetus
137 having been observed”. Altogether, these information motivated us to investigate
138 whether sofosbuvir chemical structure possesses anti-ZIKV activity.

139 MATERIAL AND METHODS

140 **Reagents.** The antiviral sofosbuvir, β -d-2'-deoxy-2'- α -fluoro-2'- β -C-methyluridine, was
141 donated by the BMK Consortium: Blanver Farmoquímica Ltda; Microbiológica
142 Química e Farmacêutica Ltda; Karin Bruning & Cia. Ltda, (Toboão da Serra, São Paulo,
143 Brazil). Ribavirin was received as donation from the Instituto de Tecnologia de
144 Farmacos (Farmanguinhos, Fiocruz). Sofosbuvir triphosphate (STP), β -d-2'-deoxy-2'- α -

145 fluoro-2'- β -C-methyluridine triphosphate, ribavirin triphosphate (RTP) and AZT-
146 triphosphate (AZT-TP) were purchased (Codontech.org, CA and Sierra Bioresearch,
147 AZ). Interferon-alpha was purchased from R&D bioscience. All small molecule
148 inhibitors were dissolved in 100 % dimethylsulfoxide (DMSO) and, subsequently,
149 diluted in culture or reaction medium minimally 10^4 -fold before each assay. The final
150 DMSO concentrations showed no cytotoxicity. Materials for cell culture were
151 purchased from Thermo Scientific Life Sciences (Grand Island, NY), unless otherwise
152 mentioned.

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154 **Cells.** Human neuroblastoma (SH-Sy5y; ATCC), baby hamster kidney (BHK-21) and
155 African green monkey kidney cells (Vero) cells were cultured in MEM:F-12 (1:1),
156 MEM and DMEM, respectively. *Aedes albopictus* cells (C6/36) were grown in L-15
157 medium supplemented with 0.3% tryptose phosphate broth, 0.75 g/L sodium
158 bicarbonate, 1.4 mM glutamine, and nonessential amino acids. The culture medium of
159 the cell types was supplemented with 10 % fetal bovine serum (FBS; HyClone, Logan,
160 Utah), 100 U/mL penicillin, and 100 μ g/mL streptomycin(17, 18). Mammals cells were
161 kept at 37 °C in 5% CO₂, whereas mosquito cells were maintained at 26 °C. Passages of
162 SH-sy5y cells included both adherent and non-adherent cells.

163

164 **Virus.** ZIKV was isolated from a serum sample of confirmed case from Rio de Janeiro,
165 Brazil. This sample was received and diagnosed by the Reference Laboratory for
166 Flavivirus, Fiocruz, Brazilian Ministry of Health, as part of the surveillance system
167 against arboviruses(3). ZIKV was originally isolated in C6/36 cells, titrated by plaque-
168 forming assay and further passaged at the multiplicity of infection (MOI) of 0.01. Virus
169 passages were performed by inoculating C6/36 cells for 1 h at 26 °C. Next, residual

170 viruses were washed out with phosphate-buffered saline (PBS) and cells were cultured
171 for an additional 9 days. After this period, cells were lysed by freezing and thawing,
172 centrifuged at $1,500 \times g$ at 4°C for 20 min to remove cellular debris.

173 ZIKV was purified in between fractions of 50 % and 20 % sucrose.
174 Sucrose gradients were made in 40 mL ultracentrifuge tubes (Ultra-clear; Beckman,
175 Fullerton, CA) in PBS without Ca^{++} and Mg^{++} (pH 7.4). Tubes were allowed to stand
176 for 2 h at room temperature. Up to 20 mL of virus was added to each tube and
177 centrifuged in a SW 28 rotor (Beckman) at 10,000 rpm for 4 h at 4°C . Fractions were
178 collected and assayed for total protein and for virus-induced hemagglutination (HA),
179 with turkey red blood cells (Fitzgerald Industries International, North Acton, MA).
180 Fractions displaying HA activity (≥ 16 UHA/50 μL) were pooled and dialyzed against
181 PBS without Ca^{++} and Mg^{++} (pH 7.4) and 10 % sucrose overnight at 4°C . Virus pools
182 were filtered through a 0.22- μm membranes (Chemicon, Millipore, Bedford, NY).
183 Infectious virus titers were determined by plaque assay in BHK-21 cells and stored at -
184 70°C for further studies.

185

186 **Cytotoxicity assay.** Monolayers of 10^4 BHK-21 or 5×10^4 SH-Sy5y cells in 96-
187 multiwell plates were treated with various concentrations of sofosbuvir or ribavirin, as
188 an additional control, for 5 days. Then, 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-
189 Tetrazolium-5-Carboxanilide (XTT) at 5 mg/ml was added in DMEM in the presence of
190 0.01 % of N-methyl- dibenzopirazina methyl sulfate (PMS). After incubation for 4 h at
191 37°C , plates were read in a spectrophotometer at 492 nm and 620 nm(19). The 50 %
192 cytotoxic concentration (CC_{50}) was calculated by non-linear regression analysis of the
193 dose-response curves.

194 **Plaque forming assay.** Monolayers of BHK-21 in 6-well plates were exposed to
195 different dilutions of the supernatant from yield-reduction assays for 1 h at 37 °C. Next,
196 cells were washed with PBS and DMEM containing 1 % FBS and 3 %
197 carboxymethylcellulose (Fluka) (overlay medium) was added to cells. After 5 days at 37
198 °C, the monolayers were fixed with 10 % formaldehyde in PBS and stained with a 0.1
199 % solution of crystal violet in 70 % methanol, and the virus titers were calculated by
200 scoring the plaque forming units (PFU).

201 **Yield-reduction assay.** Monolayers of 10^4 BHK-21, Vero or 5×10^4 SH-Sy5y cells in
202 96-multiwell plates were infected with ZIKV at indicated MOIs for 1 h at 37 °C. Cells
203 were washed with PBS to remove residual viruses and various concentrations of
204 sofosbuvir, or interferon-alpha as a positive control, in MEM with 1 % FBS were added.
205 After 24 h, cells were lysed, cellular debris was cleared by centrifugation, and virus
206 titers in the supernatant were determined by PFU/mL. Non-linear regression of the
207 dose-response curves was performed to calculate the inhibitory activity of ZIKV-
208 induced plaque formation by 50 % (EC_{50}) .

209 **Preparation of ZIKV RNA polymerase.** ZIKV RNA polymerase was obtained from
210 ZIKV-infected BHK-21 cells. Cells were infected with ZIKV at a MOI of 10 for 24 h,
211 lysed with buffer containing 0.25 M potassium phosphate (pH 7.5), 10 mM 2-
212 mercaptoethanol (2-ME), 1 mM EDTA, 0.5% Triton X-100, 0.5 mM phenylmethane
213 sulfonylfluoride (PMSF) and 20% glycerol, sonicated and centrifuged at $10,000 \times g$ for
214 10 min at 4 °C. The resulting supernatant was further centrifuged at $100,000 \times g$ for 90
215 min at 4 °C and passed through two ion-exchange columns, DEAE- and phopho-cellulose
216 (17).

217 **RNA polymerase inhibition assay.** ZIKV RNA polymerase inhibition assays was
218 adapted from previous publication (20). The reaction mixture for measurements ZIKV

219 RNA-dependent RNA-polymerase (RDRP) activity was composed of 50 mM hepes (pH
220 7.3), 0.4 mM of each ribonucleotide (ATP, GTP, CTP and labelled UTP), 0.4 mM
221 dithiothreitol, 3 mM MgCl₂, 500 ng of ZIKV genomic RNA and cell extracts. ZIKAV
222 RNA was obtained with QIAmp viral RNA mini kit (Qiagen, Duesseldorf, Germany),
223 according to manufacturer instructions, except for the use of RNA carrier. The reaction
224 mixtures were incubated for 1 h at 30 °C in the presence or absence of the drugs.
225 Reactions were stopped with addition of EDTA to make a 10 mM final solution.

226 The labelled UTP mentioned above represents an equimolar ratio between
227 biotinylated-UTP and digoxigenin-UTP (DIG-UTP) (both from Roche Life Sciences,
228 Basel, Switzerland). Detection of incorporated labeled UTP nucleotides was performed
229 by amplified luminescent proximity homogeneous assay (ALPHA; PerkinElmer,
230 Waltham, MA). In brief, streptavidin-donor and anti-DIG-acceptor beads were
231 incubated with the stopped reaction mixture for 2 h at room temperature. Then, plates
232 containing were read in the EnSpire® multimode plate reader instrument (PerkinElmer).
233 Different types blank controls were used, such as reaction mixtures without cellular
234 extracts and control reaction mixture without inhibitor and beads. In addition, the
235 extract from mock-infected cells was also assayed, to evaluate the presence of RNA-
236 dependent RNA-polymerase activity unrelated to ZIKV. Non-linear regression curves
237 were generated to calculate IC₅₀ values for the dose-response effect of the compounds.

238 **Comparative molecular modeling.** The amino acid sequence encoding of ZIKV RNA
239 polymerase (ZVRP) (UniProtKB ID: B3U3M3) was obtained from the EXPASY
240 proteomic server (21) (<http://ca.expasy.org/>). The template search was performed at the
241 Blast server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the Protein Data Bank (22)
242 (PDB; <http://www.pdb.org/pdb/home/home.do>) as database and the default options. The
243 T-COFFEE algorithm was used to provide a multiple-alignment between the amino acid

244 sequences of the template proteins and ZVRP. Subsequently, the construction of the
245 SFV-ZVRP complex was performed using MODELLER 9.16 software(23) that
246 employs spatial restriction techniques based on the 3D-template structure. The
247 preliminary model was refined in the same software, using three cycles in the default
248 optimization protocol. Thus, the model structural evaluation was carried out using two
249 independent algorithms in the SAVES server
250 (http://nihserver.mbi.ucla.edu/SAVES_3/): PROCHECK software(24) (stereochemical
251 quality analysis); and VERIFY 3D(25) (compatibility analysis between the 3D model
252 and its own amino acid sequence, by assigning a structural class based on its location
253 and environment, and by comparing the results with those of crystal structures).

254 **Metagenomics and genome assembly.** A 0.3 mL of supernatant containing the ZIKV
255 (2×10^5 PFU) was filtered through 0.22 μ m filters to remove residual culture cells. The
256 virus RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen®) with DNase
257 RNase-free (Qiagen®) treatment, omitting carrier RNA. Double-stranded cDNA
258 libraries were constructed by Truseq Stranded total RNA LT (Illumina®) with Ribo-zero
259 treatment, according to the manufacture's instruction. The library size distribution was
260 assessed using 2100 Bioanalyzer (Agilent®) with High Sensitive DNA kit (Agilent®),
261 and the quantification was performed with 7500 Real-time PCR System (Applied
262 Biosystems®) with KAPA Library Quantification Kit (Kapa Biosystems). Paired-end
263 sequencing (2 x 300 bp) was done with MiSeq Reagent kit v3 (Illumina®). The
264 sequences obtained were preprocessed using the PRINSEQ software to remove reads
265 smaller than 50 bp and sequences with scores of lower quality than a Phred quality
266 score of 20. Paired-End reAd merger (PEAR) software was used to merge and extend
267 the paired-end Illumina reads using the default parameters(26, 27). The extended reads
268 were analyzed with Deconseq program, against the Human Genome Database, with

269 Identity and Coverage cutoff of 70%, to remove human RNA sequences(28). Non-
270 human reads were analyzed against all GenBank viral genomes (65,052 sequences)
271 through BLAST software using 1e-5 e-value cutoff. The sequences rendering a genome
272 were assembled with SPAdes 3.7.1 software(29) followed by a reassemble with CAP3
273 program(30).

274 **Sequence comparisons.** Sequences encoding for the C-terminal portion of the RNA
275 polymerase form members of the *Flaviviridae* family were acquired from complete
276 sequence deposited in GenBank. Alignment was made using the ClustalW algorithm in
277 Mega 6.0 software. Sequences were analyzed using neighbor-joining, with pairwise
278 deletion, with bootstrap of 1,000 replicated and *P* distances were registered. Sequences
279 were also analyzed for mean evolutionary rate.

280 **Statistical analysis.** All assays were performed and codified by one professional.
281 Subsequently, a different professional analyzed the results before identification of the
282 experimental groups. This was done to keep the pharmacological assays as blind. All
283 experiments were carried out at least three independent times, including technical
284 replicates in each assay. The dose-response curves to calculate EC₅₀ and CC₅₀ values
285 were generated by Excel for Windows. Dose-response curve to calculate IC₅₀ values
286 were obtained by Prism graphpad software 5.0. The equations to fit the best curve were
287 considered based on the R² values ≥ 0.9 . Above mentioned are the statistical analysis
288 specific to each program software used in the bioinformatics analysis.

289 **RESULTS**

290 **Sofosbuvir fits on the ZVRP predicted structure.** The RNA polymerase structures
291 from WNV (PDB # 2HFZ) (31), JEV (PDB # 4K6M) (32), DENV (PDB # 5DTO) (33)
292 and HCV (PDB # 4WTG) (34) share 72, 70, 68, and 25 % sequence identity with ZIKV
293 orthologue enzyme, respectively. Despite that, the HCV enzyme is complexed with
294 sofosbuvir and the amino acids residues that interacts with this drug are highly

conserved over members of the *Flaviviridae* family, around 80 % (34). The region encoding for the C-terminal portion of *Flaviviridae* RNA polymerase contains around the 800 amino acid residues and identical residues are highlighted in yellow (Supplementary Material 1). The residues critical for RDRP activity are conserved among different viral species and strains, including: ZIKV African strain from the 50's and those circulating currently, DENV and different genotypes of HCV (Supplementary Material 1) (35).

Based on the HCV RNA-direct RNA-polymerase domain, we constructed a 3D model for ZIKV orthologue enzyme (Figure 1). Sofosbuvir was located among the palm and fingers region of ZIKV RNA polymerase (Figure 1A), an area important to coordinate the incorporation of incoming nucleotides into the new strand of RNA (34). Consequently, amino acid residues relevant to sofosbuvir interaction are some of those critical for natural nucleotide incorporation and thus RDRP activity (Figure 1B) (34). The amino acid residues involved with the interaction with sofosbuvir are identical or conserved among the members of the *Flaviviridae* family (Supplementary Material 2). The conserved residues are considered to be evolving slower than other residues of this enzyme (Supplementary Material 2). These information mean that residues predicted to be required for interaction with sofosbuvir tend to be conserved among members of *Flaviviridae* family.

Sofosbuvir inhibits ZVRP in a dose-dependent fashion. Next, we evaluated whether sofosbuvir triphosphate (STP), the bioactive compound, could inhibit ZIKV RDRP activity. Fractions containing the ZIKV RDRP activity were purified from infected cells (17). STP inhibited ZIKV RDRP activity with an IC_{50} value of $0.38 \pm 0.03 \mu M$ (Figure 2). Ribavirin-triphosphate (RTP) and AZT-TP were used as positive and negative controls, respectively (Figure 2). RTP and AZT-TP presented IC_{50} values of 0.21 ± 0.06 and $> 10 \mu M$, respectively (Figure 2). The data from Figure 2, confirmed the molecular modeling prediction that sofosbuvir docked onto ZVRP structure and, revealed that sofosbuvir chemical structure inhibits ZIKV RDRP activity.

Sofosbuvir inhibits ZIKV replication in cell-, MOI- and dose-dependent manner. Sofosbuvir phosphoramidate prodrug must be converted to its triphosphate analog within the cellular environment to become active. Therefore, we investigated whether sofosbuvir inhibits ZIKV replication in cellular systems. Before that, we isolated a Brazilian ZIKV strain from a confirmed case of zika fever and characterized this isolate for experimental use. The full-length virus genome was sequenced (GenBank accession

329 # KX197205) and characteristic plaque forming units (PFU) and cytopathic effect
330 (CPE) were detected in BHK-21 cells (Figure S1). Another concern was to establish
331 whether other plaque-forming viral agents was co-isolated, which could be misleading
332 to interpret the antiviral activity. Metagenomic analysis reveal that ZIKV was the only
333 full-length genome of a plaque-forming virus in BHK-21 detected (Supplementary
334 Material 3).

335 After the characterization of a Brazilian ZIKV strain for experimental virology
336 assays, we evaluated the ZIKV susceptibility to sofosbuvir. BHK-21, Vero or human
337 neuroblastoma (SH-Sy5y) cells were inoculated at different MOIs and treated with
338 various concentrations of sofosbuvir. Supernatant from these cells were collected and
339 infectious virus progeny titrated. Sofosbuvir induced a MOI- and dose-dependent
340 inhibition of ZIKV replication (Figure 3A and B, Table 1 and Figure S2). Potency and
341 efficiency to inhibit ZIKV replication were higher in SH-Sy5y than BHK-21 cells
342 (Figure 3A and B, Table 1 and Figure S2). Of note, over 10 μ M of sofosbuvir did not
343 inhibit ZIKV replication in Vero cells, indicating a cell-dependent inhibition of ZIKV
344 replication. IFN- α and ribavirin were used as positive controls to inhibit ZIKV
345 replication (Figure 3A and B, Table S1 and Figure S2).

346 Sofosbuvir cytotoxicity was also cell type-dependent (Table 1), being less
347 cytotoxic for SH-Sy5y than for BHK-21 cell line. Our results indicate that the
348 selectivity index (SI; which represents the ratio between CC₅₀ and EC₅₀ values) for
349 sofosbuvir varied from 185 to 653 (Table 1) – being safer at MOI equals to 0.5 in the
350 neuroblastoma cell line. For comparisons, SI values for sofosbuvir were up to 5 times
351 higher than for ribavirin (Table 1). Our data point out that that sofosbuvir chemical
352 structure is endowed with anti-ZIKV activity and efforts to promote its broader
353 activation in different cellular types could lead to the development to new anti-ZIKV
354 based therapies.

355

356 Discussion

357 ZIKV is a member of *Flaviviridae* family, such as other clinically relevant
358 viruses, such as DENV, WNV, JEV and HCV. Among this family, ZIKV was
359 considered to be a virus causing only mild and self-limited infections (6). However,
360 based on clinical evidence and laboratorial data, ZIKV infection was associated with
361 neurological-related morbidities, with impacts on the development of human nervous
362 system and triggering of GBS (7, 8, 14, 36-39). Antiviral treatment options are thus

363 required to block viral replication. Previous studies have demonstrated that the anti-
364 malarial drug chloroquine and novel nucleoside analogs inhibit ZIKV replication (11-
365 13). Here, we show that the clinically approved uridine nucleotide analog anti-HCV
366 drug, sofosbuvir, is endowed with anti-ZIKV activity.

367 Delvecchio et al. evaluated the pharmacological activity of chloroquine against
368 ZIKV replication (11). The authors used a clinically approved drug feasible to be used
369 in pregnant women. The pharmacological activity of chloroquine against ZIKV
370 replication in Vero cells, which produce high virus titers, and relevant cellular models
371 for studying ZIKV neurotropic replication were evaluated. The African ZIKV isolate
372 used, which is differently than the one circulating currently. Besides, chloroquine's
373 potency over ZIKV replication is around 10 μ M, whereas against different species of
374 *Plasmodium* it acts at the sub-micromolar range. This means that the 500 mg chloroquine
375 tablets approved for clinical use to treat malaria might not be enough to treat ZIKV
376 infection. The mechanism by which chloroquine inhibits ZIKV replication has not been
377 characterized. Whether it inhibits the viral life cycle directly or enhances cell survival
378 must be investigated.

379 The studies from Zmurko et al. and Eyer et al. show the anti-ZIKV activity
380 novel nucleoside analogs (12, 13). Zmurko et al. characterized their studied molecule
381 *in vitro*, indicating its ability to target the ZIKV RNA polymerase, and *in vivo* (12).
382 Eyer et al. evaluated a broad range of nucleoside/nucleotide analogs, including
383 clinically approved drugs, pointing out to another novel molecule (13). In these
384 studies, the authors also used the African ZIKV strain and the pharmacological
385 activity has not been characterized in neuronal cell lines. As these works have focused
386 on novel molecules, the translation of their data into clinical trials will require further
387 extensive studies.

388 Sofosbuvir chemical structure is endowed with anti-ZIKV activity, by different
389 approaches we observed that. Predicted ZVRP structure suggest that sofosbuvir required
390 critical amino acid residues for ribonucleotide incorporation, such as Arg473, Gly538,
391 Trp539, Lys691. These results could anticipate that genetic barrier for antiviral
392 resistance would be high. That is, changes in these residues would jeopardize enzyme
393 activity. Such a high genetic barrier is found to the emergence of sofosbuvir-resistant
394 strains of HCV (34). The fluoride radical in sofosbuvir ribosyl moiety is coordinated by
395 the Asn612, an interaction involved with the drug selectivity to RDRP, which may

avoid unspecific effects towards the cellular DNA-dependent RNA-polymerase. The Lys458 seems to be the docking residue for the uridine analog. Further enzymatic studies with site-directed mutagenesis to this residue could confirm its participation for sofosbuvir docking.

Sofosbuvir produced a dose-dependent inhibition of ZIKV replication with different magnitudes in terms of potency and efficiency in BHK-21 and SH-Sy5y cells. On the other hand, we observed no inhibition of viral replication with over 10 μ M in Vero cells. Similarly, in the recent study from Eyer et al. (13), African ZIKV susceptibility sofosbuvir was screened in Vero cells, and this compound did not emerged as a potential hit. Although Eyer et al. (13) and us used different viral strains, we reached similar results in Vero cells. Interestingly, sofosbuvir is a substrate for glycoprotein-P (40). Differently than in BHK-21 and Sh-Sy5y, proteomic data reveal that Vero cells express this multi-drug resistance ABC-transporter, which may cause sofosbuvir efflux out of the cell (41-43).

Of note, although we succeed to determine the sofosbuvir antiviral activity against ZIKV in different cell types, this drug metabolism *in vivo* occurs mainly in the liver and adjacent organs. Different experimental *in vivo* infection assays have been studied (ref). Prior to the detection of the ZIKV in the nervous system, virus is found in peripheral organs, such as in spleen and liver (12, 44-46). We believe this could be an insight from the natural history of the ZIKV infection in patients. That is, it is likely that before reaching sites of immune privilege, such as the nervous system or the placental barrier, ZIKV virions may be amplified in peripheral organs and provoke an inflammation response to disrupt the specific barriers. Although the role of the liver during ZIKV infection is currently overlooked, ZIKV could replicate in the liver before its systemic spread. Indeed, it has been reported for some ZIKV-infected patients that liver transaminase levels are enhanced during the onset of illness (47). Moreover, DENV viral loads are increased in the liver and represent one of the hallmarks of this other flavivirus pathogenesis. Naturally, liver is the main site of HCV replication. Therefore, *in vivo* experimental assays are necessary to better determine sofosbuvir capacity to acting as a therapeutic or prophylactic agent during ZIKV experimental infection.

ZIKV-associated microcephaly and GBS highlights that antiviral interventions are urgent. Our data reveal that a clinically approved drug is endowed with antiviral

activity against ZIKV. Thus, the potential second use of sofosbuvir, an anti-HCV drug, against ZIKV seems to be plausible.

AUTHORS CONTRIBUTIONS

C.Q.S., G.R.deM., N.R., L.V.B.H., M.M., C.S.deF., N.F.-R., A.M., A.C., G.B.-L., E.de M.V., D.A.T. and L.L. – contributed with experiment execution and analysis.
M.M.B., F.A.B., P.B., N.B., F.L.T., A.M.B.deF., K.B. and T.M.L.S. – Data analysis manuscript preparation and revision
K.B. and T.M.L.S. – Conceptualized the study
All authors revised and approved the manuscript.

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The authors declare no competing financial interests.

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587 **Legend for the Figures**

588 **Figure 1 – Putative ZIKV RNA polymerase in complex with sofosbuvir.** Based on
589 the crystal structure of the HCV RNA polymerase in complex with sofosbuvir
590 diphosphate (PDB accession # 4WTG), the putative structure from ZIKV RNA
591 polymerase was constructed. The amino acid sequence encoding ZIKV RNA
592 polymerase (ZVRP) (UniProtKB ID: B3U3M3) was aligned using T-COFFEE server
593 with orthologue RNA polymerases from members of *Flaviviridae* family, Hepatitis C
594 virus (HCV; PDB # 4WTG, West Nile virus (WNIV; PDB # 2HFZ), Japanese
595 Encephalitis virus (JEV; PDB # 4K6M), and Dengue virus (DENV; PDB # 5DTO). The
596 MODELLER 9.16 software was used to build a 3D-model of ZIKV RNA polymerase,

with subsequent refinement using three cycles in the default optimization protocol. The model structural evaluation was carried out using two independent algorithms, PROCHECK software and VERIFY 3D. (A) The 3D-model of ZIKV RNA polymerase is presented. (B) The residues presumably required for ZIKV RNA polymerase interaction with sofosbuvir and Mg^{++} ions.

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Figure 2 – Sofosbuvir inhibits ZIKV RDRP activity. Cell extracts from ZIKV-infected cells were assayed for RDRP activity using virus RNA as the template and labelled UTP as the tracer. biotinylated-UTP and digoxigenin-UTP were detected by ALPHA technology using the EnSpire® multimode plate reader instrument (PerkinElmer). The molecules used assayed were sofosbuvir triphosphate (STP), ribavirin triphosphate (RTP) and AZT triphosphate (AZT-TP). As a control, RNA-dependent RNA-polymerase activity was measured in extracts from mock-infected cells (mock). Data represent means $\pm$ SEM of five independent experiments.

Figure 3 – The antiviral activity of sofosbuvir against ZIKV. BHK-21 (A) or SH-Sy5y (B) cells were infected with ZIKV at indicated MOIs, exposed to various concentrations of sofosbuvir or IFN-alpha (inset), and viral replication was measured by plaque-forming assay after 24 h of infection. Data represent means $\pm$ SEM of five independent experiments.

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642 **Legend for the Supplementary Figures**

643 **Figure S1 – Plaque assay for ZIKV.** Monolayers of BHK-21 cells were infected for 1
644 h at 37 °C. After that, viruses were washed out with PBS and wells were covered with
645 overlay medium with 1 % FBS. At 5 days post-infection, plaques were fixed and
646 colored with crystal violet. (A) A representative plaque forming unit (PFU) is presented
647 at 40 x magnification. (B) The PFU and adjacent cellular monolayer is presented at 100
648 x magnification, some of these cells present ZIKV-induced cytopathic effect (CPE),
649 three examples are highlighted by the red arrows. (C) A representative closer view of
650 ZIKV-induced CPE (red arrow).

651 **Figure S2 – The antiviral activity of Sofosbuvir against ZIKV.** BHK-21 or SH-sy5y
 652 were infected with ZIKV at indicated MOIs, exposed to various concentrations of
 653 sofosbuvir (A) or IFN-alpha (B), and viral replication was measured by plaque-forming
 654 assay after 24 h of infection. Data represent means $\pm$ SEM of three independent
 655 experiments.

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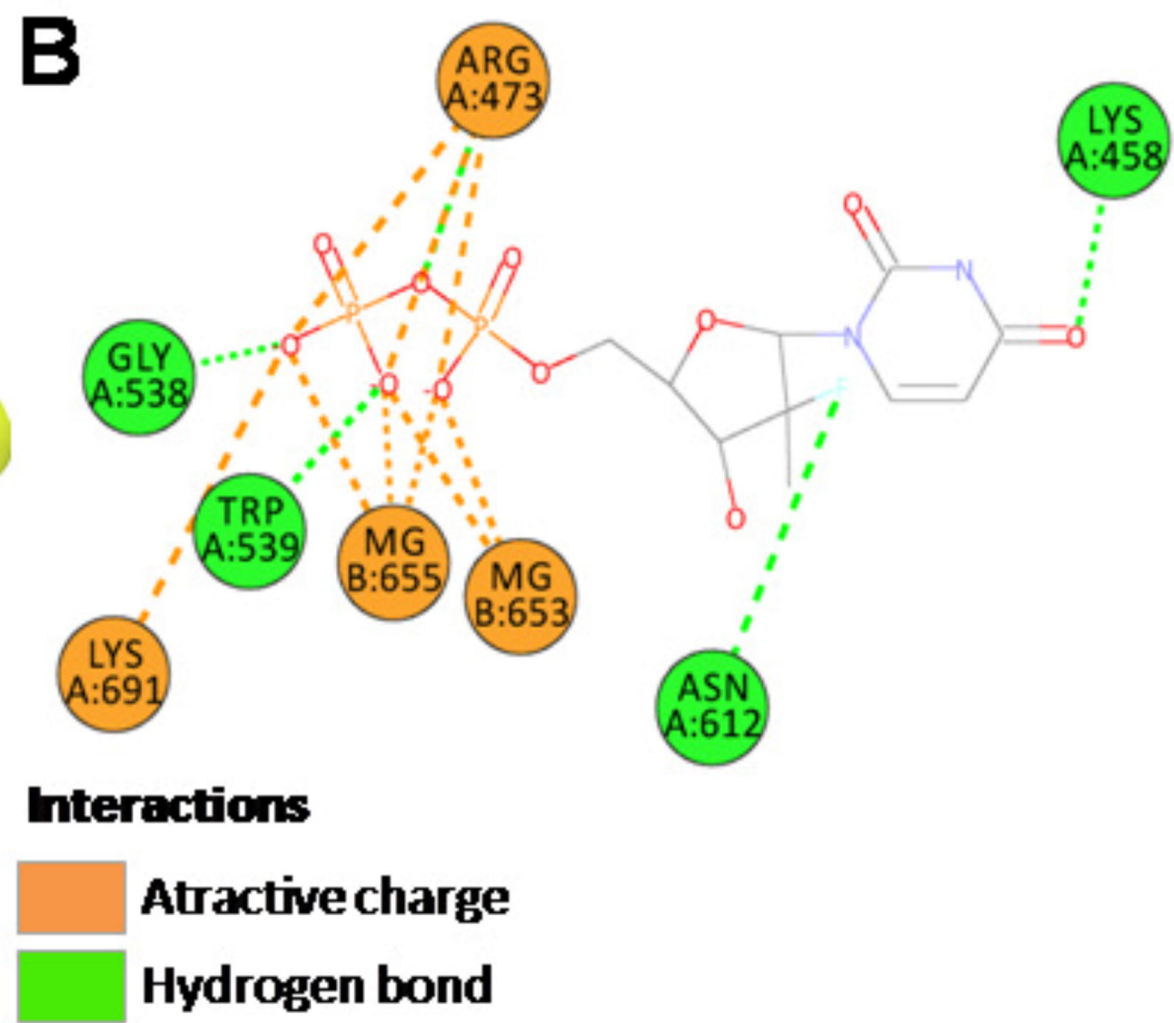
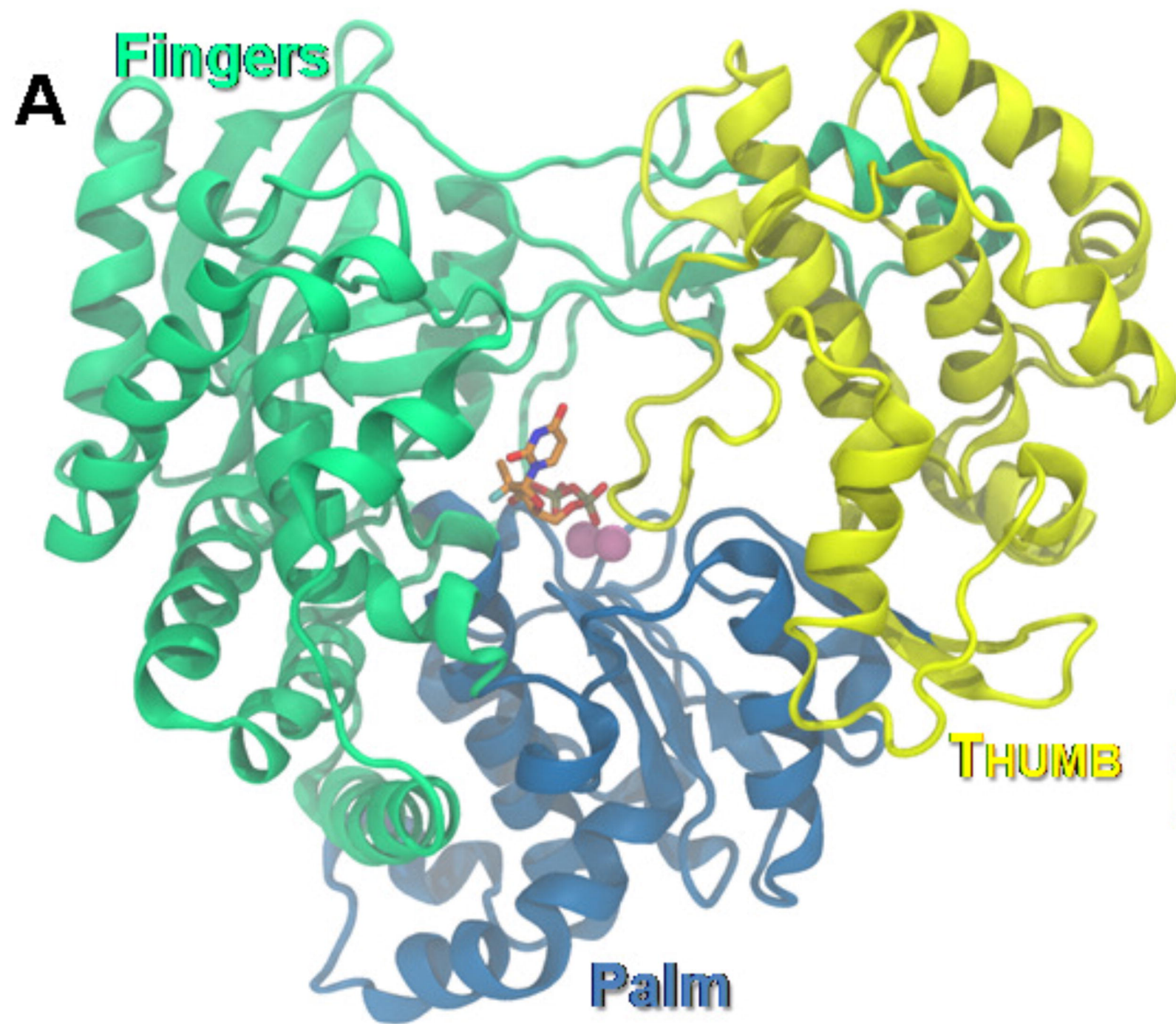
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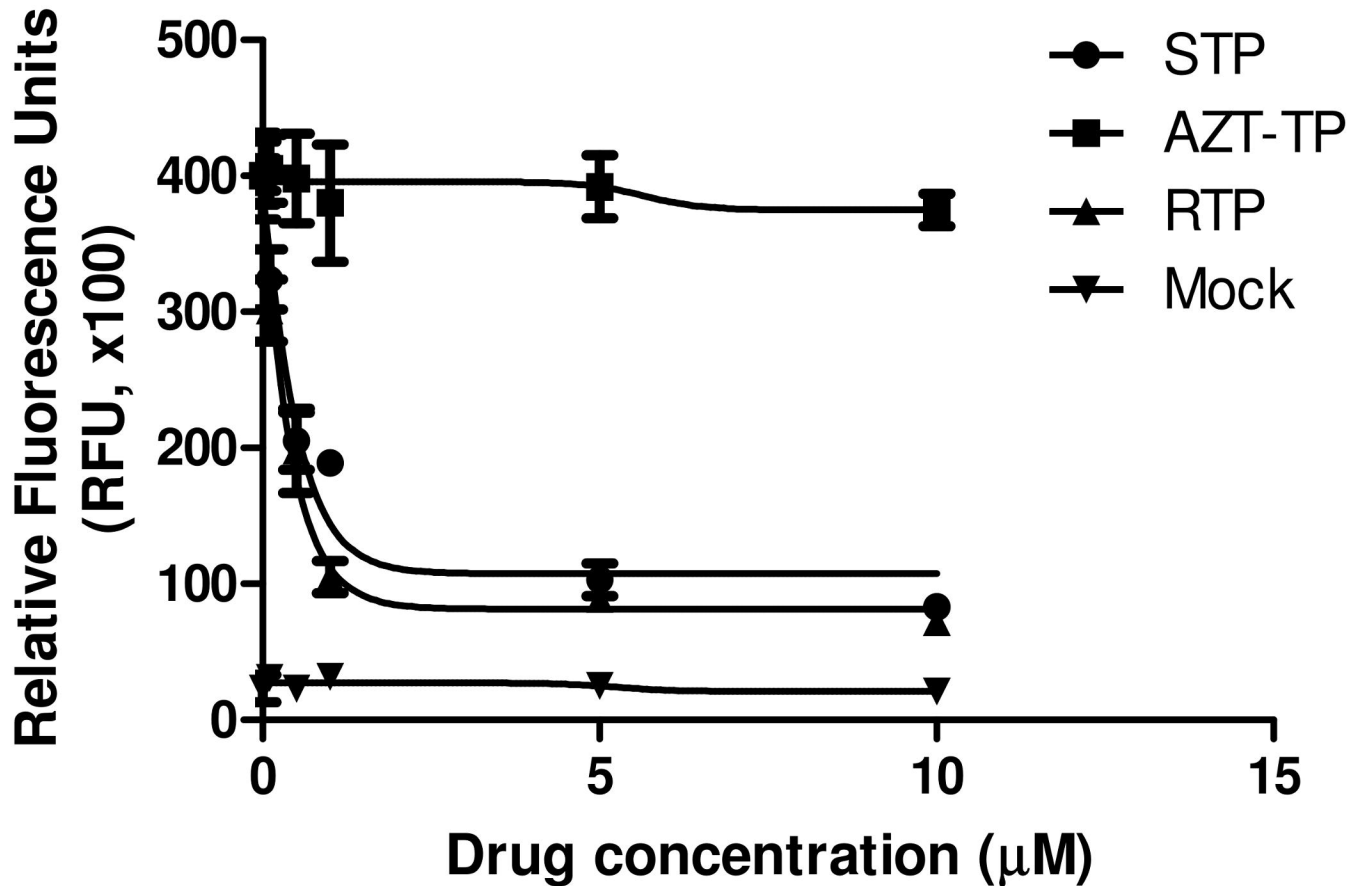
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Pharmacological															
Parameter		EC ₅₀ (μM)						CC ₅₀ (μM)			SI				
Cell type	BHK		SH-Sy5Y		Vero		BHK	SH-Sy5Y	Vero	BHK		SH-Sy5Y		Vero	
MOI	1.0	0.5	1.0	0.5	1.0	0.5				1.0	0.5	1.0	0.5	1.0	0.5
Sofosbuvir	1.9 ± 0.2	1.7 ± 0.1	1.1 ± 0.2	0.65 ± 0.08	>10	>10	360 ± 43	421 ± 34	461 ± 32	184	212	384	653	NA	NA
Ribavirin	5.3 ± 0.8	3.1 ± 0.6	2.9 ± 0.4	1.2 ± 0.2	6.8 ± 1.2	4.8 ± 0.8	177 ± 22	300 ± 21	371 ± 16	33	57	103	250	54	77
IFN-alpha*	7.3 ± 0.3	2.8 ± 0.3	9.8 ± 1.2	4.9 ± 0.6	10.2 ± 1.6	5.9 ± 1.0	ND	ND	ND	NA	NA	NA	NA	NA	NA

Table 1 – Antiviral activity and cytotoxicity of sofosbuvir

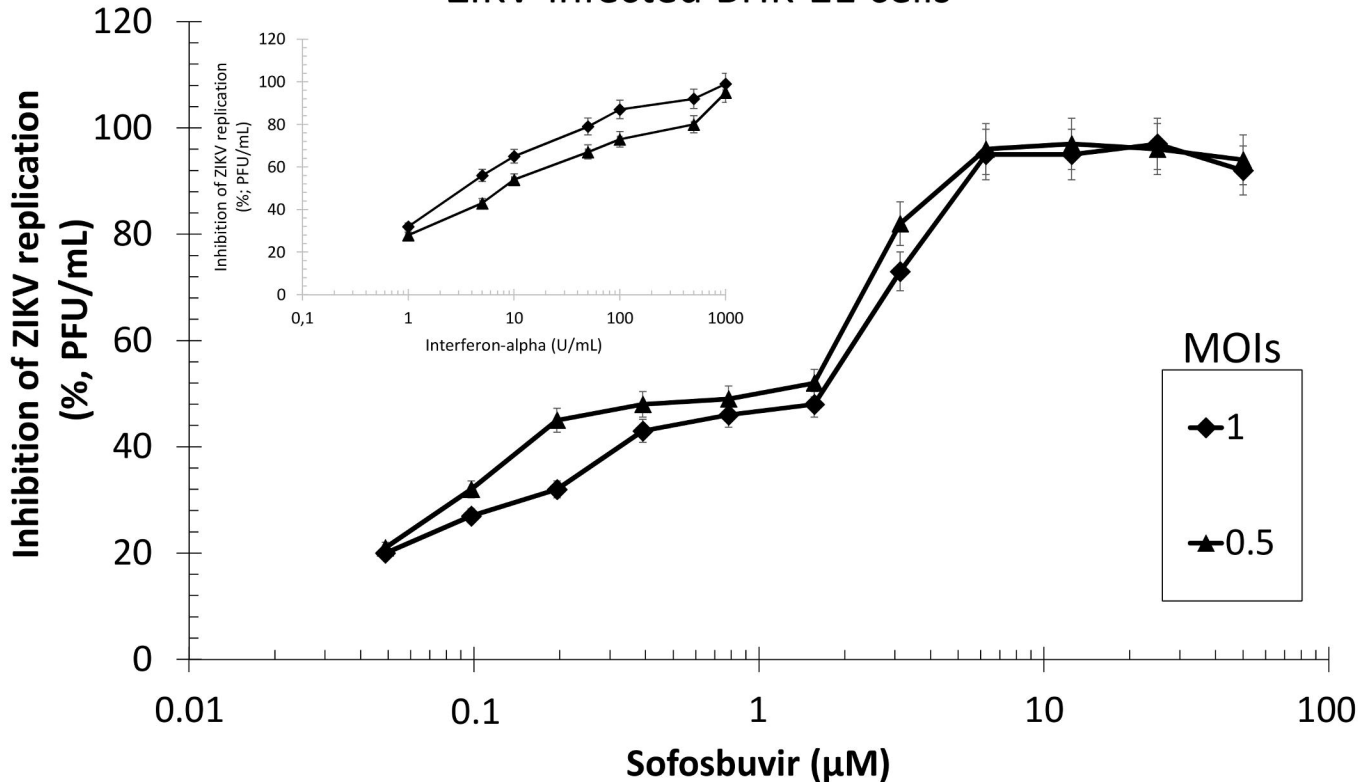
*Values are expressed as U/ml, ND – Not determined, NA – Not Applicable





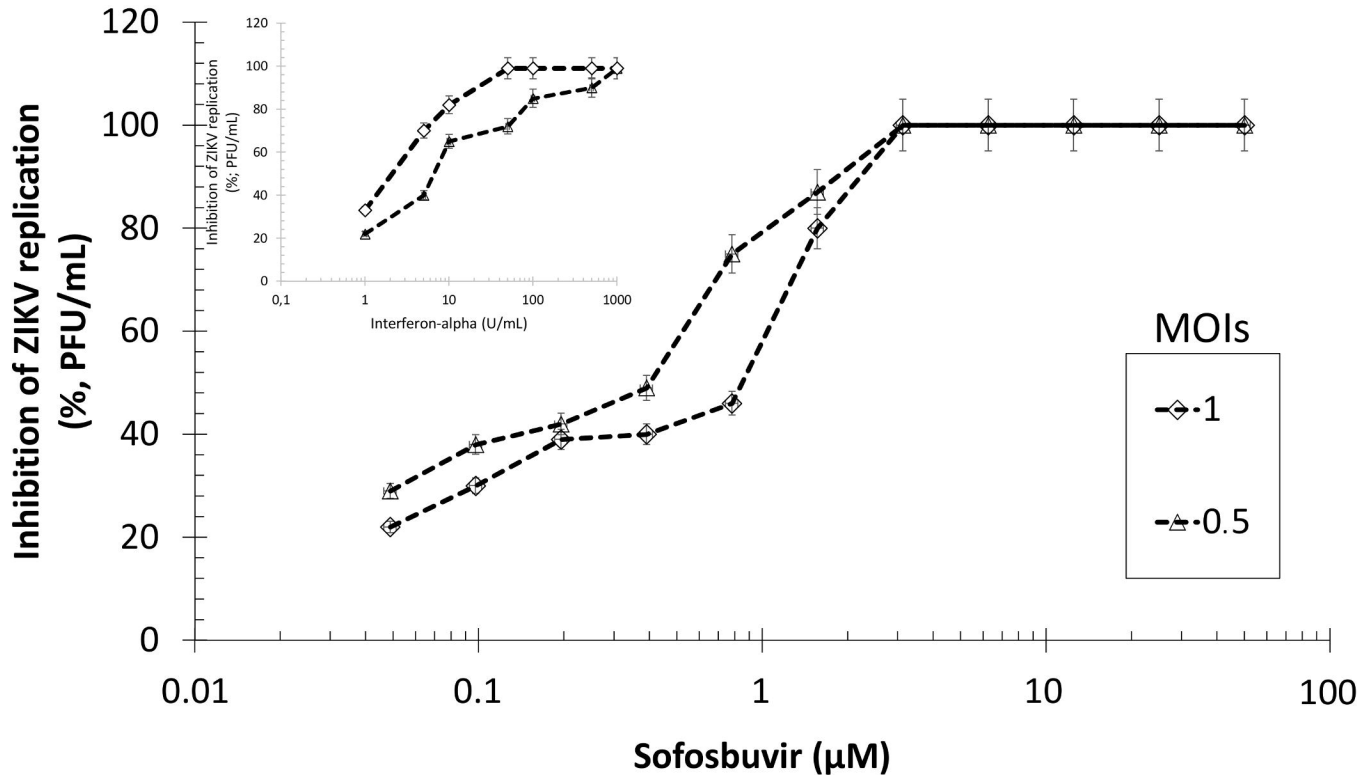
ZIKV-infected BHK-21 cells

A



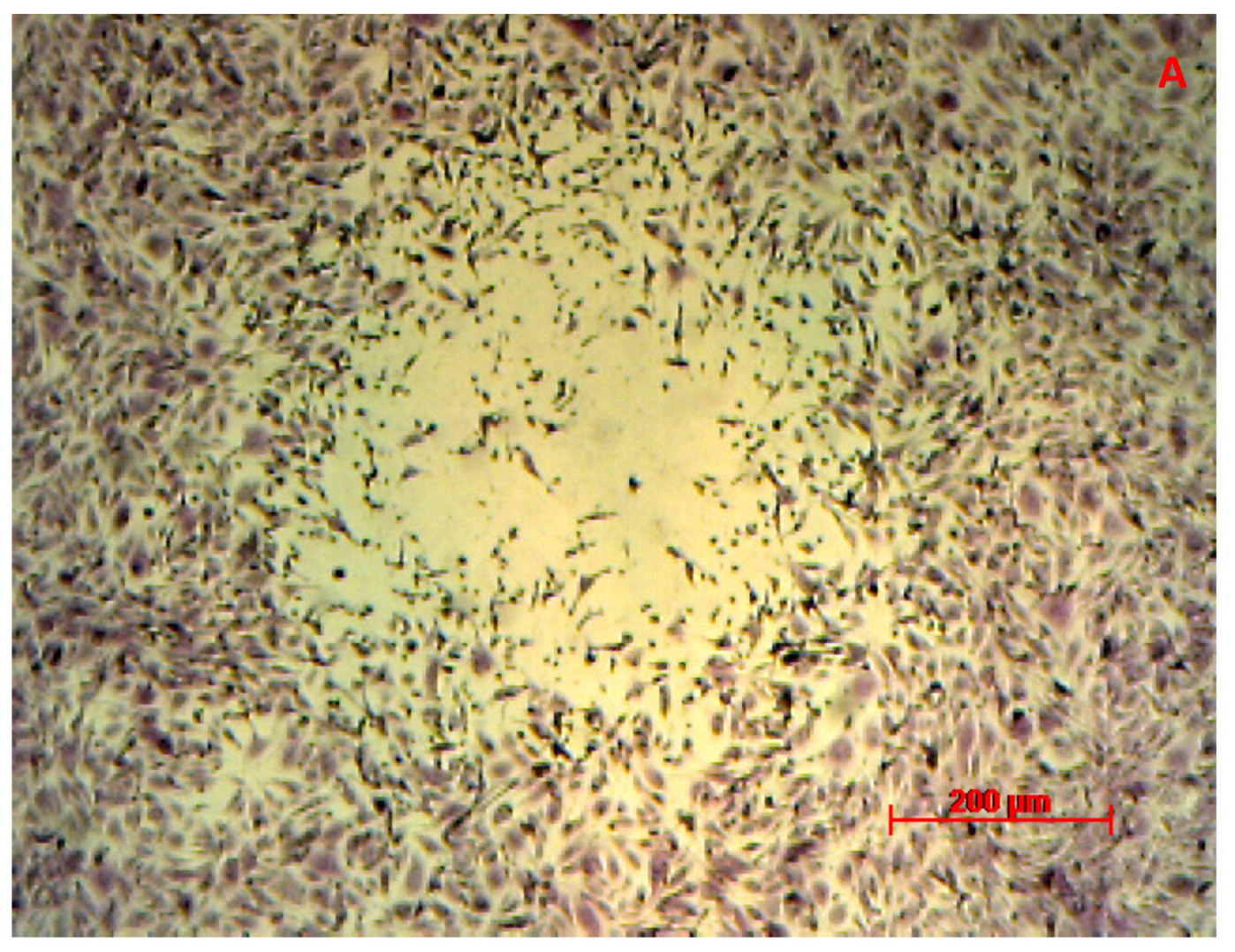
ZIKV-infected SH-sy5y

B



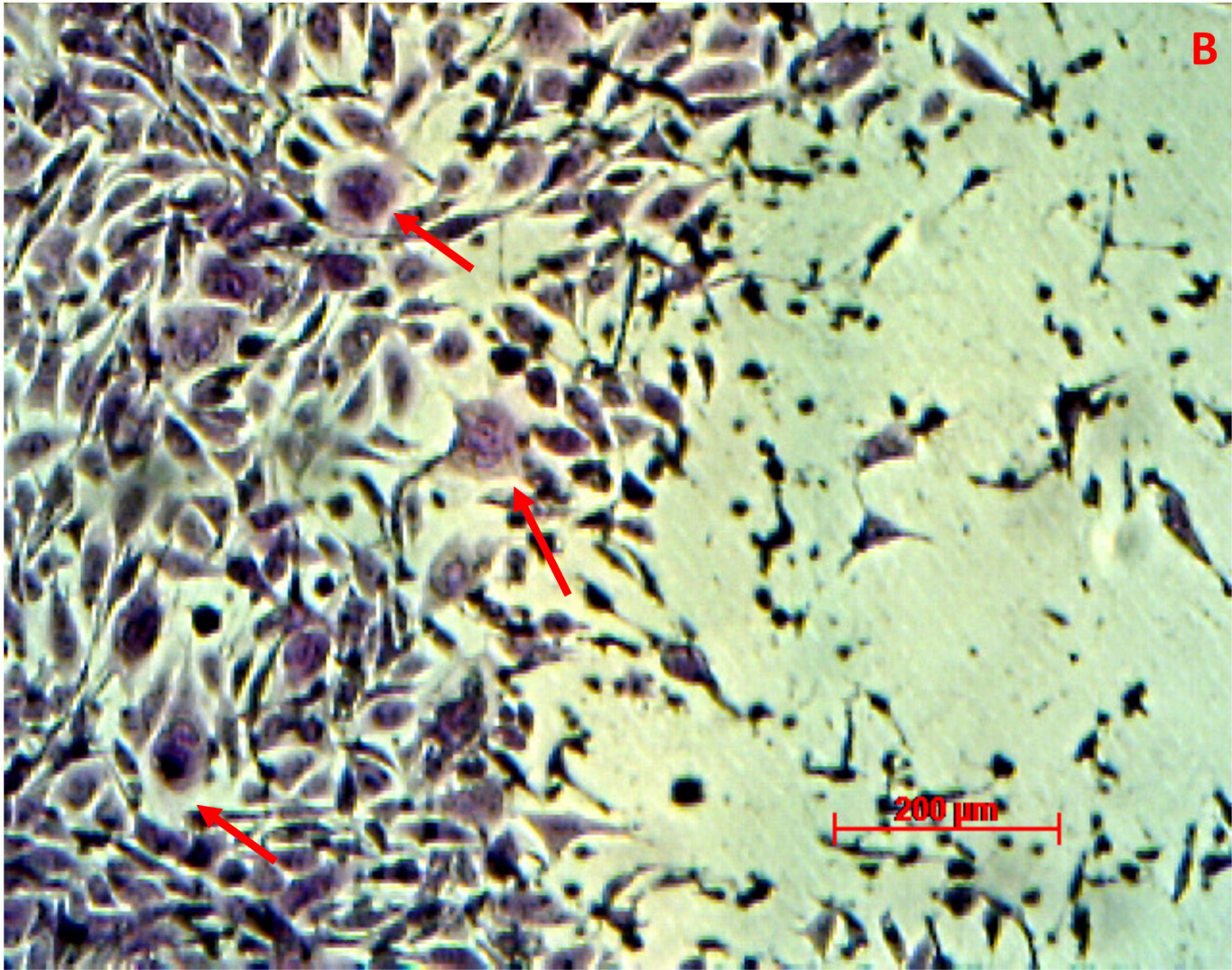
A

200 μm

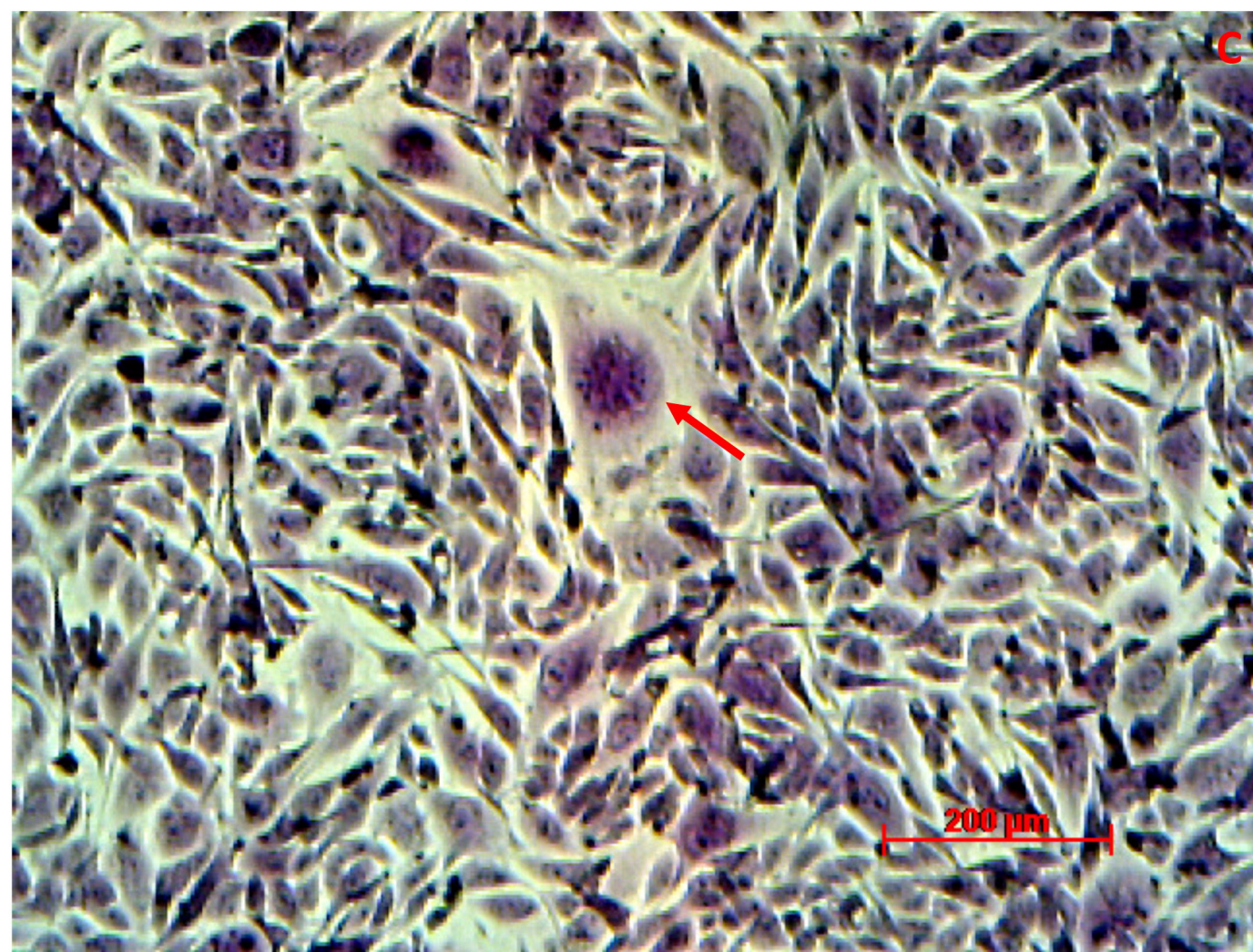


This micrograph, labeled 'A' in the top right corner, displays a dense field of cells with a yellowish-brown hue. A prominent, lighter-colored, circular region is visible in the center of the image. In the bottom right corner, a red scale bar is present, labeled '200 μm'.

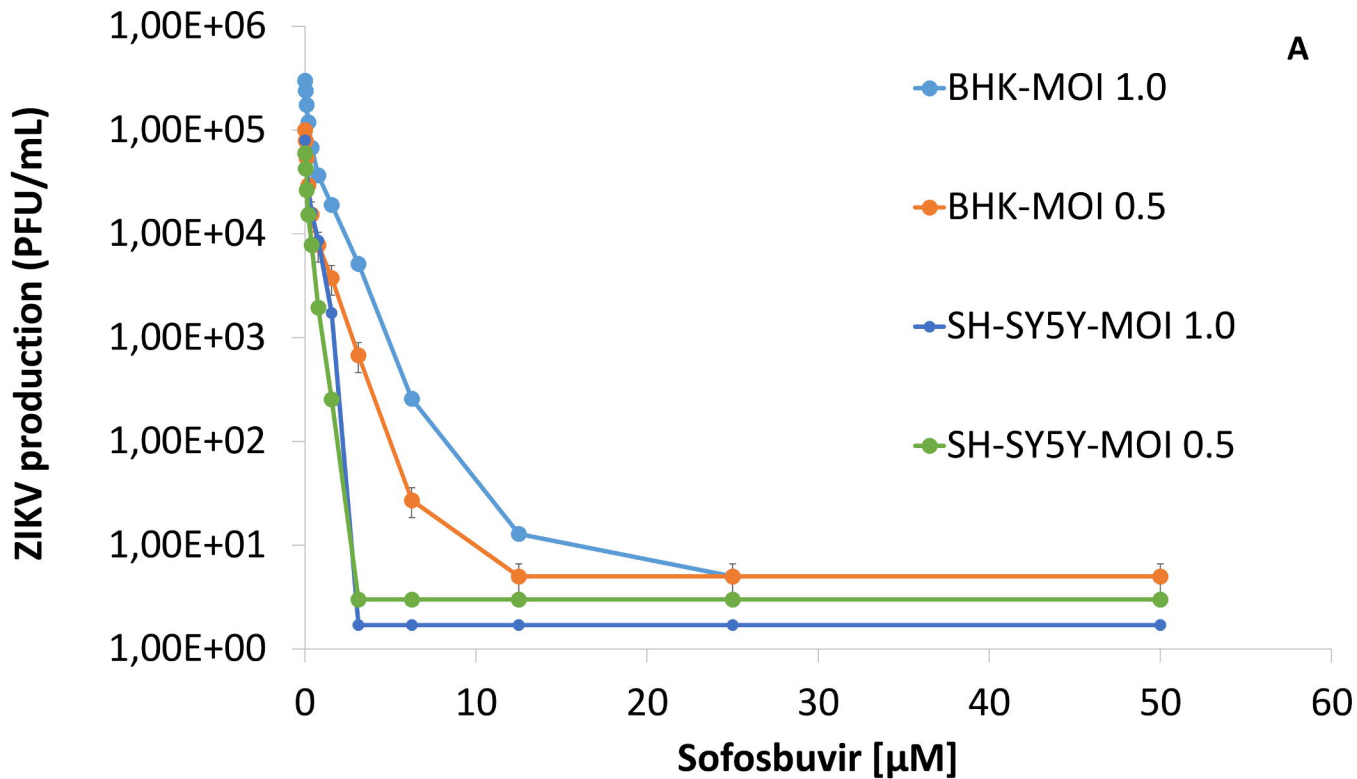
B



C



A



B