TITLE

Old drugs with new tricks: Efficacy of fluoroquinolones to suppress replication of flaviviruses

Short Title

Fluoroquinolones suppress flavivirus replication

AUTHORS

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31 **Abstract**

32 Antiviral therapies are urgently needed to treat infections with flaviviruses such as Zika
33 (ZIKV) and dengue (DENV) virus. Repurposing FDA-approved compounds could provide the
34 fastest route to alleviate the burden of flaviviral diseases. In this study, three fluoroquinolones,
35 enoxacin, difloxacin and ciprofloxacin, curtailed replication of flaviviruses ZIKV, DENV,
36 Langat (LGTV) and Modoc (MODV) in HEK-293 cells at low micromolar concentrations.
37 Time-of-addition assays revealed that enoxacin suppressed ZIKV replication when added at 6
38 hours post-infection, suggesting inhibition of an intermediate step in the virus life cycle, whereas
39 ciprofloxacin and difloxacin had a wider window of efficacy of 2, 6, and 8 hours post-infection
40 for difloxacin and 2 to 8 hours post-infection for ciprofloxacin. The efficacy of enoxacin to
41 suppress ZIKV replication in 5-week-old A129 mice was evaluated in two experiments. First,
42 mice were infected with $1x10^5$ plaque-forming units (pfu) ZIKV FSS13025 (n=20) or PBS
43 (n=11) on day 0 and subsets were treated with enoxacin at 10mg/kg or 15mg/kg or diluent orally
44 twice daily on days 1-5. Treated and control mice did not differ in weight change or virus titer in
45 serum or brain. Mice treated with enoxacin showed a significant, 5-fold decrease in ZIKV titer
46 in testes relative to controls. Second, mice were infected with $1x10^2$ pfu ZIKV (n=13) or PBS

(n=13) on day 0 and subsets were treated with 15mg/kg oral enoxacin or diluent twice daily on days 0 (pre-treatment) and 1-5. Mice treated with enoxacin showed a significant, 2.5-fold decrease in ZIKV titer in testes relative to controls, while weight and viral load in the serum, brain, and liver did not differ between treated and control mice. Enoxacin efficacy in cultured murine Sertoli cells was not enhanced compared to efficacy in HEK-293 cells. ZIKV can be sexually transmitted, so reduction of titer in the testes by enoxacin should be further investigated.

Author Summary

Flaviviruses such as Zika and dengue virus pose a significant threat to public health worldwide, and there are currently no antiviral therapies to treat any flaviviral infection. Repurposing FDA-approved drugs as anti-flaviviral therapies can accelerate clinical use. We demonstrated that fluoroquinolone antibiotics exhibit anti-flaviviral efficacy, suppressing flavivirus replication in cultured human cells. Additionally, we found that the fluoroquinolone enoxacin suppressed Zika virus replication in mouse testes. While Zika virus is primarily transmitted via mosquitoes, the virus also undergoes sexual transmission. The importance of sexual transmission for the overall epidemiology of the virus remains unclear; nonetheless all routes of potential transmission to pregnant women are of concern as fetal infection *in utero* can have devastating effects. Thus, our data indicate that fluoroquinolones hold promise for treatment of flaviviral infections, particularly infection of the testes by Zika virus, and that this class of drugs warrants further study.

Introduction

Viruses of the genus *Flavivirus* (family Flaviviridae) are major causes of morbidity and mortality worldwide (1–6). Moreover many flaviviruses, such as Zika (ZIKV), dengue (DENV),

69 tick-borne encephalitis (TBEV), West Nile (WNV), and Japanese encephalitis (JEV) virus (7–9)
70 are categorized as emerging pathogens due to rising incidence and expanding geographic range
71 (10,11). Effective antiviral drugs could abate flavivirus transmission and disease burden, but to
72 date no drugs for treatment of flavivirus infections have been brought to market because efforts
73 to develop anti-flaviviral drugs have been unsuccessful (12,13). Most anti-flaviviral drug
74 candidates have stalled at the point of hit-to-lead optimization due to poor drug-like properties
75 (14–16). This history of roadblocks in development of novel drugs suggests that repurposing
76 clinically approved drugs offers the fastest track to clinical treatments of flavivirus infections
77 (17).

78 The fluoroquinolones (FQs) are not an immediately obvious choice as anti-flavivirals.
79 The flavivirus genome comprises a single, positive-sense RNA, while FQs are primarily known
80 for inhibiting topoisomerases and gyrases in bacterial targets (18,19), neither of which play a role
81 in genome synthesis in positive-sense RNA viruses (20,21). However in the last several years, a
82 multitude of previously unsuspected effects of FQs on eukaryotic cell functions have been
83 revealed, including inhibition of cellular helicases (22,23), enhancement of RNAi (24–26),
84 attenuation of cytokines and pro-inflammatory reactive oxygen species (27–29), and
85 modification of apoptosis (30) and autophagy (31). Furthermore, FQs have been shown to
86 suppress hepatitis C virus (HCV, family *Flaviviridae*) replication *in vitro*, possibly by inhibiting
87 the viral helicase (32), but this suppression has not translated into an effective treatment for
88 patients with liver failure due to chronic HCV infection (33). Additionally, FQs suppress
89 rhinovirus infection by reducing expression of the viral receptor on cells (34). Recently, Xu and
90 colleagues demonstrated that a high concentration of enoxacin administered to human neuronal

91 progenitor cells (hNPC) and brain organoids prior to and after infection with ZIKV suppressed
92 viral replication and restored normal cellular proliferation, possibly by enhancing RNAi (35).
93 Here we evaluated the utility of repurposing FQs as anti-flavivirals by testing their ability
94 to suppress flavivirus replication in cell culture and a mouse model. We found that three FQs
95 tested, enoxacin, ciprofloxacin and difloxacin, generally suppressed replication of six
96 flaviviruses in HEK-293 cells at low micromolar concentrations. Enoxacin displayed the lowest
97 EC_{50} values in cell culture and was selected for evaluation in ZIKV-infected A129 mice.
98 Although enoxacin did not mitigate weight loss in ZIKV-infected mice or suppress ZIKV
99 replication in the serum, brain, or liver, the drug did suppress ZIKV replication in the testes.

100 Material and Methods

101 Viruses

102 The seven flaviviruses utilized in this study are listed in Table 1. Working stocks of
103 viruses were propagated in Vero cells and viral supernatants were collected either in 1X SPG
104 (2.18 mM sucrose, 38 mM potassium phosphate [monobasic], 72 mM potassium phosphate
105 [dibasic], 60 mM L-glutamic acid) [DENV-1,2 and 4, MODV, LGTV, ZIKV MEX 1-7] for
106 studies in culture or 1X DMEM supplemented with 5% heat inactivated fetal bovine serum
107 (FBS, Atlantica Biologicals, Flowery Branch, GA) and 100µg/mL penicillin/streptomycin
108 (Gibco, Life Technologies, Grand Island, NY) [ZIKV FSS13025] for studies *in vivo*.
109 Supernatants were clarified by centrifugation, aliquoted and stored at -80 °C. Viral titers were
110 determined via serial dilution onto HEK-293 cells followed by immunostaining using methods as
111 previously described (36,37). Briefly, each virus was subjected to serial tenfold dilution and
112 inoculated onto confluent HEK-293 cells in 24-well plates. After two hours of incubation at
113 37°C with occasional rocking, infected cells were overlaid with 1% methylcellulose in

114 OptiMEM (Gibco, Life Technologies, Grand Island, NY) that had been supplemented with 2%
115 FBS (Gibco, Life Technologies, Grand Island, NY), 2mM L-glutamine (Gibco, Life
116 Technologies, Grand Island, NY), and 0.05 mg/mL gentamycin (Gibco, Life Technologies,
117 Grand Island, NY). Plates were incubated for five days under maintenance conditions, after
118 which cells were fixed with ice cold methanol: acetone (1:1) for 30 minutes. Viral plaques were
119 immunostained using species-specific antibodies and peroxidase-labeled goat anti-mouse
120 secondary antibody (KPL, Gaithersburg, MD) then developed with KPL True Blue Peroxidase
121 Substrate (SeraCare, Milford, MA) and counted to calculate viral titer.
122
123 **Cells**
124 HEK-293 and murine Sertoli cells were purchased from ATCC (CRL-1573 and CRL-
125 2618, Manassas, VA). Vero cells were obtained from the lab of Stephen Whitehead (NIAID,
126 NIH). HEK-293 cells were maintained at 37°C with 5% CO_2 in DMEM/F12 medium (Gibco,
127 Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Gibco),
128 2mM L-glutamine (Gibco), and 0.5% antibiotic-antimycotic (penicillin, streptomycin, and
129 amphotericin B; Gibco). Sertoli cells were maintained at 32°C with 5% CO_2 in DMEM/F12
130 (Gibco) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and
131 penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively; Gibco). Vero cells were
132 maintained at 37˚ C with 5% CO_2 in DMEM (Gibco) supplemented with 10% heat-inactivated
133 FBS (Gibco).
134

135 **Fluoroquinolone compounds**

136 For each experiment, a fresh working stock of enoxacin (Sigma-Aldrich, E3764, St.
137 Louis, MO), difloxacin (Sigma-Aldrich, D2819, St. Louis, MO), or ciprofloxacin (Corning,
138 86393-32-0, Manassas, VA) at a concentration of 1.5 mM was sonicated in nanopore water with
139 3 mM lactic acid (Sigma-Aldrich, L1750, St. Louis, MO) and sterilized *via* passage through a 0.2
140 μm filter. The compounds were diluted to their final concentrations in cell culture media for
141 assays in cell culture, or nanopore water for *in vivo* treatments.

142

143 **Determination of half-maximal effective concentration (EC_{50}) against select flaviviruses**

144 To determine the EC_{50} of enoxacin, difloxacin, and ciprofloxacin, monolayers of 80%
145 confluent HEK-293 cells in 24-well plates were infected with either ZIKV, DENV-1, DENV-2,
146 DENV-4, LGTV, or MODV in triplicate at a multiplicity of infection (MOI) of 1. The virus was
147 allowed to adsorb for 2 hours at 37 °C after which cells were washed with 1 mL 1x phosphate
148 buffered saline (PBS) to remove unadsorbed virus. Each drug was diluted in a two-fold dilution
149 series in cell culture media, with final concentrations ranging from 150 μM to 4.7 μM, and one
150 mL was added to triplicate treatment wells. Triplicate control wells were treated with cell
151 culture media alone and another set of controls were treated with cell culture media containing
152 3mM concentration lactic acid, the drug diluent. Infected cells were incubated for five days at
153 normal conditions, after which viral supernatants were collected and viral titers were determined
154 as described above.

155 As enoxacin was found to suppress ZIKV in the mouse testes, enoxacin efficacy was
156 evaluated in one testicular cell line (murine Sertoli cells) and compared to the efficacy in HEK-
157 293 cells. The EC_{50} methods described above were repeated for ZIKV MEX 1-7 in Sertoli cells

158 and HEK-293 cells, both incubated at 32 °C to control for potential differences in enoxacin
159 activity at the lower temperature required for Sertoli cell viability. For both cell types, two MOIs
160 were tested, 0.1 and 1.0, and virus was collected at two time points, 2 days post infection (p.i.)
161 and 5 days p.i. Viral titers were determined in HEK-293 cells as described above.
162

163 **Determination of half-maximal cytotoxic concentration (CC_{50}) of fluoroquinolones**

164 To determine the toxicity of enoxacin, difloxacin, and ciprofloxacin, HEK-293 cells were
165 grown in 96-well plates until confluent at which time the media was removed. Each filter-
166 sterilized FQ was diluted two-fold, starting at 500 μM, and added to wells in triplicate at a total
167 volume of 100 μL. Control wells were treated with 100 μL of cell culture media containing 3
168 mM lactic acid. Plates were incubated at normal conditions for five days, after which the media
169 was removed and 110 μL of 10% resazurin dye (Milllipore Sigma, St. Louis, MO) diluted in cell
170 culture media was added to each well. After two hours incubation, absorbance was measured on
171 a plate reader at 600 nm and normalized to the mean absorbance of the control wells.
172

173 **Time-of-addition assay**

174 A time-of-addition assay was conducted to gain insight into the potential mechanism of
175 action of each drug (39–43). The impact of enoxacin, difloxacin, and ciprofloxacin on ZIKV
176 replication was tested at two concentrations (enoxacin: 18.1 μM and 24.4 μM; difloxacin: 35.9
177 μM and 50.0 μM; ciprofloxacin: 25.0 μM and 116.1 μM). The concentrations for the first assay
178 (24.4 μM enoxacin, 35.9 μM difloxacin, and 116.1 μM ciprofloxacin) were the EC_{50} values
179 based on an infection MOI of 0.2. Concentrations in the second assay (18.1 μM enoxacin, 50.0

180 μM difloxacin, and 25.0 μM ciprofloxacin) were chosen to give substantial but not complete
181 suppression at MOI of 1.0.
182 Eight timepoints were evaluated during both time-of-addition assays: two hours prior to
183 infection, at the time of infection (drug mixed with ZIKV), 2, 4, 6, 8, 12, and 18 hours p.i. These
184 time points capture flavivirus binding and entry (-2 and 0 hrs p.i.), translation (2 and 4 hrs p.i.),
185 genome replication (6 and 8 hrs p.i.), and virion assembly and budding (12 and 18 hrs p.i.) (44).
186 At time zero, monolayers of 80% confluent HEK-293 cells in 24-well plates were infected with
187 ZIKV MEX 1-7 at MOI 0.2 or 1. After two hours of incubation, media was removed from all
188 wells, the cells were washed with 1 mL of 1x PBS, and 1 mL of media per well was replaced. At
189 each time point, media was removed from designated triplicate wells, cells were washed with 1x
190 PBS, and 1 mL of drug at the specified concentration was added. Dilution of FQs to final
191 concentration occurred at the time of treatment. Control wells infected with ZIKV were washed
192 two hours p.i. and treated with 1 mL of media per well. At 24 hours p.i. all the viral supernatants
193 were collected, clarified, and stored as described above.
194
195 **Murine FQ dosing studies**
196 The impact of enoxacin, the FQ with the lowest EC_{50}, on ZIKV infection of A129 mice
197 was tested. Mice were infected at 5 weeks of age because our previous work shows that in this
198 age group ZIKV infection caused sustained weight loss that did not require euthanasia until 8
199 days p.i., ensuring that the majority of mice would survive a 5-day trial (45). Mice were housed
200 in sterile caging in colonies at the University of Texas Medical Branch, an AALAS-accredited
201 facility, and research was conducted in accordance with UTMB policy under IACUC Protocol
202 #1708051.

203 The efficacy of enoxacin to suppress ZIKV replication *in vivo* was tested in two separate
204 experiments (Fig. 1). In experiment 1, we tested two concentrations of enoxacin at 10 mg/kg and
205 at 15 mg/kg in A129 mice infected with $1x10^5$ pfu ZIKV, the viral dose used to in our previous
206 work to characterize ZIKV infection in 5-week-old A129 mice (45). The two concentrations, 10
207 mg/kg and 15 mg/kg, were selected because in an average sized mouse (20 g) these doses
208 correspond to peak serum concentrations (6.2 μM and 9.4 μM) that are comparable to the peak
209 serum concentrations achieved in humans receiving a typical clinical dose, wherein 200 mg and
210 400 mg oral dosages result in peak serum concentrations of 5.0 μM and 11.2 μM, respectively
211 (46,47). In experiment 2, we tested the impacts of a lower dose of virus ($1x10^2$ pfu) and a pre-
212 infection treatment of enoxacin on ZIKV infection in mice. The experiment was limited to a
213 single concentration of enoxacin, 15 mg/kg, in order to utilize the minimum number of mice.

214 Experiment 1 (Fig. 1a): Mice were intradermally injected on day 0 with $1x10^5$ pfu ZIKV
215 FSS13025 diluted in 1x PBS (n=19) or 1x PBS as a control (n=11) in a total volume of 100 μL
216 and subsets of infected and uninfected mice were treated with oral enoxacin or drug diluent (3
217 mM lactic acid) (Table 6) twice daily on days 1-6 p.i. Weight and body condition were recorded
218 twice daily. Two days p.i., 70 μL of blood was collected from the retro-orbital sinus, clarified by
219 centrifugation (5 minutes at 3380 x g), and serum was stored at -80 °C. Six days p.i., mice were
220 euthanized and brain and testes were collected. Each tissue, along with a sterile steel ball, were
221 placed into a 2 mL Eppendorf tube containing 500 μL DMEM supplemented with 2% FBS and
222 penicillin/streptomycin and homogenized in a Qiagen TissueLyser II shaking at 26 pulses/second
223 for 5 minutes. Homogenates were clarified by centrifugation at 3380 x g for 5 minutes and
224 stored at -80 ˚C. Viral titers from serum and tissues were determined in Vero cells in 12 well
225 plates essentially as described above (45).

226 Experiment 2 (Fig. 1b): Mice received a pre-treatment of 15 mg/kg enoxacin (n= 14) or drug
227 diluent (n = 13) and were intradermally injected with $1x10^2$ pfu ZIKV FSS13025 or 1x PBS 8
228 hours later as specified in Table 2. Subsets of infected and uninfected mice were treated with
229 oral enoxacin or lactic acid diluent twice daily on days 1-5. Weight and body condition were
230 recorded daily. Serum and organs were collected, and viral titers determined as described above.
231

232 **Statistical analysis**

233 EC_{50} values were calculated using nonlinear regression of inhibition dose response for log
234 drug concentration and viral titer and CC_{50} values were calculated using nonlinear regression of
235 inhibition dose response for log drug concentration and cell viability in GraphPad Prism (version
236 5 for Mac OS X, GraphPad Software, La Jolla California USA). The selectivity index for each
237 FQ and virus combination was calculated by dividing the CC_{50} by the EC_{50} values. Mean viral
238 titers at each time point from the time-of-addition assays were tested for normality then analyzed
239 using ANOVAs. If the overall ANOVA was significant, t-tests were used to detect pairwise
240 differences. Mean viral titers from mice were assessed for normality and compared using
241 ANOVAs or t-tests as appropriate, and differences in ZIKV replication in Sertoli cells and HEK-
242 293 cells at 32 °C were evaluated using general linear models in R (48).

243 ## Results

244 **Fluoroquinolones effectively suppress flavivirus replication in cultured human cells**

245 As shown in Table 3, the EC_{50} values of enoxacin, ciprofloxacin and difloxacin against
246 DENV, ZIKV, LGTV and MODV in HEK-293 cells at 37 °C were all in the range of 4.3-56.8
247 μM, except for the EC_{50} of difloxacin for MODV, for which the EC_{50} value was greater than the

248 limit of detection in our assay (Table 3). CC_{50} values for enoxacin, ciprofloxacin, and difloxacin
249 were all substantially higher than the EC_{50} values (Table 3). Excluding MODV, difloxacin
250 yielded the highest selectivity indices for all viruses tested.
251
252 **Fluoroquinolone suppression of different life cycle stages of ZIKV**
253 A time-of-addition study was used to discern the viral life stage(s) inhibited by each of
254 the three fluoroquinolones. DENV-infected monolayers of HEK-293 cells were treated with
255 each of the three drugs at one lower and one higher concentration. While significance varied
256 between the two concentration treatments for each drug, the overall patterns were consistent. As
257 seen in Fig. 2a and 2b, enoxacin suppressed virus replication most strongly when added at 2, 4 or
258 6 hours p.i. Virus replication was suppressed by about 25% by 18.1 μM enoxacin and 50% by
259 24.4 μM enoxacin. At both concentrations, differences between viral titers by time of treatment
260 were significant (ANOVA: $F(8, 18) = 2.6$, $P = 0.04$ and $F(8,18) = 3.6$, $P = 0.01$, respectively).
261 Pairwise t-tests indicated that enoxacin treatment at 2, 4, and 6 hours p.i. significantly reduced
262 ZIKV titer compared to the media control (pairwise t-test $P < 0.05$; full statistics in Table S1). At
263 both higher and lower drug concentrations of difloxacin, differences between viral titers by time
264 of treatment were significant (ANOVA: $F(8,18) = 2.8$, $P = 0.04$ and $F(8,18) = 12.8$, $P = 5.2 \times 10^{-}$
265 ^{6}, respectively). However, the higher concentration of difloxacin suppressed virus replication
266 when added two hours before infection, at the time of infection, 2, 4, 6, or 8 hours p.i. (Fig. 2d)
267 while the lower concentration of difloxacin suppressed virus replication when added 2, 6, 8, 12,
268 and 18 hrs p.i. (Fig. 2c) as detected by pairwise t-tests (full statistics in Table S1). Virus was
269 suppressed 37% by 35.9 μM difloxacin 2, 6, and 8 hrs p.i. and 50% by 50.0 μM difloxacin 2,4,
270 and 6 hours p.i.. As seen in Fig. 2e and 2f, ciprofloxacin most strongly and consistently

271 suppressed virus replication when added 2, 4, 6, or 8 hours p.i.. Virus was suppressed 40% by
272 25.0 μM ciprofloxacin and below the level of detection by 116.1 μM ciprofloxacin. The
273 differences in viral titers by time of treatment were significant for both the low and high
274 concentrations of ciprofloxacin (ANOVA: $F(8,18) = 16.2$, $P = 2.3 \times 10^{-7}$ and $F(8,18) = 14.7$, $P =$
275 1.9×10^{-6}, respectively). Pairwise comparisons with t-tests that ciprofloxacin treatment of 25.0
276 μM at 2 hours before infection, at the time of infection, and up until 8 hours p.i. significantly
277 reduced viral replication compared to the media control while treatment with 116.1 μM
278 ciprofloxacin reduced replication at 2, 4, 6, and 8 hours p.i. (full statistics in Table S1).
279

280 **Enoxacin treatment of ZIKV-infected mice did not alleviate or exacerbate weight loss**

281 In both experiments 1 and 2, all mice lost weight, irrespective of treatment (Fig. 3). Loss
282 of weight by control mice was unexpected, and likely resulted from the effects of dosing these
283 small (average 19.3 g) animals twice daily with 100 μL volume of liquid. In experiment 1, mean
284 percent of initial weight of ZIKV-infected mice treated with 10 mg/kg and 15 mg/kg were not
285 significantly different from each other (repeated measures ANOVA: $F(5,55) = 0.7$, $P = 0.61$)
286 and mean percent of initial weight of uninfected mice treated with 10 mg/kg and 15 mg/kg were
287 also not significantly different from each other (repeated measures ANOVA: $F(6,54) = 0.6$, $P =$
288 0.70); therefore, we combined the data on weight for infected mice treated with enoxacin and the
289 data for uninfected mice treated with enoxacin. After pooling by infection status, there was a
290 significant interaction between treatment group and day post infection (repeated measures
291 ANOVA: $F(12, 194) = 3.1$, $P = 0.0006$). Pairwise comparisons with t-tests identified
292 differences in percent of initial weight on days 1, 3, 4, 5, and 6 p.i. ($P < 0.05$). On days 1, 3, 4,
293 and 5 p.i. the percent of initial weight for the sham infected mice treated with enoxacin was

294 lower compared to the ZIKV-infected mice treated with enoxacin (pairwise t-test $P<0.05$; for full
295 statistics see Table S2). On day 6 p.i. the mean percent of initial weight of the uninfected mice
296 treated with enoxacin was greater than that of the infected mice regardless of treatment (pairwise
297 t-test $P<0.05$; for full statistics see Table S2). Additionally, at no point during the experiment
298 did the percent of initial weight differ between the ZIKV infected mice treated with enoxacin
299 compared to the diluent control mice (Figure 3a, Table S2). In experiment 2, weight loss by day
300 6 was less drastic than, and significantly different from, experiment 1 (mean percent of initial
301 weight (SE): experiment 1 = 84.0 (1.0), experiment 2 = 93.0 (0.9); $t = -5.7$, $df = 55$, $P = 2.6\times10^{-}$
302 ^{7}), likely due to improved technical facility in dosing these very small mice, and weight loss was
303 not significantly different among treatments (Fig. 3b, repeated measures ANOVA: $F(18,132) =$
304 1.4, $P = 0.10$).
305

306 **Enoxacin suppressed ZIKV replication in mouse testes, but not serum, brain, or liver**

307 Experiment 1. In this experiment, mice were infected with 1×10^{5} pfu ZIKV and subsequently
308 treated with enoxacin. ZIKV titer in the serum of mice treated with 15 mg/kg enoxacin was 0.6
309 $\log_{10}$ pfu/mL, significantly higher than those mice treated with 10 mg/kg enoxacin (Fig 4a;
310 ANOVA $F(2,17) = 4.7$, $P = 0.02$; pairwise t-test $P < 0.05$). However, neither dose of enoxacin
311 altered ZIKV titers in serum significantly relative to control mice (Fig 4a; pairwise t-test $P =$
312 0.22 for both). Similarly ZIKV titer in the brains of mice treated with 15 mg/kg enoxacin was
313 1.2 $\log_{10}$ pfu/g higher than those mice treated with 10 mg/kg enoxacin (Fig. 4b; ANOVA $F(2,17)$
314 $= 4.2$, $P = 0.03$; pairwise t-test $P < 0.05$) but these titers did not differ from the brains of control
315 mice (pairwise t-test, $P = 0.73$ for 10 mg/kg enoxacin and $P = 0.06$ for 15 mg/kg enoxacin). In
316 contrast to serum and brain, mean ZIKV titers in the testes of mice treated with 10 mg/kg and 15

317 mg/kg were not significantly different from each other (Fig. 4c; 5.4 $\log_{10}$ pfu/g (± 0.1 SE) vs 5.7
318 $\log_{10}$ pfu/g (± 0.1 SE); t = -1.6, df = 8, P = 0.14), and were both lower than the control group (6.2
319 $\log_{10}$ pfu/g (± 0.5 SE)), albeit only the decrease from 10mg/kg treatment was significant
320 (ANOVA F(3,19) = 3.0, P = 0.05; pairwise t-test P < 0.05). To compensate for the decrease in
321 sample size inherent in analyzing only males, the data from the two enoxacin concentrations
322 were pooled. The mean ZIKV titer in testes for mice treated with any dosage of enoxacin was 5.5
323 $\log_{10}$ pfu/g (± 0.1 SE), which was significantly lower than 6.2 $\log_{10}$ pfu/g (± 0.5 SE) in the
324 control group (pairwise t-test P < 0.05).
325
326 Experiment 2. In experiment 2 mice were pre-treated with enoxacin after which they were
327 infected with $1x10^2$ pfu ZIKV and subsequently dosed daily with enoxacin. As expected, ZIKV
328 titers in serum and brain were two orders of magnitude lower than those in experiment 1,
329 however titers in the testes were similar between the two experiments. Consistent with
330 experiment 1, ZIKV titers in sera, brains, and livers of enoxacin-treated mice were not different
331 from control mice (Fig. 5a-c, all P-values > 0.05), while ZIKV titer in testes of treated mice were
332 2-fold lower than those of control mice, and this difference was significant (Fig. 5d, pairwise t =
333 -5.4, df = 5, P = 0.003).
334
335 **Anti-ZIKV efficacy of enoxacin is not enhanced in mouse Sertoli cells**
336 To investigate why the effect of enoxacin on ZIKV infection in mice was limited to the
337 testes, the EC_{50} of this drug was quantified in both mouse Sertoli cells and HEK-293 cells at each
338 of two MOI: 0.1 and 1.0. As expected, at 32 °C, higher initial MOI generally resulted in higher
339 ZIKV titers, particularly at early timepoints p.i., in both cell lines. Sertoli cells must be cultured

340 at 32 °C, so in this experiment, for a fair comparison, the efficacy of enoxacin in HEK-293 cells
341 was re-tested at 32 °C. Moreover, to extend the window for comparison, virus was harvested at
342 both 2 days (Fig. 6a) and at 5 days p.i. (Fig. 6b).

343 As expected, at 32 °C, higher initial MOI generally resulted in higher ZIKV titers,
344 particularly at early timepoints p.i., in both cell lines. Kumar *et al*., Siemann *et al*., and Mlera
345 and Bloom have previously tested the replication of ZIKV in Sertoli cells and found them to be
346 highly susceptible to ZIKV infection (49–51). In our study, in the absence of enoxacin, there
347 was no difference in ZIKV replication in HEK-293 and Sertoli cells 2 days p.i. (linear model, β =
348 -0.2, P = 0.59), but 5 days p.i. ZIKV titers in Sertoli cells were significantly lower than in HEK-
349 293 cells (linear model, β = -1.4 , P = 7.4×10^{-7}).

350 Unlike the dose response curve in HEK-293 cells incubated at 37 ° C, increasing
351 concentration of enoxacin in both cell lines at 32 °C did not result in a sharp inflection in ZIKV
352 titer, making it difficult to accurately quantify the EC_{50} via non-linear regression. Instead,
353 general linear models were used to test the relative efficacy in the two cell types at 32 °C.
354 Efficacy at 32 °C in either cell line was detectable at the higher concentrations of enoxacin on
355 day 2, but not on day 5 (Fig. 6a and 6b).

356 We tested two hypotheses for greater impact of the drug in testes: first that this effect
357 may have been due to the lower temperature of the testes and second that it may have been due
358 to a greater efficacy in testis cells. To test the first hypothesis, we compared enoxacin efficacy in
359 HEK-293 cells infected with ZIKV at MOI 1 incubated at 32 °C or 37 °C and harvested on day 5
360 (data at 37 °C was collected as part of the initial EC_{50} analysis). Counter to the hypothesis,
361 enoxacin efficacy was greater at 37 °C compared to 32 °C in this cell line (linear model, β = -0.2,
362 P = 4.7×10^{-15}).

363 We tested the second hypothesis by comparing the impact of enoxacin in HEK-293 and
364 Sertoli cells at 32 °C. We used Sertoli cells as our model testis cell, while acknowledging that the
365 testes are composed of many cell types and results from Sertoli cells cannot be generalized to the
366 testes as a whole. In this analysis enoxacin concentration, cell type, and MOI and their
367 interactions were all included in the model. On day 2 pi, interaction between enoxacin
368 concentration and cell type was not significant ($\beta = 0.01$, $P = 0.83$), while on day 5 p.i. there was
369 a significant interaction ($\beta = 0.7$, $P = 0.0001$). On both days, enoxacin concentration and Sertoli
370 cells continue to negatively impact ZIKV replication (day 2 linear model, $\beta = -0.4$, $P = 3.2\text{x}10^{-12}$;
371 $\beta = -0.2$, $P = 0.03$, respectively; day 5 linear model, $\beta = -0.8$, $P = 4.9\text{x}10^{-11}$; $\beta = -1.4$, $P = 1.9\text{x}10^{-}$
372 ^{8}, respectively) meaning that ZIKV titer decreased as enoxacin concentration increased and
373 ZIKV infection of Sertoli cells resulted in lower titers overall compared to HEK-293 cells.
374 However, on day 5 the interaction between enoxacin concentration and Sertoli cells had a
375 positive impact on ZIKV titer, meaning that enoxacin was less effective in Sertoli cells than in
376 HEK-293 cells.
377

378 **Discussion**

379 Flavivirus infections are acute, and rapid initiation of treatment must occur for a therapy
380 to be effective (6,52,53). However, individuals infected with different flaviviruses often present
381 with similar symptoms (52,54), and in many places where flavivirus infections are common,
382 diagnostic capacity is limited (52,55). Thus, the ideal antiflaviviral drug will have broad efficacy
383 across different members of the genus (12,56,57). We found that the three FQs used in this
384 study, enoxacin, ciprofloxacin and difloxacin, all suppressed replication of the six flaviviruses
385 tested at low micromolar concentrations, with the exception that difloxacin lacked efficacy for

386 MODV. These six flaviviruses, DENV-1, DENV-2, DENV-4, ZIKV, LGTV, and MODV, span
387 the diversity of human pathogenic flaviviruses (58,59). While enoxacin consistently
388 demonstrated the lowest EC_{50} values, difloxacin exhibited the best selectivity indexes. The *in*
389 *vivo* half-life of difloxacin (3 hours) and ciprofloxacin (3 hours) are slightly longer than enoxacin
390 (1.75 hours) which could explain the differences in selectivity indexes (60–62). These findings
391 suggest that FQs could offer broad-spectrum anti-flaviviral activity, a very desirable property.
392 Though the anti-flaviviral mechanism of action of FQs remains unknown, FQs are
393 derived from the original quinolone, nalidixic acid, which is a biproduct of synthesizing
394 chloroquine, an antimalarial drug (63). Chloroquine has shown efficacy against ZIKV and
395 DENV in cultured mammalian cells, including Vero, HuH-7, U937, human neural progenitor
396 (hNPC), and human brain microvascular endothelial cells (hBMEC) cells, with a range of EC_{50}
397 values from 1 μM to 14 μM against ZIKV (64–68). Moreover initial studies in mouse and
398 monkey models were promising and demonstrated a reduction in viremia in the serum, placenta,
399 and fetal head of infected individuals as well as the duration of viremia (65,69,70). However,
400 one human clinical trial failed to find a reduction in DENV viremia following chloroquine
401 treatment (71). Another clinical trial did not detect a difference between chloroquine and
402 placebo groups in duration of DENV infection, but did find an improvement in some dengue-
403 associated symptoms, such as pain (72).
404 FQs and chloroquine share a 4-quinolone structure, and we speculate that this could be
405 the shared basis of their anti-flaviviral efficacy (18). As a weak base, chloroquine is known to
406 inhibit acidification during endocytosis (73). Studies that have evaluated the antiviral properties
407 of chloroquine have suggested that the compound inhibits viral entry by increasing the pH of
408 vesicles required for endocytosis-mediated cellular entry (66,73–75). Additionally, chloroquine

409 has demonstrated broad-spectrum pH-based antiviral activity against RNA viruses other than
410 flaviviruses, including chikungunya virus (75) and poliovirus (76,77). If chloroquine and FQs
411 share similar antiviral mechanisms of action, then results from studies of chloroquine could
412 offer insight into what modifications could be made to the FQs to increase their antiviral
413 efficacy.

414 The suppression of viral replication by FQs could also arise from alternate effects, such
415 as RNAi enhancement (RNAi) (25,26), a key antiviral defense in invertebrates whose role in
416 vertebrate antiviral immunity has been suggested (78–80) but not yet conclusively demonstrated
417 (81). Enoxacin is known to enhance RNAi by binding the trans-activation response region RNA
418 binding protein (TRBP) (25). However RNAi enhancement is not equal across all FQs;
419 compared to enoxacin, difloxacin has little to no effect on RNAi, while ciprofloxacin has a
420 moderate effect (25). Here we have demonstrated that all three FQs tested, enoxacin,
421 ciprofloxacin, and difloxacin, are potent inhibitors of the six flaviviruses tested, with the
422 exception of difloxacin and MODV, suggesting that the antiviral action of FQs is not exclusively
423 due to enhancement of RNAi. Khan et al. (32) demonstrated that many FQs, including enoxacin
424 and difloxacin, suppressed HCV replication and inhibited the viral helicase *in vitro* (32). Further
425 studies will be needed to assess the generality and *in vivo* relevance of this result.

426 To distinguish among some of these potential mechanisms of action, we conducted a
427 time-of-addition study of all three drugs. This approach has previously been used to reveal that
428 the action of chloroquine on ZIKV is mediated suppressing early life cycle stages, likely cellular
429 entry (66). In our study, we found that enoxacin suppression was restricted to 2 to 6 hours p.i.,
430 while the window of difloxacin and ciprofloxacin suppression activity was wider, encompassing
431 2 to 8 hours p.i. across assays, and prior to infection in a subset of assays. These results indicate

432 that difloxacin and ciprofloxacin may impact early and intermediate viral life cycle stages
433 whereas enoxacin's effect is limited to intermediate stages. All combined, these data suggest
434 that ciprofloxacin and difloxacin may share a mechanism-of-action that is different than that of
435 enoxacin. Like chloroquine, all three are bases, but difloxacin and ciprofloxacin are slightly
436 stronger bases than enoxacin (82) and could potentially be more effective than enoxacin at
437 inhibiting pH-mediated endocytosis during entry. All three FQs suppress ZIKV when added
438 during translation and replication time periods, consistent with both helicase inhibition or RNAi
439 enhancement as a mechanism of action.

440 In light of enoxacin's low EC_{50}, we tested the ability of this drug mitigate weight loss
441 (45) and suppress serum viremia and virus titer in key tissues including liver, brain and testes
442 (45) in ZIKV-infected A129 mice via two experiments. First, we infected A129 mice with a high
443 titer of ZIKV and then treated immediately after infection with drug diluent or 10 or 15 mg/kg of
444 enoxacin, which for the average weight of a 5-week-old mouse corresponds to 6 or 10 μM
445 enoxacin. These concentrations were selected because they are close, although slightly lower, to
446 the EC_{50} value for enoxacin in HEK-293 cells (18.1 μM) and also close to the peak human serum
447 concentration after oral consumption of clinically available dosages of enoxacin, which ranges
448 from 5 to 11 μM (46,47). Unexpectedly, all of the mice, including the sham-infected mice, lost
449 weight in this experiment. Two likely, non-exclusive explanations for the weight loss is that oral
450 administration of 100 μL liquid twice a day caused satiety and prevented the mice from eating
451 normally or that the drug diluent or enoxacin treatments caused stomach discomfort which also
452 prevented the mice from eating. Despite this, none of the mice lost more than 20% of their
453 weight and therefore did not reach the cutoff for euthanasia. Enoxacin treatment had no impact
454 on serum viremia or on virus titer in liver or brain. As FQs readily cross the blood-brain barrier

455 (83) and are metabolized in the liver (84), the absence of a drug effect in these tissues is unlikely
456 to be due to lack of enoxacin availability. Indeed, ZIKV titer was significantly greater in the
457 serum and brain of mice treated with 15mg/kg of enoxacin compared to 10 mg/kg. At present
458 we have no explanation for this finding, as we did not repeat the 10 mg/kg treatment. Given the
459 small sample sizes of this study, it is possible that this effect is due to random sampling, but
460 certainly further investigation of the dose effects of this drug *in vivo* are warranted. The most
461 intriguing result of this experiment, however, was that male mice treated with enoxacin showed a
462 significant 50% decrease in ZIKV titer in the testes.

463 To assess the reproducibility of these findings under a different treatment regimen, we
464 next tested the effect of pre-treating mice with enoxacin and then infecting them with ZIKV. As
465 in the first experiment, all mice lost weight (though weight loss was less than in the first
466 experiment) and ZIKV titers in serum, liver and brain did not differ between enoxacin-treated
467 and control mice. Importantly, consistent with the first experiment, ZIKV was significantly
468 suppressed in the testes of enoxacin-treated mice relative to controls.

469 The restriction of enoxacin's efficacy to the testes *in vivo* could be due to higher efficacy
470 in specific cell types in the testes or due to the lower temperature of the testes. However, counter
471 to these explanations, we found that enoxacin was less effective against ZIKV at 32˚C than 37˚C
472 and less effective against ZIKV in Sertoli cells than in human kidney cells. However, Sertoli
473 cells are one of many cell types in the testes, which also contain stem cells, spermatozoa and
474 Leydig cells, which vary in their susceptibility to ZIKV (50,51,85–87), thus our findings in
475 Sertoli cells do not reveal the action of enoxacin in the testes as a whole. Immunohistochemical
476 staining of ZIKV-infected mouse testes 7 days p.i. revealed the presence of viral antigen
477 primarily in the stem cells of the seminiferous tubules and in the seminal fluid from the vas

478 deferens (87). Using *in situ* hybridization, viral RNA of a mouse-adapted strain of ZIKV was
479 detected in the stem cells (spermatogonia and primary spermatocytes), Sertoli cells, and
480 spermatozoa of ZIKV-infected mouse testes at 7 days p.i. (86). ZIKV-infected germ cells were
481 detected in the basal layer of the seminiferous tubules of ZIKV-infected Olive Baboons via
482 immunofluorescence 11 days p.i. (88).

483 Recently, Xu *et al*. (35) reported that ZIKV infection of hNPCs activates the RNAi
484 antiviral response and elicits the production of virus-derived small interfering RNAs (vsi-RNA),
485 but infection of human neurons does not, indicating that cellular differentiation degrades the
486 functionality of RNAi. Additionally, Xu et al. (35) demonstrated that treatment with enoxacin, a
487 known enhancer of RNAi (25,26), significantly suppressed ZIKV replication in hNPCs. The
488 HEK-293 cells in which we demonstrated enoxacin efficacy against ZIKV have stem-cell like
489 properties (89). Our time-of-addition assays indicate that enoxacin, ciprofloxacin, and difloxacin
490 all suppress an intermediate life stage of ZIKV, which is consistent with enhancement of RNAi.
491 Furthermore, we detected an impact of enoxacin in testes, which are rich in stem cells, but not in
492 differentiated brain or liver cells. Thus, our results are generally consistent with those of Xu et
493 al. (35), which implicate RNAi enhancement as a key antiviral mechanism of enoxacin.

494 However, multiple alternative explanations for our *in vivo* findings must be considered.
495 First and foremost, Xu et al. (35) used 10-fold more enoxacin (100 μM vs 10 μM) in their study
496 than we used in ours. Additionally, testicular ZIKV infection results in oxidative stress, and
497 antioxidants such as ebselen have been shown to reduce oxidative stress, lessen testicular
498 damage, and prevent sexual transmission in mice (90). Like ebselen, FQs are also known to act
499 as antioxidants (27–29). FQs may also damage the testes and thereby restrict flavivirus
500 replication. Demir *et al*. (91) demonstrated that rats treated with oral ciprofloxacin at 150 mg/kg

501 for ten days experienced testicular damage to the germinal epithelium, tubular atrophy, and
502 swelling. As we did not evaluate the histopathological effects of enoxacin treatment in our study
503 we cannot discount the possibility that such damage occurred. However, Demir et al. (91) used a
504 much higher concentration of ciprofloxacin than we did in our study (150 mg/kg ciprofloxacin vs
505 15 mg/kg enoxacin). More research on the *in vivo* testicular toxicity of FQs is needed, especially
506 since ZIKV infection itself damages testicular tissues (86,92,93) although damage to human
507 Sertoli cells is minimal (50).

508 More generally, several caveats pertain to our study. First, although use of the A129
509 immunocompromised mouse model to initially test compounds for *in vivo* efficacy against ZIKV
510 has become a standard practice (65,94,95), nonetheless, these mice do lack an interferon
511 response, and the interplay between interferon and ZIKV clearly shapes pathogenesis (96–102).
512 Thus, the reproducibility of our findings in other, immunocompetent animal models, such as the
513 human STAT2 knockin mouse model or C57B1/6 mice treated with the anti-type I IFN receptor
514 antibody (102–104), should be tested. Second, as we only tested enoxacin across two
515 concentrations *in vivo*, a wider range of FQs and FQ concentrations should be investigated *in*
516 *vivo* for efficacy in suppressing ZIKV. Third, our investigation of the testes-specific efficacy of
517 enoxacin focused only on Sertoli cells, but efficacy in other testicular cells should also be
518 evaluated, particularly stem cells.

519 In summary, we found that FQs had reasonable efficacy against six flaviviruses in
520 cultured cells and enoxacin demonstrated efficacy against ZIKV in mouse testes. Limiting
521 replication of the virus in the testes is important, as ZIKV is capable of sexual transmission
522 (105–108). The results from this study and of Xu et al. (35) also suggest that testing the ability

523 of FQs to alleviate the teratogenic effects of ZIKV in relevant mouse models (103,109,110)
524 should be a priority.
525

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821 # Tables

822 **Table 1**. Passage history for flaviviruses utilized in this study

Virus	Strain	Obtained from	Passage history
Zika virus (ZIKV)	MEX 1-7	World Reference Center for Emerging Viruses and Arboviruses (WRCEVA)	C6/36 (x3)
Zika virus (ZIKV)	FSS13025		C6/36 (x1), Vero (x1)
Dengue virus-1 (DENV-1)	Thailand 160087-1A	Laboratory of Dr. Stephen Whitehead, NIAID, NIH	Vero (x5)
Dengue virus-2 (DENV-2)	NGC proto		C6/36 (x3), Vero (x2)
Dengue virus-4 (rDENV-4)	Dominica p4-3b (36)		Vero (x4)
Langat virus (LGTV)	E5 (38)	Laboratory of Dr. Alexander Pletnev, NIAID, NIH	Vero (x4)
Modoc virus (MODV)	7/26/61	WRCEVA	IC suckling mice (x9), Vero (x4)

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834 **Table 2.** Number of ZIKV-infected and control mice treated with enoxacin or drug diluent

	Experiment 1		Experiment 2	
	$1x10^5$ pfu ZIKV	PBS Control	$1x10^2$ pfu ZIKV	PBS Control
Drug diluent	7 (4 female, 3 male)	NA	7 (3 female, 4 male)	6 (3 female, 3 male)
Enoxacin (10 mg/kg)	7 (3 female, 4 male)	6 (2 female, 4 male)	NA	NA
Enoxacin (15 mg/kg)	6 (0 female, 6 male)	5 (3 female, 2 male)	6 (3 female, 3 male)	7 (5 female, 2 male)

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852 **Table 3.** Efficacy and toxicity of enoxacin, difloxacin, and ciprofloxacin against flaviviruses in

853 HEK-293 cells

FQ	CC_{50} µM (95% CI)	Virus	EC_{50} µM (95% CI)	Selectivity Index [a]
Enoxacin	537.6 (378.8-763.0)	ZIKV	18.1 (14.6-22.4)	29.7
		DENV-1	6.6 (6.0-7.3)	81.5
		DENV-2	4.7 (3.5-6.2)	114.4
		DENV-4	7.6 (7.1-8.2)	70.7
		LGTV	4.3 (2.8-6.5)	125.0
		MODV	14.6 (7.4-29.0)	36.8
Difloxacin	763.0 (579.3-1005.0)	ZIKV	25.4 (20.8-30.9)	59.2
		DENV-1	10.9 (9.2-12.9)	138.0
		DENV-2	5.7 (4.8-6.9)	263.9
		DENV-4	10.1 (9.1-11.3)	148.9
		LGTV	8.2 (6.3-10.6)	183.4
		MODV	>150	n.d.
Ciprofloxacin	1504.0 (730.0-3099.0)	ZIKV	56.8 (39.6-81.5)	13.4
		DENV-1	27.8 (22.1-34.9)	27.4
		DENV-2	8.0 (5.0-12.9)	188.0
		DENV-4	19.6 (16.5-23.2)	38.9
		LGTV	7.4 (3.9-14.0)	103.1
		MODV	11.2 (3.8-32.6)	68.1

854 a: Selectivity Index: CC_{50} divided by EC_{50}

855 n.d.: not determined

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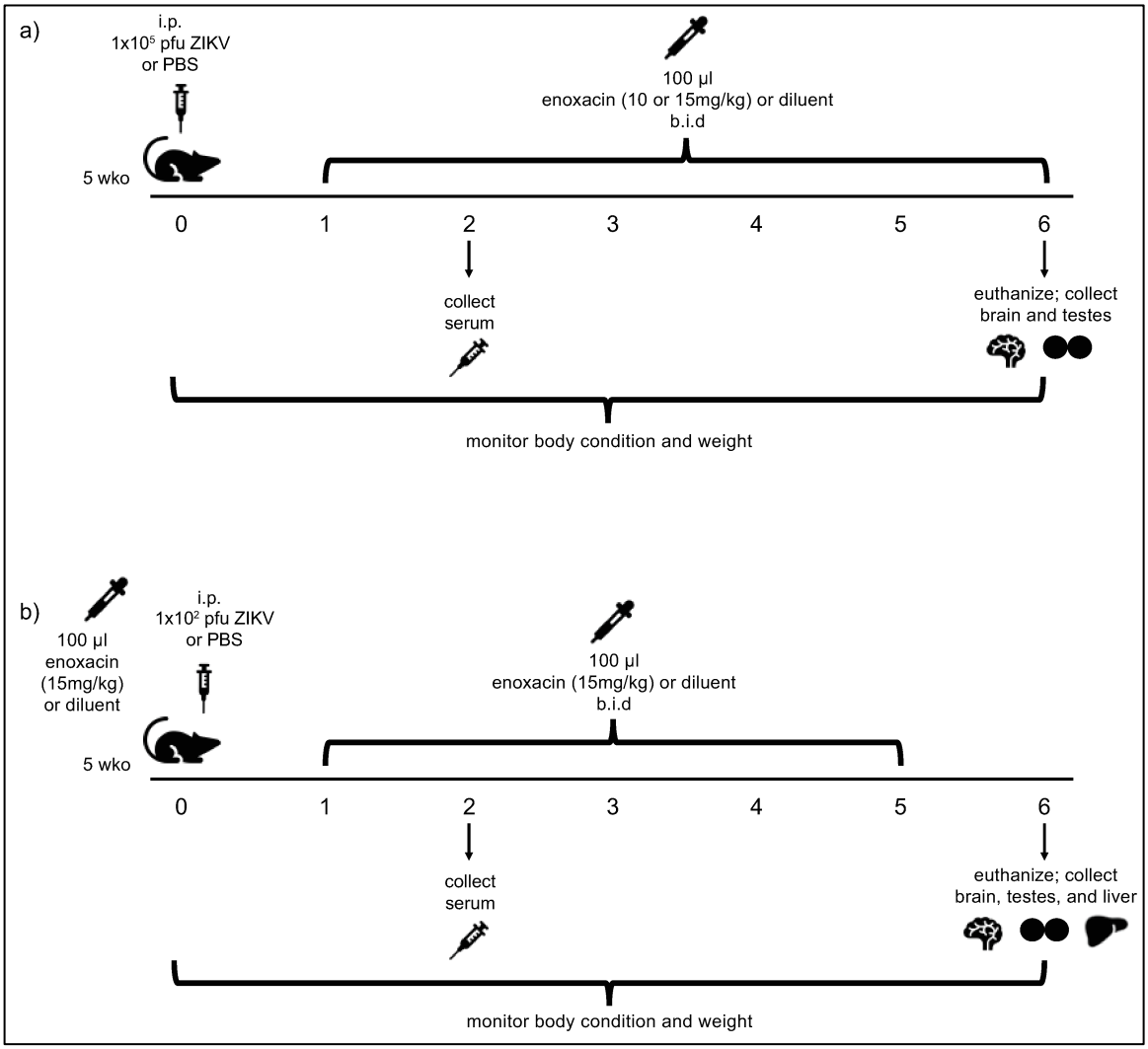
865 **Figures**



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867 **Fig 1. Experimental design.** a) In experiment 1, 5-week-old A129 mice were injected with
868 ZIKV ($1x10^5$ pfu) or PBS then treated orally with enoxacin (10 or 15 mg/kg) or drug diluent
869 twice daily on days 1-6. b) In experiment 2, 5-week-old A129 mice were pre-treated with
870 enoxacin (15 mg/kg) or diluent 8 hours before injection with ZIKV ($1x10^2$ pfu) or PBS and then
871 were treated orally with enoxacin (15 mg/kg) or diluent twice daily on days 1-5 p.i.

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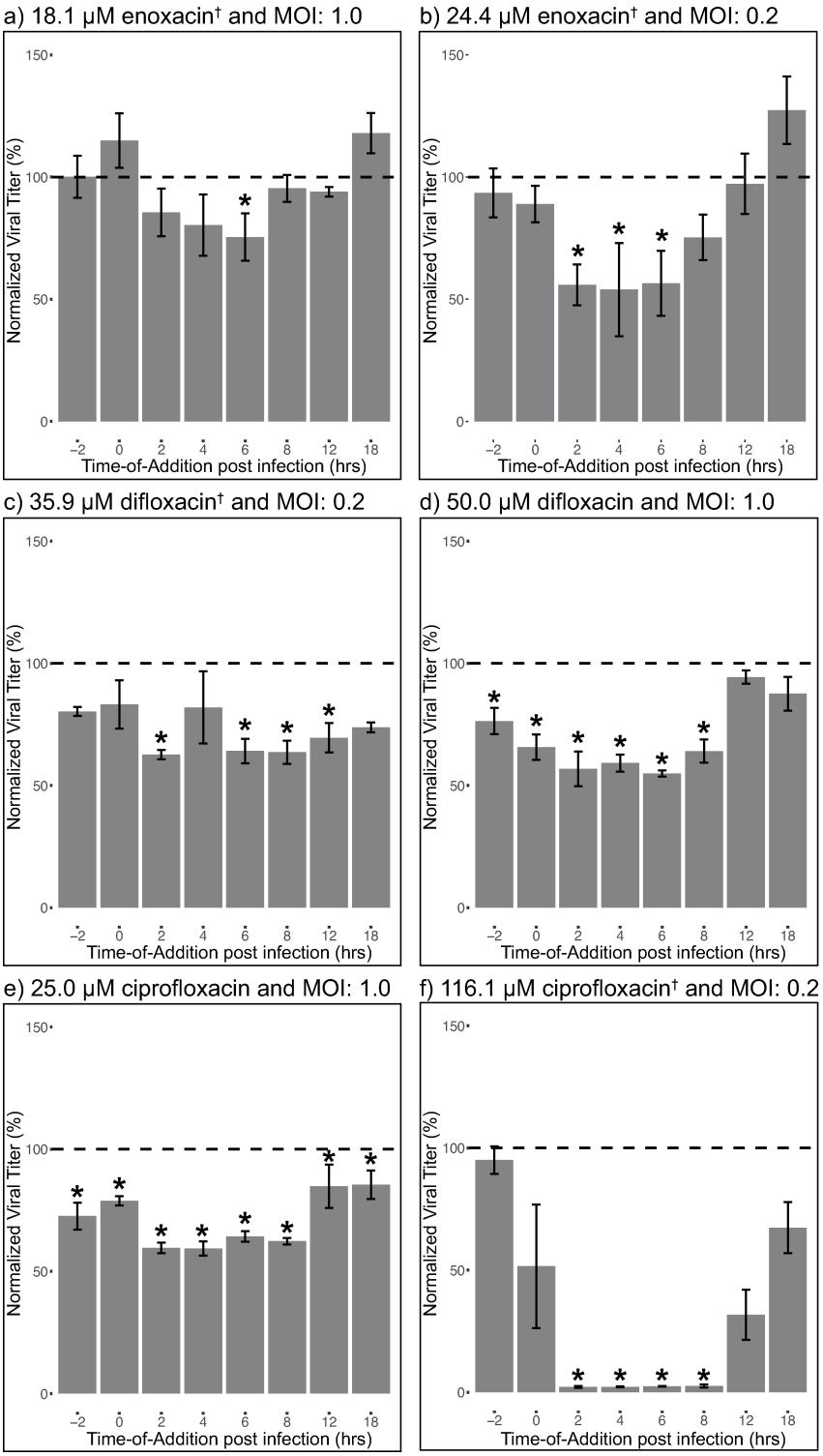

Fig 2. Enoxacin suppresses intermediate life cycle stages of ZIKV while difloxacin and ciprofloxacin suppress early and intermediate life cycle stages of ZIKV

877 Impact on replication of ZIKV at designated MOI by specified drug at designated concentration
878 when added at designated timepoint (a) 18.1 μM enoxacin, MOI 1.0; (b) 24.4 μM enoxacin, MOI
879 0.2; (c) 35.9 μM difloxacin, MOI 0.2; (d) 50.0 μM difloxacin, MOI 1.0; (e) 25.0 μM
880 ciprofloxacin, MOI 1.0; (f) 116.1 μM ciprofloxacin, MOI 0.2. † drug EC_{50} in Table 3. Viral
881 titers (n = 3 replicates per drug per time point) for each time point were normalized to the
882 average viral titer with media treatment and reported as average percent (titer at time
883 point/average media titer*100). Differences in mean viral titers (log_{10} pfu/mL) were detected
884 with ANOVA and pairwise t-tests; full pairwise statistics in Table S1. * $P < 0.05$ compared to
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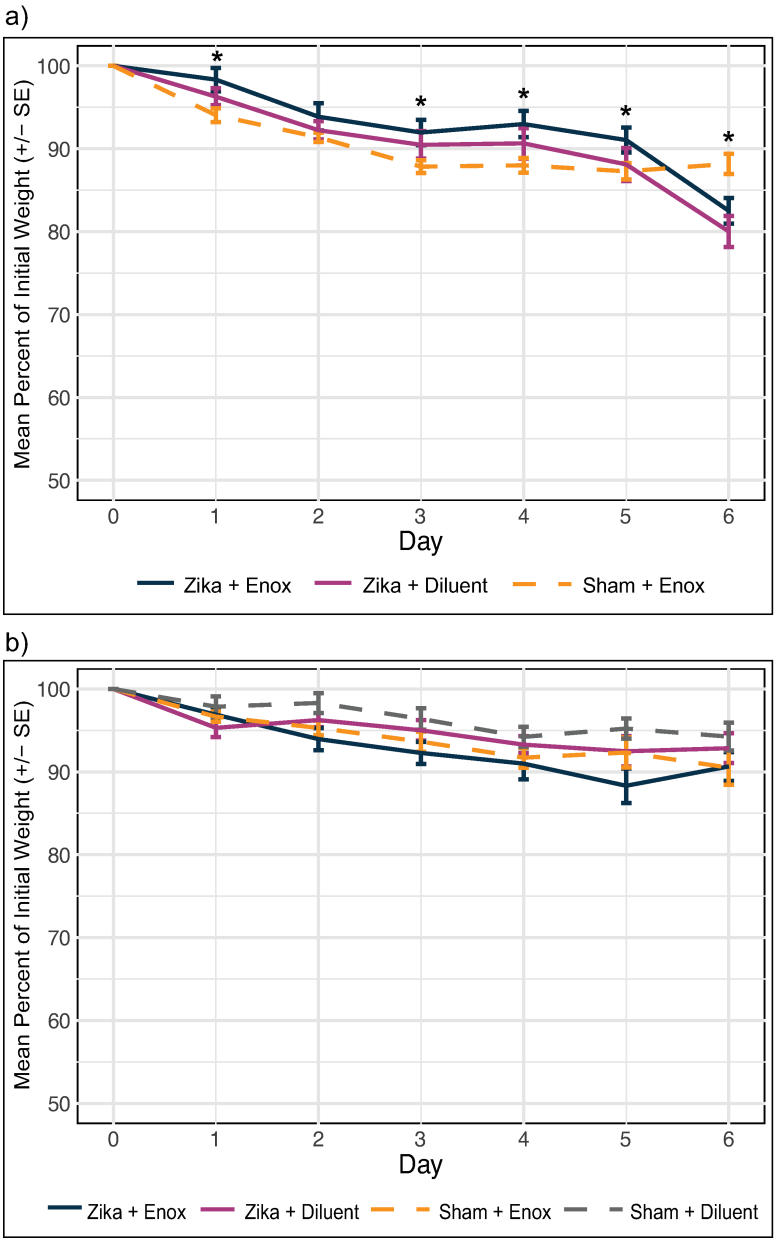

Fig 3. **Treatment had no significant effect on mouse weight loss**. (a) Daily percent of initial weight for experiment 1 was the same for ZIKV infected mice treated with enoxacin (10 mg/kg and 15 mg/kg combined) or diluent and uninfected mice treated with enoxacin (10 mg/kg and 15 mg/kg combined) until day 6 p.i. when the infected mice, regardless of treatment, lost significantly more weight than the uninfected controls (repeated measures ANOVA: F (12, 194) = 3.1, P = 0.0006; sample sizes in Table 2; full pairwise t-test statistics in Table S2); * at least

907 one group is different at $P < 0.05$. (b) Percent of initial weight for experiment 2 was not different
908 among treatment groups (repeated measures ANOVA: $F(18, 132) = 1.4$, $P = 0.12$; sample sizes
909 in Table 2)
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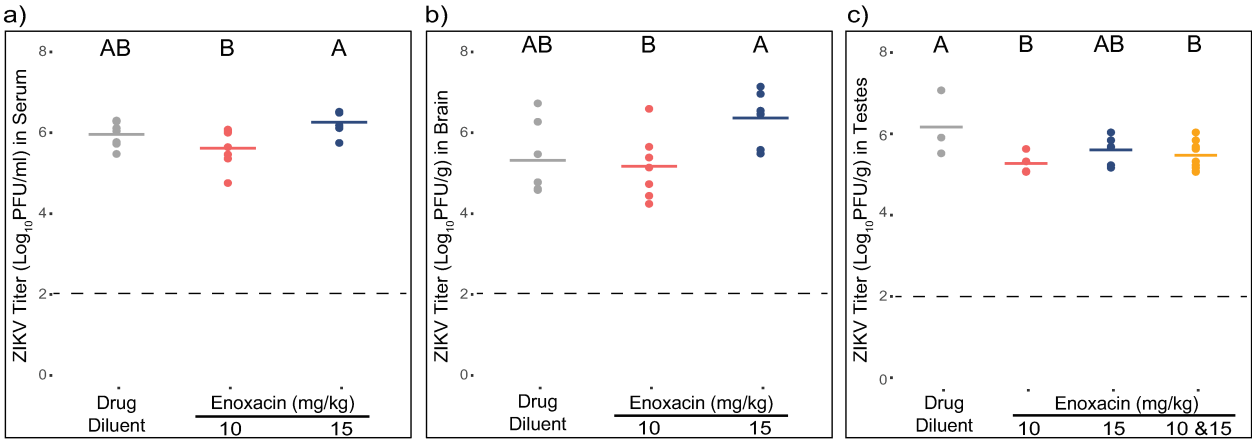

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931 **Fig 4**. **Treatment with enoxacin following high-titer infection suppresses ZIKV replication**

932 **in mouse testes but not in sera or brain.** Individual (dots) and mean (line) ZIKV titers of mice

933 treated with the drug diluent or enoxacin from (a) sera, (b) brain, and (c) testes. Sample sizes for

934 each treatment are listed in Table 2.

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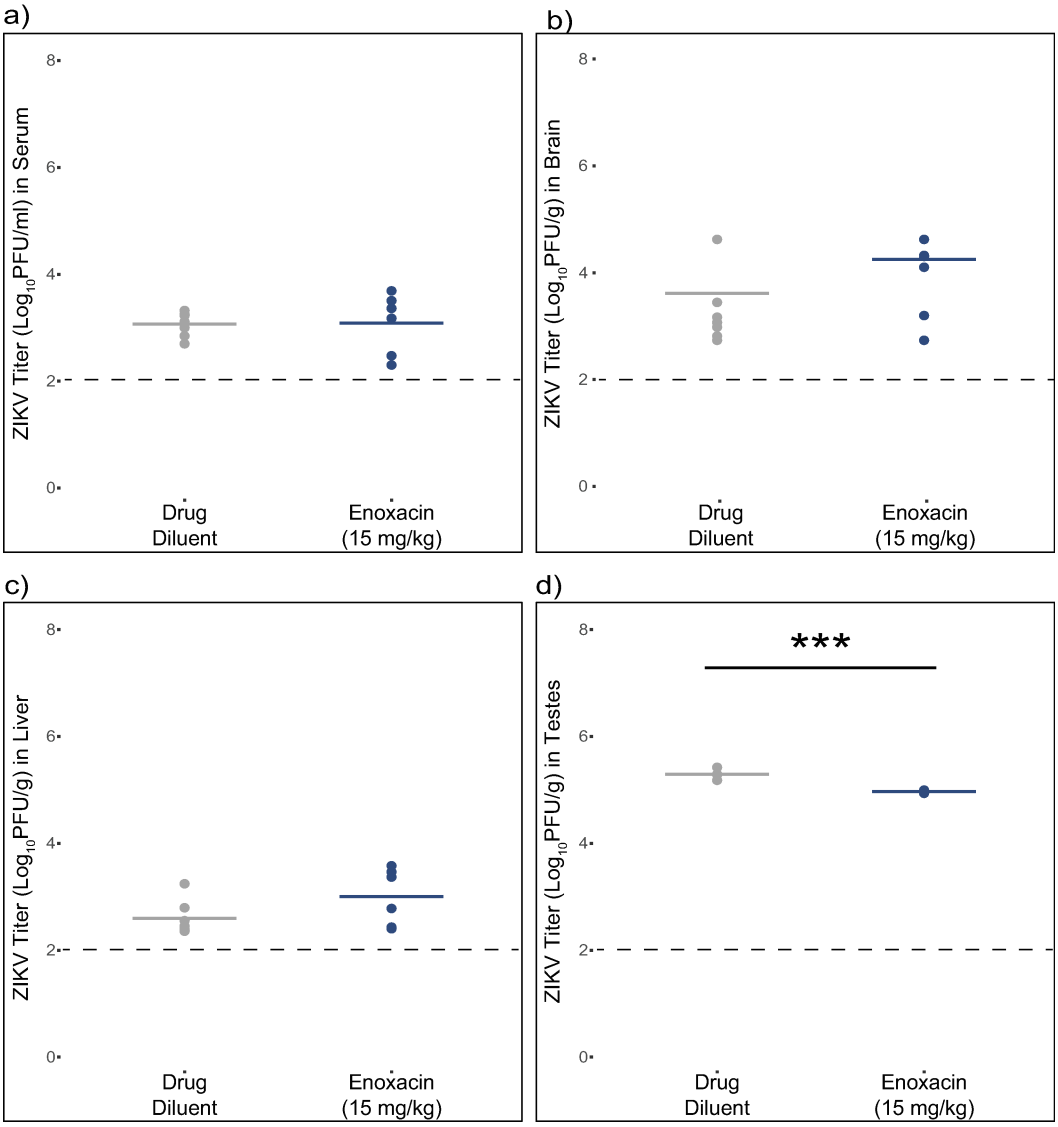

Fig 5. **Treatment with enoxacin prior to and following low-titer ZIKV infection suppresses viral replication in the testes but not in serum, brain or liver.** Individual (dots) and mean (line) ZIKV titers of mice treated with the drug diluent or enoxacin from (a) sera, (b) brain, (c) liver, and (d) testes. Sample sizes for each treatment are listed in Table 2.

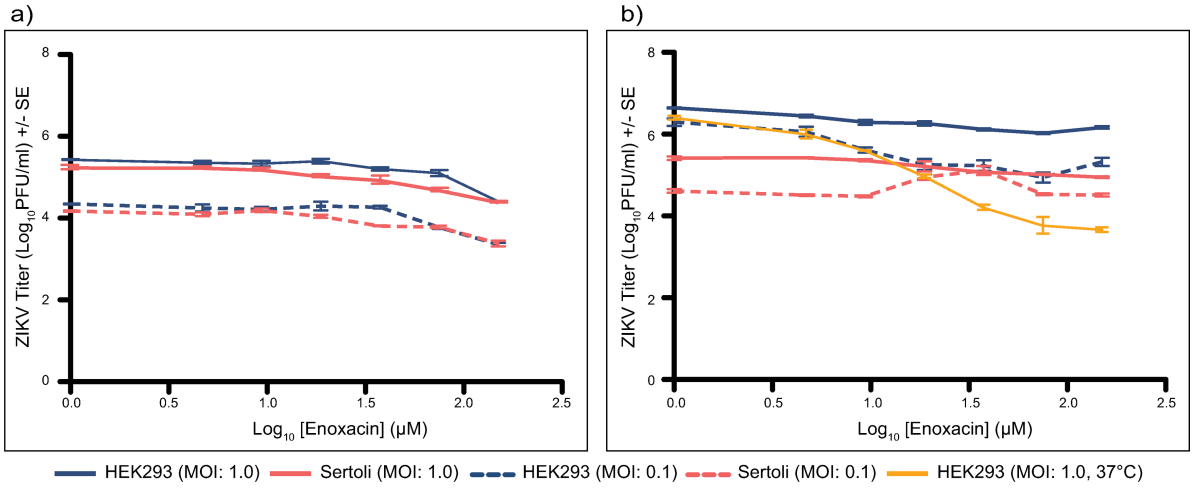

Fig 6. **Enhancement of enoxacin efficacy to suppress ZIKV was not detected in Sertoli cells two days pi (a) or five days pi (b).** Dose-response curves for enoxacin and ZIKV titer at 32 °C in Sertoli cells (pink) and HEK-293 cells (blue) at two MOI, 0.1 (dashed) and 1.0 (solid). Dose-response curve for enoxacin and ZIKV titer in HEK-293 cells at 37 °C (yellow) is included for comparison on day 5 p.i. See the text for statistics.

972 **Supporting Information**

973 **S1 Table.** ANOVA results and pairwise t-test comparisons for ZIKV titer after treatment with

974 enoxacin, difloxacin and ciprofloxacin from the time-of-addition assays in Fig. 2.

	Enoxacin		Difloxacin		Ciprofloxacin	
Concentration (μM)	18.1	24.4	35.9	50.0	25.0	116.1
MOI	1.0	0.2	0.2	1.0	1.0	0.2
F (df), P	2.6 (8, 18), 0.04	3.6, (8,18), 0.01	2.8 (8, 18), 0.04	12.8 (8, 18), 5.2e-6	16.2 (8, 18), 2.3E-7	14.7 (8, 18), 1.9e-6
Time of addition (hour p.i.)						
Media	AB	AB	A	A	A	A
-2	AB	AB	AB	BC	CD	A
0	A	ABC	AB	CD	BC	A
2	BC	CD	B	CD	E	B
4	BC	D	AB	D	E	B
6	C	CD	B	D	DE	B
8	ABC	BCD	B	CD	DE	B
12	ABC	AB	B	A	BC	A
18	A	A	B	AB	B	A

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988 **S2 Table**. Pairwise t-test comparisons of mean percent weight change from *in vivo* ZIKV

989 infection in Fig 3a

Virus + Treatment	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Sham + enoxacin	JK	FHI	DEG	BC	BC	B	BC
Zika + lactic acid	K	IJ	EFGH	BCDEFGH	BCDEFGH	BCD	A
Zika + enoxacin	JK	JK	GHI	DEFGH	EFGHI	CDEF	A

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