1 **TITLE**

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

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4 **Short Title**

5 Fluoroquinolones suppress flavivirus replication

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29

Abstract

31   Antiviral therapies are urgently needed to treat infections with flaviviruses such as Zika

32   (ZIKV) and dengue (DENV) virus. Repurposing FDA-approved compounds could provide the

33   fastest route to alleviate the burden of flaviviral diseases. In this study, three fluoroquinolones,

34   enoxacin, difloxacin and ciprofloxacin, curtailed replication of flaviviruses ZIKV, DENV,

35   Langat (LGTV) and Modoc (MODV) in HEK-293 cells at low micromolar concentrations.

36   Time-of-addition assays revealed that enoxacin suppressed ZIKV replication when added at 6

37   hours post-infection, suggesting inhibition of an intermediate step in the virus life cycle, whereas

38   ciprofloxacin and difloxacin had a wider window of efficacy of 2, 6, and 8 hours post-infection

39   for difloxacin and 2 to 8 hours post-infection for ciprofloxacin. The efficacy of enoxacin to

40   suppress ZIKV replication in 5-week-old A129 mice was evaluated in two experiments. First,

41   mice were infected with 1×10^5 plaque-forming units (pfu) ZIKV FSS13025 (n=20) or PBS

42   (n=11) on day 0 and subsets were treated with enoxacin at 10mg/kg or 15mg/kg or diluent orally

43   twice daily on days 1-5. Treated and control mice did not differ in weight change or virus titer in

44   serum or brain. Mice treated with enoxacin showed a significant, 5-fold decrease in ZIKV titer

45   in testes relative to controls. Second, mice were infected with 1×10^2 pfu ZIKV (n=13) or PBS

46   (n=13) on day 0 and subsets were treated with 15mg/kg oral enoxacin or diluent twice daily on

47   days 0 (pre-treatment) and 1-5. Mice treated with enoxacin showed a significant, 2.5-fold

48   decrease in ZIKV titer in testes relative to controls, while weight and viral load in the serum,

49   brain, and liver did not differ between treated and control mice. Enoxacin efficacy in cultured

50   murine Sertoli cells was not enhanced compared to efficacy in HEK-293 cells. ZIKV can be

51   sexually transmitted, so reduction of titer in the testes by enoxacin should be further investigated.

Author Summary

53   Flaviviruses such as Zika and dengue virus pose a significant threat to public health

54   worldwide, and there are currently no antiviral therapies to treat any flaviviral infection.

55   Repurposing FDA-approved drugs as anti-flaviviral therapies can accelerate clinical use. We

56   demonstrated that fluoroquinolone antibiotics exhibit anti-flaviviral efficacy, suppressing

57   flavivirus replication in cultured human cells. Additionally, we found that the fluoroquinolone

58   enoxacin suppressed Zika virus replication in mouse testes. While Zika virus is primarily

59   transmitted via mosquitoes, the virus also undergoes sexual transmission.   The importance of

60   sexual transmission for the overall epidemiology of the virus remains unclear; nonetheless all

61   routes of potential transmission to pregnant women are of concern as fetal infection *in utero* can

62   have devastating effects. Thus, our data indicate that fluoroquinolones hold promise for

63   treatment of flaviviral infections, particularly infection of the testes by Zika virus, and that this

64   class of drugs warrants further study.

Introduction

66   Viruses of the genus flavivirus (family *Flaviviridae*) are major causes of morbidity and

67   mortality worldwide (1–6). Moreover many flaviviruses, such as Zika (ZIKV), dengue (DENV),

68   tick-borne encephalitis (TBEV), West Nile (WNV), and Japanese encephalitis (JEV) virus (7–9)

69   are categorized as emerging pathogens due to rising incidence and expanding geographic range

70   (10,11). Effective antiviral drugs could abate flavivirus transmission and disease burden, but to

71   date no drugs for treatment of flavivirus infections have been brought to market because efforts

72   to develop anti-flaviviral drugs have been unsuccessful (12,13). Most anti-flaviviral drug

73   candidates have stalled at the point of hit-to-lead optimization due to poor drug-like properties

74   (14–16). This history of roadblocks in development of novel drugs suggests that repurposing

75   clinically approved drugs offers the fastest track to clinical treatments of flavivirus infections

76   (17).

77   The fluoroquinolones are not an immediately obvious choice as anti-flavivirals. The

78   flavivirus genome comprises a single, positive-sense RNA, while fluoroquinolones are primarily

79   known for inhibiting topoisomerases and gyrases in bacterial targets (18,19), neither of which

80   play a role in genome synthesis in positive-sense RNA viruses (20,21). However in the last

81   several years, a multitude of previously unsuspected effects of fluoroquinolones on eukaryotic

82   cell functions have been revealed, including enhancement of RNAi (22–24), inhibition of cellular

83   helicases (25,26), attenuation of cytokines and pro-inflammatory reactive oxygen species (27–

84   29), and modification of apoptosis (30) and autophagy (31). Furthermore, fluoroquinolones have

85   been shown to suppress hepatitis C virus (HCV, family *Flaviviridae*) replication *in vitro*,

86   possibly by inhibiting the viral helicase (32), but this suppression has not translated into an

87   effective treatment for patients with liver failure due to chronic HCV infection (33).

88   Additionally, fluoroquinolones suppress rhinovirus infection by reducing expression of the viral

89   receptor on cells (34). Recently, Xu and colleagues demonstrated that a high concentration of

90   enoxacin administered to human neuronal progenitor cells (hNPC) and brain organoids prior to

91   and after infection with ZIKV suppressed viral replication and restored normal cellular

92   proliferation, possibly by enhancing RNAi (35).

93   Here we evaluated the utility of repurposing fluoroquinolones as anti-flavivirals by

94   testing their ability to suppress flavivirus replication in cell culture and a mouse model. This

95   study was initially motivated by our interest in the ability of fluoroquinolones to enhance RNAi,

96   and thus we focused on three fluoroquinolones, enoxacin, ciprofloxacin and difloxacin, that have

97   high, moderate and little impact on RNAi, respectively (23). We found that all three drugs

98   suppressed replication of six flaviviruses in HEK-293 cells at low micromolar concentrations.

99   Enoxacin displayed the lowest EC_{50} values in cell culture and was selected for evaluation in

100   ZIKV-infected A129 mice. Although enoxacin did not mitigate weight loss in ZIKV-infected

101   mice or suppress ZIKV replication in the serum, brain, or liver, the drug did suppress ZIKV

102   replication in the testes.

Material and Methods

Viruses

105   The seven flaviviruses utilized in this study are listed in Table 1. Working stocks of

106   viruses were propagated in Vero cells and viral supernatants were collected either in 1X SPG

107   (2.18 mM sucrose, 38 mM potassium phosphate [monobasic], 72 mM potassium phosphate

108   [dibasic], 60 mM L-glutamic acid) [DENV-1,2 and 4, MODV, LGTV, ZIKV MEX 1-7] for

109   studies in culture or 1X DMEM supplemented with 5% heat inactivated fetal bovine serum

110   (FBS, Atlantica Biologicals, Flowery Branch, GA) and 100μg/mL penicillin/streptomycin

111   (Gibco, Life Technologies, Grand Island, NY) [ZIKV FSS13025] for studies *in vivo*.

112   Supernatants were clarified by centrifugation, aliquoted and stored at -80 °C. Viral titers were

113   determined via serial dilution onto HEK-293 cells followed by immunostaining using methods as

114   previously described (36,37). Briefly, each virus was subjected to serial tenfold dilution and

115   inoculated onto confluent HEK-293 cells in 24-well plates. After two hours of incubation at

116   37°C with occasional rocking, infected cells were overlaid with 1% methylcellulose in

117   OptiMEM (Gibco, Life Technologies, Grand Island, NY) that had been supplemented with 2%

118   FBS (Gibco, Life Technologies, Grand Island, NY), 2mM L-glutamine (Gibco, Life

119   Technologies, Grand Island, NY), and 0.05 mg/mL gentamycin (Gibco, Life Technologies,

120   Grand Island, NY). Plates were incubated for five days under maintenance conditions, after

121   which cells were fixed with ice cold methanol: acetone (1:1) for 30 minutes. Viral plaques were

122   immunostained using species-specific antibodies and peroxidase-labeled goat anti-mouse

123   secondary antibody (KPL, Gaithersburg, MD) then developed with KPL True Blue Peroxidase

124   Substrate (SeraCare, Milford, MA) and counted to calculate viral titer.

125

126   **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		Vero (x5)
Dengue virus-2 (DENV-2)	NGC proto	Laboratory of Dr. Stephen Whitehead, NIAID, NIH	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)

127

128

129 **Cells**

130   HEK-293 and murine Sertoli cells were purchased from ATCC (CRL-1573 and CRL-

131 2618, Manassas, VA). Vero cells were obtained from the lab of Stephen Whitehead (NIAID,

132 NIH). HEK-293 cells were maintained at 37°C with 5% CO_2 in DMEM/F12 medium (Gibco,

133 Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Gibco),

134 2mM L-glutamine (Gibco), and 0.5% antibiotic-antimycotic (penicillin, streptomycin, and

135 amphotericin B; Gibco). Sertoli cells were maintained at 32°C with 5% CO_2 in DMEM/F12

136 (Gibco) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and

137 penicillin/streptomycin (100 units/mL and 100 µg/mL, respectively; Gibco). Vero cells were

138 maintained at 37° C with 5% CO_2 in DMEM (Gibco) supplemented with 10% heat-inactivated

139 FBS (Gibco). All cell culture efficacy and toxicity experiments were conducted with HEK-293

140 cells, an interferon competent human cell line that supports flavivirus replication and is often

141 used to evaluate potency and toxicity of potential antivirals (39–45).

142

143 **Fluoroquinolone compounds**

144   For each experiment, a fresh working stock of enoxacin (Sigma-Aldrich, E3764, St.

145 Louis, MO), difloxacin (Sigma-Aldrich, D2819, St. Louis, MO), or ciprofloxacin (Corning,

146 86393-32-0, Manassas, VA) at a concentration of 1.5 mM was sonicated in nanopore water with

147 3 mM lactic acid (Sigma-Aldrich, L1750, St. Louis, MO) and sterilized *via* passage through a 0.2

148 µm filter. The compounds were diluted to their final concentrations in cell culture media for

149 assays in cell culture, or nanopore water for *in vivo* treatments.

150

Viral replication kinetics in cell culture

151

152   To quantify replication kinetics of particular viruses, triplicate 25-cm^2 flasks of HEK-293

153   cells were grown to ~80% confluence, washed with 3mL cell culture media, and infected with a

154   specified virus at a multiplicity of infection (MOI) of 0.05 in 1mL total volume. Cells were

155   incubated at 37°C for 2 hours with occasional rocking. Virus inoculum was then removed and

156   cells washed twice with 3mL of 1x PBS to remove any unadsorbed virus. Six mL of cell culture

157   media was then added to each flask. At time 0, 1mL of cell culture supernatant was removed and

158   SPG was added at a final concentration of 1X. Cell culture supernatants were clarified by

159   centrifugation, aliquoted and stored at -80°C. Samples were collected on days 1 through 8 by

160   removing 1mL of supernatant as described above and 1 mL of cell culture media was added back

161   to the flask. Viral titers were determined in HEK-293 cells as described above. ZIKV was

162   added to this project after the replication kinetics assays were completed, in response to the

163   Public Health Emergency of International Concern declared on February 1st, 2016, thus this

164   assay was not conducted with ZIKV.

165

Determination of half-maximal effective concentration (EC$_{50}$) against select flaviviruses

167   To determine the EC$_{50}$ of enoxacin, difloxacin, and ciprofloxacin, monolayers of 80%

168   confluent HEK-293 cells in 24-well plates were infected with either ZIKV, DENV-1, DENV-2,

169   DENV-4, LGTV, or MODV in triplicate at a multiplicity of infection (MOI) of 1. The assay was

170   repeated for all three fluoroquinolones with ZIKV at an MOI of 0.2. The virus was allowed to

171   adsorb for 2 hours at 37 °C after which cells were washed with 1 mL 1x phosphate buffered

172   saline (PBS) to remove unadsorbed virus. Each drug was diluted in a two-fold dilution series in

173   cell culture media, with final concentrations ranging from 150 μM to 4.7 μM, and one mL was

174   added to triplicate treatment wells. Triplicate control wells were treated with cell culture media

175   alone and another set of controls were treated with cell culture media containing 3mM

176   concentration lactic acid, the drug diluent. Infected cells were incubated for five days at normal

177   conditions, after which viral supernatants were collected and viral titers were determined as

178   described above.

179   As enoxacin was found to suppress ZIKV in the mouse testes, enoxacin potency was

180   evaluated in one testicular cell line (murine Sertoli cells) and compared to the potency in HEK-

181   293 cells. The EC_{50} methods described above were repeated for ZIKV MEX 1-7 in Sertoli cells

182   and HEK-293 cells, both incubated at 32 °C to control for potential differences in enoxacin

183   activity at the lower temperature required for Sertoli cell viability. For both cell types, two MOIs

184   were tested, 0.1 and 1.0, and virus was collected at two time points, 2 days post infection (p.i.)

185   and 5 days p.i. Viral titers were determined in HEK-293 cells as described above.

186

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

188   To determine the toxicity of enoxacin, difloxacin, and ciprofloxacin, HEK-293 cells were

189   grown in 96-well plates until confluent at which time the media was removed. Each filter-

190   sterilized fluoroquinolone was diluted two-fold, starting at 500 μM, and added to wells in

191   triplicate at a total volume of 100 μL. Control wells were treated with 100 μL of cell culture

192   media containing 3 mM lactic acid. Plates were incubated at normal conditions for five days,

193   after which the media was removed and 110 μL of 10% resazurin dye (Millipore Sigma, St.

194   Louis, MO) diluted in cell culture media was added to each well. After two hours incubation,

195   absorbance was measured on a plate reader at 600 nm and normalized to the mean absorbance of

196   the control wells.

197

Time-of-addition assays

199   Time-of-addition assays were conducted to gain insight into the potential mechanism of

200 action of each drug against ZIKV (46–50). All assays were conducted in triplicate; MOI and

201 drug concentration were varied in order to enhance statistical power to discern time-specific

202 effects. First, the impact of enoxacin, ciprofloxacin and difloxacin were tested at 24.4 µM, 116.1

203 µM, and 35.9 µM, respectively, against ZIKV at an MOI of 0.2. These drug concentrations

204 represent the EC_{50} values determined in HEK-293 cells infected with ZIKV at an MOI of 0.2.

205 Next, the time-of-addition assays were conducted using 18.1 µM enoxacin, 56.8 µM

206 ciprofloxacin and 25.4 µM difloxacin against ZIKV at an MOI of 1.0. These drug

207 concentrations represent the EC_{50} values determined in HEK-293 cells with ZIKV at an MOI of

208 1.0. Finally, a third assay was conducted using 25.0 µM ciprofloxacin and 50.0 µM difloxacin

209 against ZIKV at an MOI of 1.0. These concentrations were chosen to ameliorate suppression of

210 ZIKV by ciprofloxacin and increase suppression by difloxacin.

211   Eight timepoints were evaluated during all time-of-addition assays: two hours prior to

212 infection, at the time of infection (drug mixed with ZIKV), 2, 4, 6, 8, 12, and 18 hours p.i. These

213 time points capture flavivirus binding and entry (-2 and 0 hrs p.i.), translation (2 and 4 hrs p.i.),

214 genome replication (6 and 8 hrs p.i.), and virion assembly and budding (12 and 18 hrs p.i.) (51).

215 At time zero, monolayers of 80% confluent HEK-293 cells in 24-well plates were infected with

216 ZIKV MEX 1-7 at MOI 0.2 or 1. After two hours of incubation, the virus was removed from all

217 wells, the cells were washed with 1 mL of 1x PBS, and 1 mL of media per well was replaced. At

218 each time point, media was removed from designated triplicate wells, cells were washed with 1x

219 PBS, and 1 mL of drug at the specified concentration was added. Dilution of fluoroquinolones to

220   final concentration occurred at the time of treatment. For the wells treated at -2 hrs p.i., the drug

221   was removed and replaced with virus at the time of infection (time 0) then, after 2hrs incubation,

222   the virus was removed, the wells were washed with 1xPBS, and media was added to the wells.

223   For the wells treated at infection (0 hr p.i.), the media was removed and replaced with ZIKV

224   diluted in the drug at the time of infection. After 2 hrs incubation the virus and drug were both

225   removed, the wells were washed, and media was added. For the post-infection time points, the

226   drug was added to the wells at the specified time points and remained in the wells until 24 hrs

227   p.i. It should be noted that the half-life of enoxacin is 1.75 hrs of ciprofloxacin and difloxacin is

228   3 hours (52–54). Control wells infected with ZIKV were washed two hours p.i. and treated with

229   1 mL of media per well. At 24 hours p.i. all the viral supernatants were collected, clarified, and

230   stored as described above.

231

Determination of *in vivo* efficacy of enoxacin

233   The impact of enoxacin, the fluoroquinolone with the lowest EC_{50}, on ZIKV infection of

234   A129 mice was tested. Mice were infected at 5 weeks of age because our previous work showed

235   that in this age group ZIKV infection caused sustained weight loss that did not require euthanasia

236   until 8 days p.i., ensuring that the majority of mice would survive a 5-day trial (55). Mice were

237   housed in sterile caging in colonies at the University of Texas Medical Branch, an AALAS-

238   accredited facility, and research was conducted in accordance with UTMB policy under IACUC

239   Protocol #1708051.

240   The efficacy of enoxacin to suppress ZIKV replication *in vivo* was tested in two separate

241   experiments (Fig. 1). An *a priori* power analysis was used to determine the minimum number of

242   mice required to achieve 80% power to detect a difference of 0.3 log (i.e. 50%) decrease in viral

243   replication of the serum of 5-week-old- A129 mice. In experiment 1, we tested two

244   concentrations of enoxacin at 10 mg/kg and at 15 mg/kg in A129 mice infected with $1x10^5$ pfu

245   ZIKV, the viral dose used to in our previous work to characterize ZIKV infection in 5-week-old

246   A129 mice (55). The two concentrations, 10 mg/kg and 15 mg/kg, were selected because in an

247   average sized mouse (20 g) these doses correspond to peak serum concentrations (6.2 µM and

248   9.4 µM) that are comparable to the peak serum concentrations achieved in humans receiving a

249   typical clinical dose, wherein 200 mg and 400 mg oral dosages result in peak serum

250   concentrations of 5.0 µM and 11.2 µM, respectively (56,57). In experiment 2, we tested the

251   impacts of a lower dose of virus ($1x10^2$ pfu) and a pre-infection treatment of enoxacin on ZIKV

252   infection in mice. The experiment was limited to a single concentration of enoxacin, 15 mg/kg,

253   in order to utilize the minimum number of mice.

254   Experiment 1 (Fig. 1a): Mice were intradermally injected on day 0 with $1x10^5$ pfu ZIKV

255   FSS13025 diluted in 1x PBS (n=19) or with 1x PBS as a control (n=11) in a total volume of 100

256   µL and subsets of infected and uninfected mice were treated with oral enoxacin or drug diluent

257   (3 mM lactic acid) (Table 6) twice daily on days 1-6 p.i. Weight and body condition were

258   recorded twice daily. Two days p.i., 70 µL of blood was collected from the retro-orbital sinus,

259   clarified by centrifugation (5 minutes at 3380 x g), and serum was stored at -80 °C. Six days p.i.,

260   mice were euthanized and brain and testes were collected. Each tissue, along with a sterile steel

261   ball, were placed into a 2 mL Eppendorf tube containing 500 µL DMEM supplemented with 2%

262   FBS and penicillin/streptomycin and homogenized in a Qiagen TissueLyser II shaking at 26

263   pulses/second for 5 minutes. Homogenates were clarified by centrifugation at 3380 x g for 5

264   minutes and stored at -80 ˚C. Viral titers from serum and tissues were determined in Vero cells

265   in 12 well plates essentially as described above (55).

266   Experiment 2 (Fig. 1b): Mice received a pre-treatment of 15 mg/kg enoxacin (n= 14) or drug

267   diluent (n = 13) and were intradermally injected with 1×10^2 pfu ZIKV FSS13025 or 1x PBS 8

268   hours later as specified in Table 2. Subsets of infected and uninfected mice were treated with

269   oral enoxacin or lactic acid diluent twice daily on days 1-5. Weight and body condition were

270   recorded daily. Serum, brain and liver were collected, and viral titers determined as described

271   above.

272

273   **Fig 1. Experimental design.** a) In experiment 1, 5-week-old A129 mice were injected with

274   ZIKV (1×10^5 pfu) or PBS then treated orally with enoxacin (10 or 15 mg/kg) or drug diluent

275   twice daily on days 1-6. b) In experiment 2, 5-week-old A129 mice were pre-treated with

276   enoxacin (15 mg/kg) or diluent 8 hours before injection with ZIKV (1×10^2 pfu) or PBS and then

277   were treated orally with enoxacin (15 mg/kg) or diluent twice daily on days 1-5 p.i.

278

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

| | Experiment 1 | | Experiment 2 | |
	1×10^5 pfu ZIKV	PBS Control	1×10^2 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)

280

281   **Statistical analysis**

282   EC_{50} values were calculated using nonlinear regression of inhibition dose response for log

283   drug concentration and viral titer and CC_{50} values were calculated using nonlinear regression of

284   inhibition dose response for log drug concentration and cell viability in GraphPad Prism (version

285   5 for Mac OS X, GraphPad Software, La Jolla California USA). The selectivity index for each

286   fluoroquinolone and virus combination was calculated by dividing the CC_{50} by the EC_{50} values.

287   Mean viral titers at each time point from the time-of-addition assays were tested for normality

288   using the Shapiro-Wilk test then analyzed using ANOVAs. If the overall ANOVA was

289   significant, pairwise t-tests with a Bonferroni correction were used to detect pairwise differences.

290   Viral titers were first log-transformed then mean viral titers from mice were assessed for

291   normality using the Shapiro-Wilk test and compared using ANOVAs or t-tests as appropriate,

292   and differences in ZIKV replication in Sertoli cells and HEK-293 cells at 32 °C were evaluated

293   using general linear models in R (58).

294   **Results**

295   **Flavivirus replication curves in cultured human cells**

296   Replication curves for DENV-1, DENV-2, DENV-4, LGTV, and MODV in HEK-293

297   cells are shown in Fig. S1. Following infection at MOI 0.05, DENV-1 and DENV-4 titer rose

298   steadily through day 8 p.i., the last day of sampling, while DENV-2 peaked on day 7, LGTV

299   peaked on day 3 and MODV plateaued around 4 days p.i. On day 5 p.i. the viral titer for all 5

300   viruses ranged from 5.3 to 6.7 log10 PFU/mL. In light of the variation in replication dynamics

301   among the different viruses, subsequent evaluations of drug potency were conducted at a higher

302   MOI (MOI:1) on day 5 post-infection to capture the plateau of viral titer.

303

304   **Fluoroquinolones suppress flavivirus replication in cultured human cells**

305   As shown in Table 3 and Fig. S2, the EC_{50} values of enoxacin, ciprofloxacin and

306   difloxacin against DENV, ZIKV, LGTV and MODV in HEK-293 cells at 37 ° C were all in the

307   range of 4.3-56.8 µM, except for the EC_{50} of difloxacin for MODV, for which the EC_{50} value

308   was greater than the limit of detection in our assay (Table 3). CC_{50} values for enoxacin,

309   ciprofloxacin, and difloxacin were all substantially higher than the EC_{50} values (Table 3). In

310   general, difloxacin yielded the highest selectivity indices across viruses tested.

311

312   **Table 3.** Potency and toxicity of enoxacin, difloxacin, and ciprofloxacin against flaviviruses in

313   HEK-293 cells

Drug	CC_{50} µM (95% CI)	Virus	EC_{50} µM (95% CI)	Selectivity Index [a]
Enoxacin	537.8 (430.1-700.0)	ZIKV (MOI: 0.2)	24.4 (17.3-34.1)	22.0
		ZIKV (MOI: 1.0)	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)	70.8
		LGTV	4.3 (2.8-6.5)	125.1
		MODV	14.6 (7.4-29.0)	36.8
Difloxacin	1479.0 (1087.0-2272.0)	ZIKV (MOI: 0.2)	35.9 (19.0-67.5)	41.2
		ZIKV (MOI: 1.0)	25.4 (20.8-30.9)	58.2
		DENV-1	10.9 (9.2-12.9)	135.7
		DENV-2	5.7 (4.8-6.9)	259.5
		DENV-4	10.1 (9.1-11.3)	146.4
		LGTV	8.2 (6.3-10.6)	180.4
		MODV	>150	n.d.
Ciprofloxacin	759.6 (649.3-912.9)	ZIKV (MOI: 0.2)	116.1(68.9-179.0)	6.5
		ZIKV (MOI: 1.0)	56.8 (39.6-81.5)	13.4
		DENV-1	27.8 (22.1-34.9)	27.3
		DENV-2	8.0 (5.0-12.9)	95.0
		DENV-4	19.6 (16.5-23.2)	38.8
		LGTV	7.4 (3.9-14.0)	102.6
		MODV	11.2 (3.8-32.6)	67.8

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

315   n.d.: not determined

316

317   **Fluoroquinolone suppression of different life cycle stages of ZIKV**

318   Three sets of time-of-addition assays were used to discern the viral life stage(s) inhibited

319   by each of the three fluoroquinolones. First, monolayers of HEK-293 cells were infected at MOI

320   0.2 and treated with each of the three drugs at the EC_{50} value determined using MOI: 0.2. Next

321   monolayers of HEK-293 cells were infected at MOI 1.0 and treated with each of the three drugs

322   at the EC_{50} value determined using MOI: 1.0. In this second set of assays, ciprofloxacin

323   suppressed virus replication below the level of detection and difloxacin had little effect (Fig. S3),

324   so these assays were run again with ciprofloxacin at half its EC_{50} and difloxacin at twice its EC_{50}.

325   While significance varied somewhat among the assays, the overall patterns were

326   consistent. As seen in Fig. 2a and 2b, enoxacin suppressed virus replication most strongly when

327   added at 2, 4 or 6 hours p.i. Virus replication was suppressed by about 25% by 18.1 µM

328   enoxacin and 50% by 24.4 µM enoxacin. At both concentrations, differences between viral titers

329   by time of treatment were significant. Pairwise t-tests indicated that enoxacin treatment at 2, 4,

330   and 6 hours p.i. significantly reduced ZIKV titer compared to the media control (pairwise t-test P

331   <0.05; full statistics in Table S1).

332   Difloxacin at 35.9 µM suppressed virus replication when added 2, 6, 8, 12, and 18 hrs p.i.

333   (Fig. 2c) while 50.0µM difloxacin suppressed virus replication when added 2 hours before

334   infection, at the time of infection, 2, 4, 6, or 8 hours p.i. (Fig. 2d), as detected by pairwise t-tests

335   (full statistics in Table S1). At most, ZIKV replication was suppressed 37% by 35.9 µM

336   difloxacin and 50% by 50.0 µM difloxacin. At 25.4 µM difloxacin, the EC_{50} of this drug against

337   ZIKV at MOI 0.2 (Table 3) no difference in viral titer was detected by time (Fig. S3a, Table S1).

338   As seen in Fig. 2e and 2f and Fig. S3b, ciprofloxacin most strongly and consistently

339   suppressed virus replication when added 2, 4, 6, or 8 hours p.i. At these time points, virus was

340   suppressed an average of 40% by 25.0 µM ciprofloxacin, 71% by 56.8 µM ciprofloxacin, and

341   below the level of detection by 116.1 µM ciprofloxacin. The differences in viral titers by time of

342   treatment were significant for all three concentrations of ciprofloxacin tested (Table S1).

343   Pairwise comparisons revealed that 25.0 μM ciprofloxacin added 2 hours before infection, at the

344   time of infection, and up until 18 hours p.i. significantly reduced ZIKV replication compared to

345   the media control; 56.8 μM ciprofloxacin added 2, 4, and 6 hours p.i. significantly reduced

346   replication, and 116.1 μM ciprofloxacin added at 2, 4, 6, and 8 hours p.i. significantly reduced

347   replication (full statistics in Table S1).

348

349   **Fig 2**. **Enoxacin suppresses intermediate life cycle stages of ZIKV while difloxacin and**

350   **ciprofloxacin suppress early and intermediate life cycle stages of ZIKV**

351   Results of time-of-addition assays of each of three fluoroquinolones against ZIKV at designated

352   drug concentrations and virus MOIs (see text for justification of drug concentration and MOI

353   pairings): for enoxacin (a,b), difloxacin (c,d), and ciprofloxacin (e,f). Viral titers (n = 3

354   replicates per drug per time point) for each time point were normalized to the average viral titer

355   with media treatment and reported as average percent (titer at time point/average media

356   titer*100). Differences in mean viral titers ($\log_{10}$ pfu/mL) were detected with ANOVA and

357   pairwise t-tests; full pairwise statistics in Table S1. * P < 0.05 compared to media control.

358

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

360   To evaluate the *in vivo* efficacy of enoxacin to suppress ZIKV, A129 mice were infected

361   with ZIKV and treated with enoxacin in two independent experiments described in Fig. 1 and

362   Table 2. In both experiments, all mice lost weight, irrespective of treatment (Fig. 3). Loss of

363   weight by control mice was unexpected, and likely resulted from the effects of dosing these

364   small (average 19.3 g) animals twice daily with 100 μL volume of liquid. In experiment 1,

365   weight loss, quantified as the percent of initial weight, did not differ between ZIKV-infected

366   mice treated with 10 mg/kg and 15 mg/kg (repeated measures ANOVA: $F_{(5,55)} = 0.7$, $P = 0.61$)

367   and mean percent weight lost by uninfected mice treated with 10 mg/kg and 15 mg/kg also did

368   not differ significantly different from each other (repeated measures ANOVA: $F_{(6,54)} = 0.6$, P

369    $= 0.70$); therefore, we pooled the weight data by enoxacin treatment regardless of dosage for the

370   ZIKV-infected and uninfected mice. There was a significant interaction between group (ZIKV-

371   infected and enoxacin treated, ZIKV-infected and diluent treated, or Sham-infected and enoxacin

372   treated) and day post infection (repeated measures ANOVA: $F_{(12, 194)} = 3.1$, $P = 0.0006$).

373   Pairwise comparisons with t-tests identified differences in weight loss on days 1, 3, 4, 5, and 6

374   p.i. ($P < 0.05$). On days 1, 3, 4, and 5 p.i. the percent of initial weight for the sham infected mice

375   treated with enoxacin was lower compared to the ZIKV-infected mice treated with enoxacin

376   (pairwise t-test $P<0.05$; for full statistics see Table S2). On day 6 p.i. the mean percent of initial

377   weight of the uninfected mice treated with enoxacin was greater than that of the infected mice

378   regardless of treatment (pairwise t-test $P<0.05$; for full statistics see Table S2). Additionally, at

379   no point during the experiment did weight loss differ between the ZIKV infected mice treated

380   with enoxacin and the diluent control mice (Fig. 3a, Table S2). In experiment 2, weight loss by

381   day 6 was less drastic than, and significantly different from, experiment 1 (mean percent of

382   initial weight (SE): experiment 1 = 84.0 (1.0), experiment 2 = 93.0 (0.9); t = -5.7, df = 55, P =

383   2.6×10^{-7}), likely due to improved technical facility in dosing these very small mice, and weight

384   loss was not significantly different among treatments (Fig. 3b, repeated measures ANOVA: F

385    $(18,132) = 1.4$, $P = 0.10$).

386

387   **Fig 3**. **Mouse weight loss did not differ among treatments**. (a) Daily percent of initial weight

388   for experiment 1 was the same for ZIKV infected mice treated with enoxacin (10 mg/kg and 15

389   mg/kg combined) or diluent and uninfected mice treated with enoxacin (10 mg/kg and 15 mg/kg

390   combined) until day 6 p.i. when the infected mice, regardless of treatment, lost significantly

391   more weight than the uninfected controls. (b) Percent of initial weight for experiment 2 was not

392   different among treatment groups. * at least one group is different at P <0.05. Sample sizes in

393   Table 2; full statistics in Table S2.

394

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

396   Experiment 1. In this experiment, mice were infected with 1×10^5 pfu ZIKV and subsequently

397   treated with enoxacin. ZIKV titer in the serum of mice treated with 15 mg/kg enoxacin was X-

398   fold higher than those mice treated with 10 mg/kg enoxacin, a significant difference (Fig 4a;

399   ANOVA F (2,17) = 4.7, P = 0.02; pairwise t-test P < 0.05). However, neither dose of enoxacin

400   altered ZIKV titers in serum significantly relative to control mice (Fig 4a; pairwise t-test P =

401   0.22 for both). Similarly ZIKV titer in the brains of mice treated with 15 mg/kg enoxacin was

402   approximately tenfold higher than that of mice treated with 10 mg/kg enoxacin (Fig. 4b;

403   ANOVA F(2,17) = 4.2, P = 0.03; pairwise t-test P < 0.05), but these titers did not differ from the

404   virus titer in brains of control mice (pairwise t-test, P = 0.73 for 10 mg/kg enoxacin and P = 0.06

405   for 15 mg/kg enoxacin). Given the small sample sizes of this study, it is possible that this effect

406   is due to random sampling. In contrast to serum and brain, mean ZIKV titers in the testes of

407   mice treated with 10 mg/kg and 15 mg/kg were not significantly different from each other (Fig.

408   4c; 5.4 $\log_{10}$ pfu/g (± 0.1 SE) vs 5.7 $\log_{10}$ pfu/g (± 0.1 SE); t = -1.6, df = 8, P = 0.14), and were

409   both lower than the control group (6.2 $\log_{10}$ pfu/g (± 0.5 SE)), albeit only the decrease from

410   10mg/kg treatment was significant (ANOVA F(3,19) = 3.0, P = 0.05; pairwise t-test P < 0.05).

411   To compensate for the decrease in sample size inherent in analyzing only males, the data from

412   the two enoxacin concentrations were pooled. The mean ZIKV titer in testes for mice treated

413   with any dosage of enoxacin was 5.5 $\log_{10}$ pfu/g ($\pm$ 0.1 SE), which was significantly lower than

414   6.2 $\log_{10}$ pfu/g ($\pm$ 0.5 SE) in the control group (pairwise t-test P < 0.05).

415

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

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

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

419   each treatment are listed in Table 2 and statistical analysis is described in the text.

420

421   Experiment 2. In experiment 2 mice were pre-treated with enoxacin after which they were

422   infected with 1x10^2 pfu ZIKV and subsequently dosed daily with enoxacin. As expected, ZIKV

423   titers in serum and brain were two orders of magnitude lower than those in experiment 1,

424   however titers in the testes were similar between the two experiments. Consistent with

425   experiment 1, ZIKV titers in sera, brains, and livers of enoxacin-treated mice were not different

426   from control mice (Fig. 5a-c, all P-values > 0.05), while ZIKV titer in testes of treated mice were

427   2-fold lower than those of control mice, and this difference was significant (Fig. 5d, pairwise t =

428   -5.4, df = 5, P = 0.003).

429

430   **Fig 5**. **Treatment with enoxacin prior to and following low-titer ZIKV infection suppresses**

431   **viral replication in the testes but not in serum, brain or liver.** Individual (dots) and mean

432   (line) ZIKV titers of mice treated with the drug diluent or enoxacin from (a) sera, (b) brain, (c)

433   liver, and (d) testes. Sample sizes for each treatment are listed in Table 2 and statistical analysis

434   is described in the text.

435

Enoxacin does not inhibit ZIKV replication in mouse Sertoli cells

436

437   To investigate why the effect of enoxacin on ZIKV infection in mice was limited to the

438   testes, the EC_{50} of this drug was quantified in both mouse Sertoli cells and HEK-293 cells at each

439   of two MOI: 0.1 and 1.0. As expected, at 32 ° C, higher initial MOI generally resulted in higher

440   ZIKV titers, particularly at early timepoints p.i., in both cell lines. Sertoli cells must be cultured

441   at 32 ° C, so, for a fair comparison, the potency of enoxacin in HEK-293 cells was re-tested at 32

442   ° C. Moreover, to extend the window for comparison, virus was harvested at both 2 days (Fig.

443   6a) and at 5 days p.i. (Fig. 6b).

444   As expected, at 32 ° C, higher initial MOI generally resulted in higher ZIKV titers,

445   particularly at early timepoints p.i., in both cell lines. Kumar *et al*., Siemann *et al*., and Mlera

446   and Bloom have previously tested the replication of ZIKV in Sertoli cells and found them to be

447   highly susceptible to ZIKV infection (59–61). In our study, in the absence of enoxacin, there

448   was no difference in ZIKV replication in HEK-293 and Sertoli cells 2 days p.i. (linear model, β =

449   -0.2, P = 0.59), but at 5 days p.i. ZIKV titers in Sertoli cells were significantly lower than in

450   HEK-293 cells (linear model, β = -1.4 , P = $7.4x10^{-7}$).

451   Unlike the dose response curve in HEK-293 cells incubated at 37 ° C, increasing

452   concentration of enoxacin in both cell lines at 32 ° C did not result in a sharp inflection in ZIKV

453   titer, making it difficult to accurately quantify the EC_{50} via non-linear regression. Instead,

454   general linear models were used to test the relative potency in the two cell types at 32 ° C.

455   Potency at 32 ° C in either cell line was detectable at the higher concentrations of enoxacin on

456   day 2, but not on day 5 (Fig. 6a and 6b).

457   We tested two hypotheses for greater impact of the drug in testes: first that this effect

458   may have been due to the lower temperature of the testes and second that it may have been due

459   to a greater potency in testis cells. To test the first hypothesis, we compared enoxacin potency in

460   HEK-293 cells infected with ZIKV at MOI 1 incubated at 32 ° C or 37 ° C and harvested on day

461   5 (data at 37 ° C was collected as part of the initial EC_{50} analysis). Counter to the hypothesis,

462   enoxacin potency was greater at 37 ° C compared to 32 ° C in this cell line (linear model, β = -

463   0.2, P = $4.7\text{x}10^{-15}$).

464   We tested the second hypothesis by comparing the impact of enoxacin in HEK-293 and

465   Sertoli cells at 32 ° C. We used Sertoli cells as our model testis cell, while acknowledging that

466   the testes are composed of many cell types and results from Sertoli cells cannot be generalized to

467   the testes as a whole. In this analysis enoxacin concentration, cell type, and MOI and their

468   interactions were all included in the model. On day 2 pi, interaction between enoxacin

469   concentration and cell type was not significant (β = 0.01, P = 0.83), while on day 5 p.i. there was

470   a significant interaction (β = 0.7, P = 0.0001). On both days, enoxacin concentration and Sertoli

471   cells continue to negatively impact ZIKV replication (day 2 linear model, β = -0.4, P = $3.2\text{x}10^{-12}$;

472   β = -0.2, P = 0.03, respectively; day 5 linear model, β = -0.8, P = $4.9\text{x}10^{-11}$; β = -1.4, P = $1.9\text{x}10^{-8}$

473   , respectively) meaning that ZIKV titer decreased as enoxacin concentration increased and

474   ZIKV infection of Sertoli cells resulted in lower titers overall compared to HEK-293 cells.

475   However, on day 5 the interaction between enoxacin concentration and Sertoli cells had a

476   positive impact on ZIKV titer, meaning that enoxacin was less effective in Sertoli cells than in

477   HEK-293 cells.

478

479   **Fig 6**. **Enoxacin does not suppress ZIKV in mouse Sertoli cells two days pi (a) or five days**

480   **pi (b).** Dose-response curves for enoxacin and ZIKV titer at 32 ° C in Sertoli cells (pink) and

481   HEK-293 cells (blue) at two MOI, 0.1 (dashed) and 1.0 (solid). Dose-response curve for

482   enoxacin and ZIKV titer in HEK-293 cells at 37 ° C (yellow) is included for comparison on day

483   5 p.i. Statistical analysis is described in the text.

484

Discussion

486   Flavivirus infections are acute, and treatment must be initiated rapidly to be effective

487   (6,62,63). However, individuals infected with different flaviviruses often present with similar

488   symptoms (62,64), and in many places where flavivirus infections are common, diagnostic

489   capacity is limited (62,65). Thus, the ideal antiflaviviral drug will have broad efficacy across

490   different members of the genus (12,66,67). We found that the three fluoroquinolones used in this

491   study, enoxacin, ciprofloxacin and difloxacin, all suppressed replication of the six flaviviruses

492   tested at low micromolar concentrations, with the exception that difloxacin lacked potency for

493   MODV. These six flaviviruses, DENV-1, DENV-2, DENV-4, ZIKV, LGTV, and MODV, span

494   the diversity of human pathogenic flaviviruses (68,69). While enoxacin consistently

495   demonstrated the lowest EC_{50} values, difloxacin exhibited the best selectivity indexes. These

496   findings suggest that fluoroquinolones could offer broad-spectrum anti-flaviviral activity, a very

497   desirable property.

498   Though the anti-flaviviral mechanism of action of fluoroquinolones remains unknown,

499   several possible mechanisms merit exploration. First, suppression of flaviviral replication by

500   fluoroquinolones could be mediated by enhancement of RNAi (23,24). The current study was

501   motivated by the discovery of this effect, and the drugs we chose to evaluate span the range from

502   high (enoxacin) to low (difloxacin) impact on RNAi (23). Our finding that all three

503   fluoroquinolones tested inhibited replication of the six flaviviruses tested suggests that the

504   antiviral action of fluoroquinolones cannot be attributed solely to enhancement of RNAi.

505   Second, fluoroquinolones could prevent endocytosis-mediated viral entry.

506   Fluoroquinolones are derived from the original quinolone, nalidixic acid, which is a biproduct of

507   synthesizing chloroquine, an antimalarial drug (70). Consequently, fluoroquinolones and

508   chloroquine share a 4-quinolone structure. As a weak base, chloroquine is known to inhibit viral

509   entry by increasing the pH of vesicles required for endocytosis-mediated cellular entry (71–80).

510   Chloroquine has been shown to suppress ZIKV and DENV in cultured mammalian cells,

511   including Vero, HuH-7, U937, human neural progenitor (hNPC), and human brain microvascular

512   endothelial cells (hBMEC) cells, with a range of EC_{50} values from 1 µM to 14 µM against ZIKV

513   (79,81–84) which is quite similar to the range of ciprofloxacin EC_{50} values against ZIKV (6.5-

514   13.4 µM) determined in this study. Initial studies of chloroquine in mouse and monkey models

515   were promising (82,85,86), however, results from two human clinical trials with DENV found

516   improvement in some dengue-associated symptoms, such as pain, but no reduction in viremia or

517   infection duration (87,88). We speculate that the structural similarities of fluoroquinolones and

518   chloroquine could be the shared basis of their anti-flaviviral efficacy (18). If chloroquine and

519   fluoroquinolones share similar antiviral mechanisms of action, then results from studies of

520   chloroquine could offer insight into what modifications could be made to the fluoroquinolones to

521   increase their antiviral efficacy.

522   Third, fluoroquinolones could suppress the viral helicase as their mechanism of action.

523   Khan et al. (32) demonstrated that many fluoroquinolones, including enoxacin and difloxacin,

524   suppressed HCV replication and inhibited the viral helicase *in vitro* (32). Further studies will be

525   needed to assess the generality and *in vivo* relevance of this result.

526   To distinguish among some of these potential mechanisms of action, we conducted a

527   time-of-addition study of all three drugs. This approach has previously been used to reveal that

528   50 µM chloroquine, which is approximately 5 times the EC_{50} value, reduces viral RNA 64-fold

529   when added at the time of infection, likely reflecting inhibition of viral entry. (79). In our study,

530   we found that difloxacin and ciprofloxacin suppression activity was wider, encompassing 2 to 8

531   hours p.i. across assays, and in a subset of assays these drugs showed potency when cells were

532   treated prior to infection, similar to chloroquine. In contrast, enoxacin suppression was restricted

533   to 2 to 6 hours p.i. These results indicate that difloxacin and ciprofloxacin may impact early and

534   intermediate viral life cycle stages whereas enoxacin's effect is limited to intermediate stages.

535   Combined, these data suggest that ciprofloxacin and difloxacin may share a mechanism of action

536   that is different than that of enoxacin.

537   In light of enoxacin's low EC_{50}, we tested the ability of this drug mitigate weight loss

538   (55) and suppress serum viremia and virus titer in key tissues including liver, brain and testes

539   (55) in ZIKV-infected A129 mice via two experiments. First, we infected A129 mice with a high

540   titer of ZIKV and then treated immediately after infection with drug diluent or 10 or 15 mg/kg of

541   enoxacin, which for the average weight of a 5-week-old mouse corresponds to 6 or 10 µM

542   enoxacin. These concentrations were selected because they are close to the EC_{50} value for

543   enoxacin in HEK-293 cells (18.1 µM) and also within the range of peak human serum

544   concentration after oral consumption of clinically available dosages of enoxacin, (5 to 11 µM)

545   (56,57). Unexpectedly, all of the mice, including the sham-infected mice, lost weight in this

546   experiment. Two non-exclusive explanations for the weight loss are that oral administration of

547   100 µL liquid twice a day caused satiety and prevented the mice from eating normally or that

548   some component of the dose caused stomach discomfort which also prevented the mice from

549   eating. Despite this, none of the mice lost more than 20% of their weight and therefore did not

550   reach the cutoff for euthanasia. Enoxacin treatment had no impact on serum viremia or on virus

551   titer in liver or brain. As fluoroquinolones readily cross the blood-brain barrier (89) and are

552   metabolized in the liver (90), the absence of a drug effect in these tissues is unlikely to be due to

553   lack of enoxacin availability. The most intriguing result of this experiment, however, was that

554   male mice treated with enoxacin showed a significant 50% decrease in ZIKV titer in the testes.

555   To assess the reproducibility of these findings under a different treatment regimen, we

556   next tested the effect of pre-treating mice with enoxacin and then infecting them with ZIKV. As

557   in the first experiment, all mice lost weight (though weight loss was less than in the first

558   experiment) and ZIKV titers in serum, liver and brain did not differ between enoxacin-treated

559   and control mice. Importantly, consistent with the first experiment, ZIKV was significantly

560   suppressed in the testes of enoxacin-treated mice relative to controls.

561   We initially hypothesized that the restriction of enoxacin's efficacy to the testes *in vivo*

562   was due to higher efficacy in specific cell types in the testes or the lower temperature of the

563   testes. However, counter to these explanations, we found that enoxacin was less effective

564   against ZIKV at 32˚C than 37˚C and less effective against ZIKV in Sertoli cells than in human

565   kidney cells. However, Sertoli cells are one of many cell types in the testes, which also contain

566   stem cells, spermatozoa and Leydig cells, which vary in their susceptibility to ZIKV (60,61,91–

567   93), thus our findings in Sertoli cells do not reveal the action of enoxacin in the testes as a whole.

568   Immunohistochemical staining of ZIKV-infected mouse testes 7 days p.i. has revealed the

569   presence of viral antigen primarily in the stem cells of the seminiferous tubules and in the

570   seminal fluid from the vas deferens (93). Using *in situ* hybridization, viral RNA of a mouse-

571   adapted strain of ZIKV was detected in the stem cells (spermatogonia and primary

572   spermatocytes), Sertoli cells, and spermatozoa of ZIKV-infected mouse testes at 7 days p.i. (92).

573   ZIKV-infected germ cells were detected in the basal layer of the seminiferous tubules of ZIKV-

574   infected olive baboons via immunofluorescence 11 days p.i. (94).

575   Recently, Xu *et al*. (35) reported that ZIKV infection of hNPCs activates the RNAi

576   antiviral response and elicits the production of virus-derived small interfering RNAs (vsi-RNA),

577   but infection of human neurons does not, indicating that cellular differentiation degrades the

578   functionality of RNAi. Additionally, Xu et al. (35) demonstrated that treatment with enoxacin, a

579   known enhancer of RNAi (23,24), significantly suppressed ZIKV replication in hNPCs. The

580   HEK-293 cells in which we demonstrated enoxacin efficacy against ZIKV have stem-cell like

581   properties (95). Our time-of-addition assays indicate that enoxacin, ciprofloxacin, and difloxacin

582   all suppress an intermediate life stage of ZIKV, which is consistent with enhancement of RNAi.

583   Furthermore, we detected an impact of enoxacin in testes, which are rich in stem cells, but not in

584   differentiated brain or liver cells. Thus, our results are generally consistent with those of Xu et

585   al. (35), which implicate RNAi enhancement as an antiviral mechanism of enoxacin.

586   However, multiple alternative explanations for our *in vivo* findings must be considered.

587   First and foremost, Xu et al. (35) used 10-fold more enoxacin (100 µM vs 10 µM) in their study

588   than we used in ours. Additionally, testicular ZIKV infection results in oxidative stress, and

589   antioxidants such as ebselen have been shown to reduce oxidative stress, lessen testicular

590   damage, and prevent sexual transmission in mice (96). Like ebselen, fluoroquinolones are also

591   known to act as antioxidants (27–29). Fluoroquinolones may also damage the testes and thereby

592   restrict flavivirus replication. More research on the *in vivo* testicular toxicity of fluoroquinolones

593   is needed, especially since ZIKV infection itself damages testicular tissues (92,97–99) although

594   damage to human Sertoli cells is minimal (60).

595   More generally, several caveats pertain to our study. First, although use of the A129

596   immunocompromised mouse model to initially test compounds for *in vivo* efficacy against ZIKV

597   has become a standard practice (82,100,101), nonetheless, these mice do lack an interferon

598   response, and the interplay between interferon and ZIKV clearly shapes pathogenesis (102–

599   108). Thus, the reproducibility of our findings in other, immunocompetent animal models, such

600   as the human STAT2 knockin mouse model or C57B1/6 mice treated with the anti-type I IFN

601   receptor antibody (108–110), should be tested. Second, as we only tested enoxacin across two

602   concentrations *in vivo*, a wider range of fluoroquinolones and fluoroquinolone concentrations

603   should be investigated *in vivo* for efficacy in suppressing ZIKV. Third, our investigation of the

604   testes-specific efficacy of enoxacin focused only on Sertoli cells, but efficacy in other testicular

605   cells should also be evaluated, particularly stem cells.

606   In summary, we found that three fluoroquinolones had reasonable potency against six

607   flaviviruses in cultured cells and enoxacin suppressed ZIKV titer in mouse testes. These results

608   offer a foundation for further attempts to optimize fluoroquinolones to increase potency.

609   Limiting replication of the virus in the testes is important, as ZIKV is capable of sexual

610   transmission (111–114). The results from this study and that of Xu et al. (35) also suggest that

611   testing the ability of fluoroquinolones to alleviate the teratogenic effects of ZIKV in relevant

612   mouse models (109,115,116) should be a priority.

613

614

615

616 **Funding**

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Supporting Information

Table S1. ANOVA results and pairwise t-test comparisons for ZIKV titer after treatment with enoxacin, difloxacin and ciprofloxacin from the time-of-addition assays in Fig. 2 and Fig. S3.

	Enoxacin		Difloxacin			Ciprofloxacin		
Concentration (µM)	18.1	24.4	35.9	25.4	50.0	116.1	56.8	25.0
MOI	1.0	0.2	0.2	1.0	1.0	0.2	1.0	1.0
F	2.6	3.6	2.8	1.0	12.8	14.7	41.2	16.2
DF	8, 18	8, 18	8, 18	8, 18	8, 18	8, 18	8, 18	8, 18
P	0.04	0.01	0.04	0.45	5.2e-6	1.9e-6	5.2e-10	2.3e-7
Time-of-addition (hour p.i.)								
Media	AB	AB	A	A	A	A	AB	A
-2	AB	AB	AB	A	BC	A	B	CD
0	A	ABC	AB	A	CD	A	B	BC
2	BC	CD	B	A	CD	B	D	E
4	BC	D	AB	A	D	B	CD	E
6	C	CD	B	A	D	B	C	DE
8	ABC	BCD	B	A	CD	B	B	DE
12	ABC	AB	B	A	A	A	A	BC
18	A	A	B	A	AB	A	AB	B

951 **Table S2**. Pairwise t-test comparisons of mean percent weight change from *in vivo* ZIKV
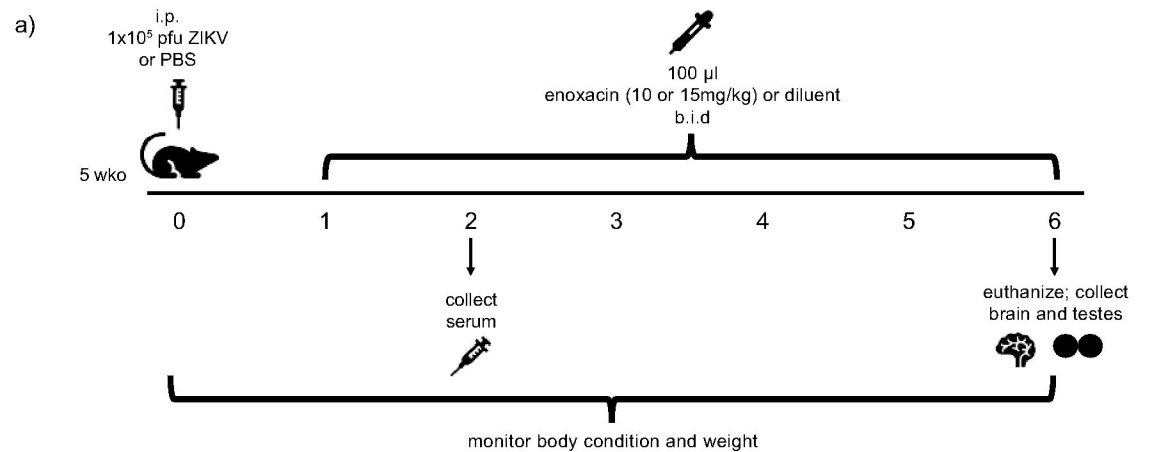
952 infection in Fig 3a

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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972   **Figure S1.** Replication kinetics of DENV-1, DENV-2, DENV-4, LGTV, and MODV at MOI

973   0.05 in HEK-293 cells.

974

975   **Figure S2.** Dose response curves and EC_{50} for ciprofloxacin (pink), difloxacin (yellow), and

976   enoxacin (blue) inhibition of DENV-1, DENV-2, DENV-4, ZIKV, LGTV, and MODV.

977

978   **Figure S3.** Impact on ZIKV replication by 25.4 µM difloxacin (a) and 56.8 µM ciprofloxacin (b)

979   when added at designated timepoints. Viral titers (n = 3 replicates per drug per time point) for

980   each time point were normalized to the average viral titer with media treatment and reported as

981   average percent (titer at time point/average media titer*100). Differences in mean viral titers

982   ($\log_{10}$ pfu/mL) were detected with ANOVA and pairwise t-tests; full pairwise statistics in Table

983   S1. * P < 0.05 compared to media control.
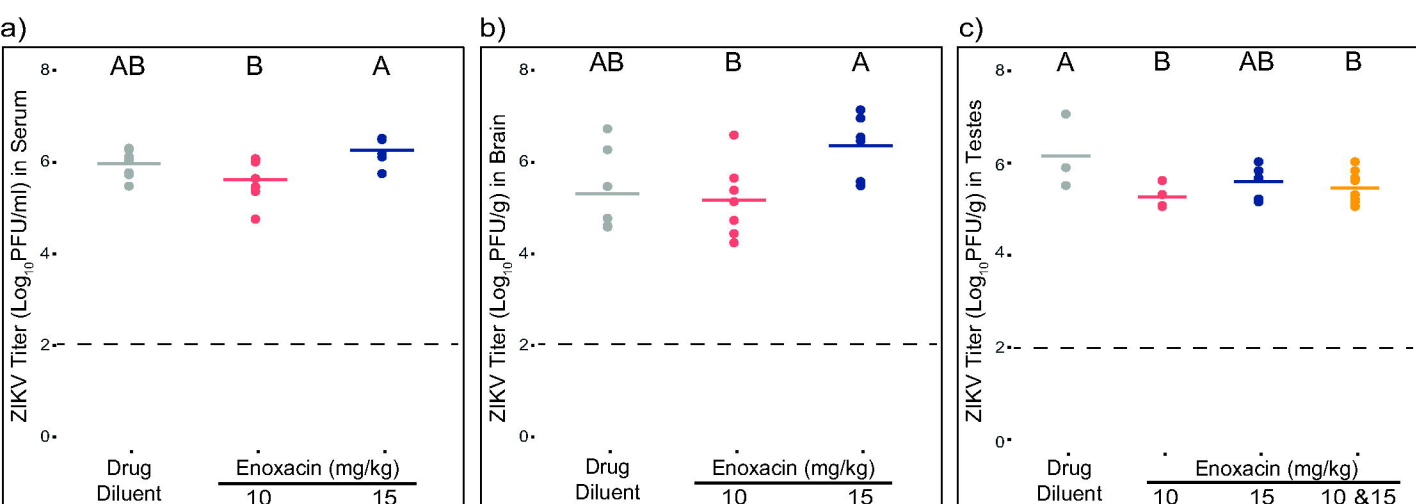
984

985   **Data S1.** Excel spreadsheet containing the underlying data for Figures 2-6, Figures S1-S3, Table

986   3, Table S1 and S2.
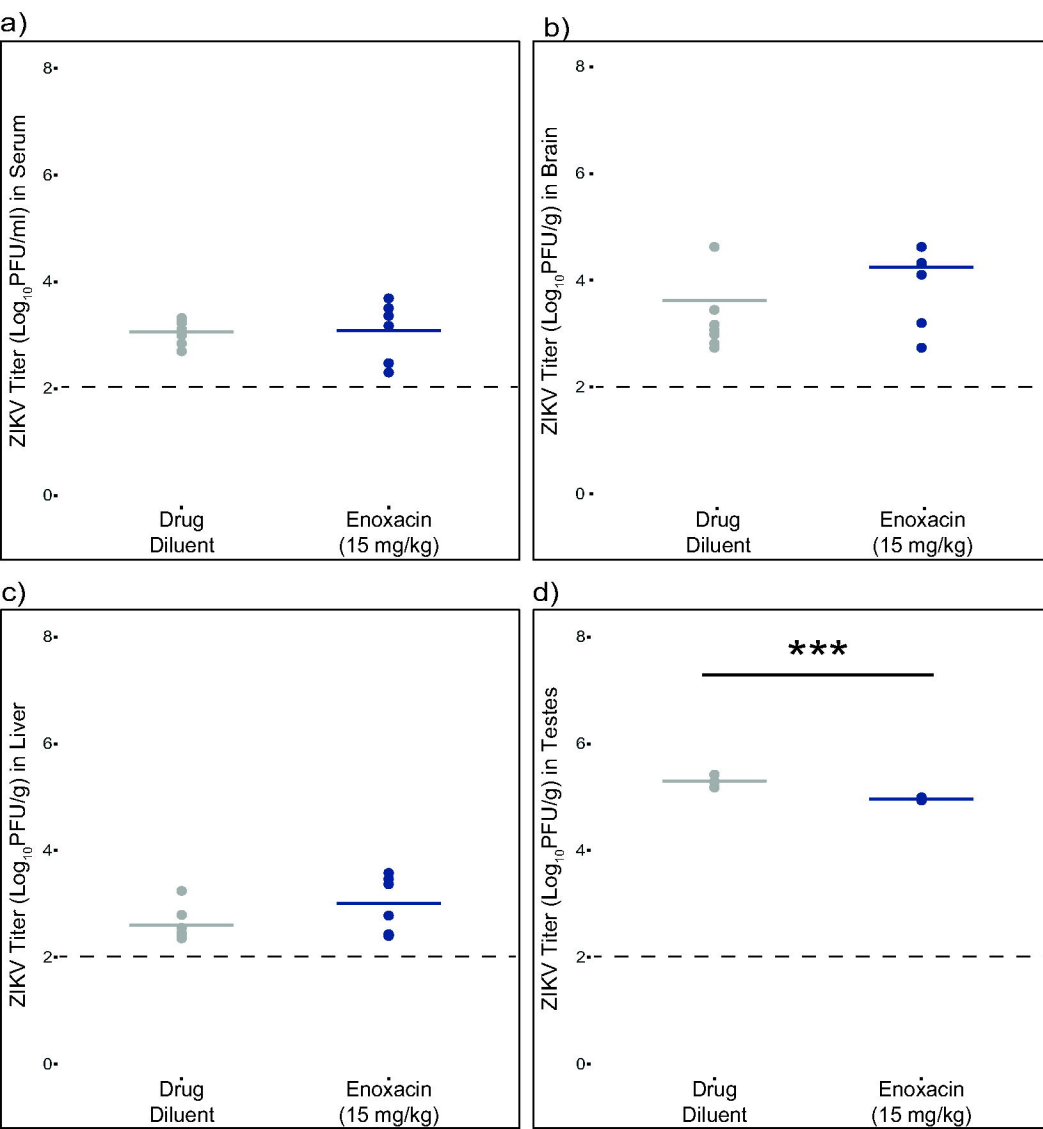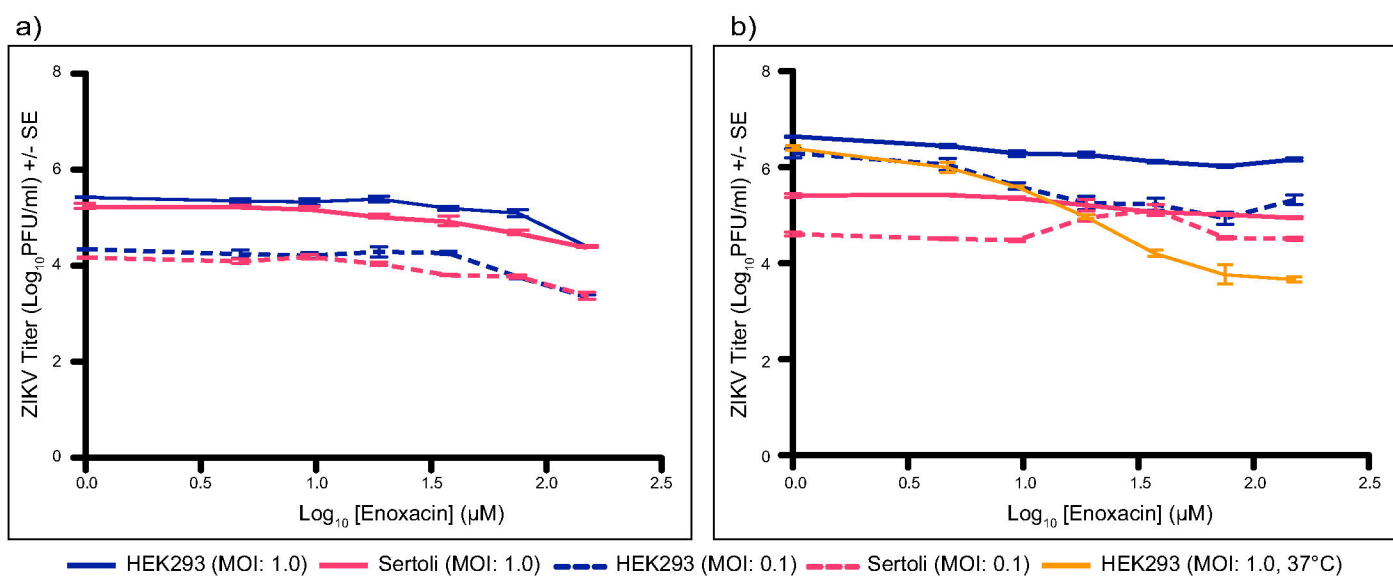
a)

i.p.
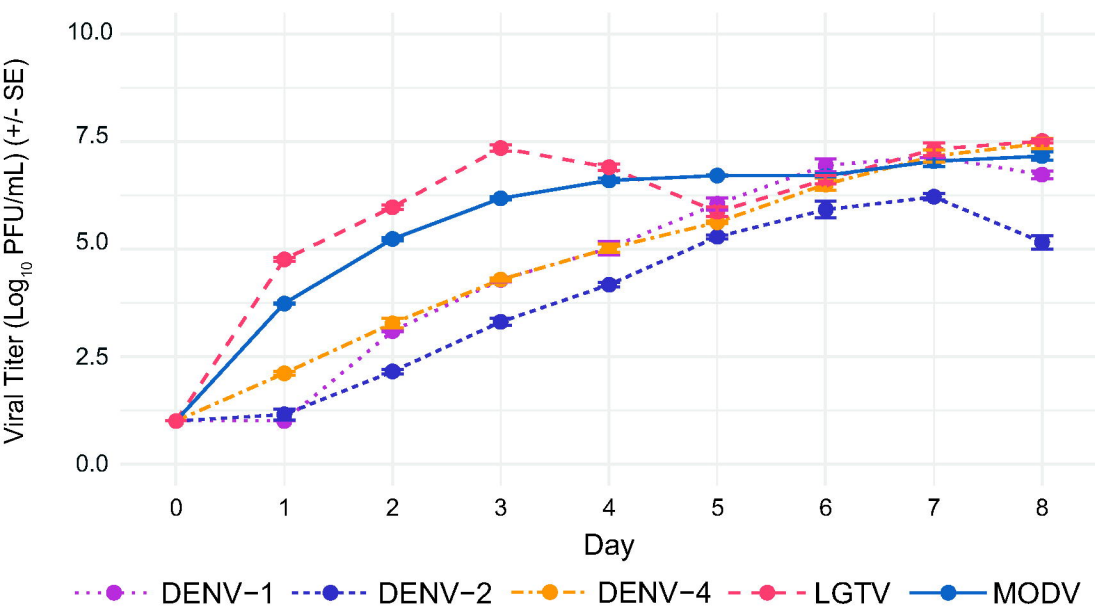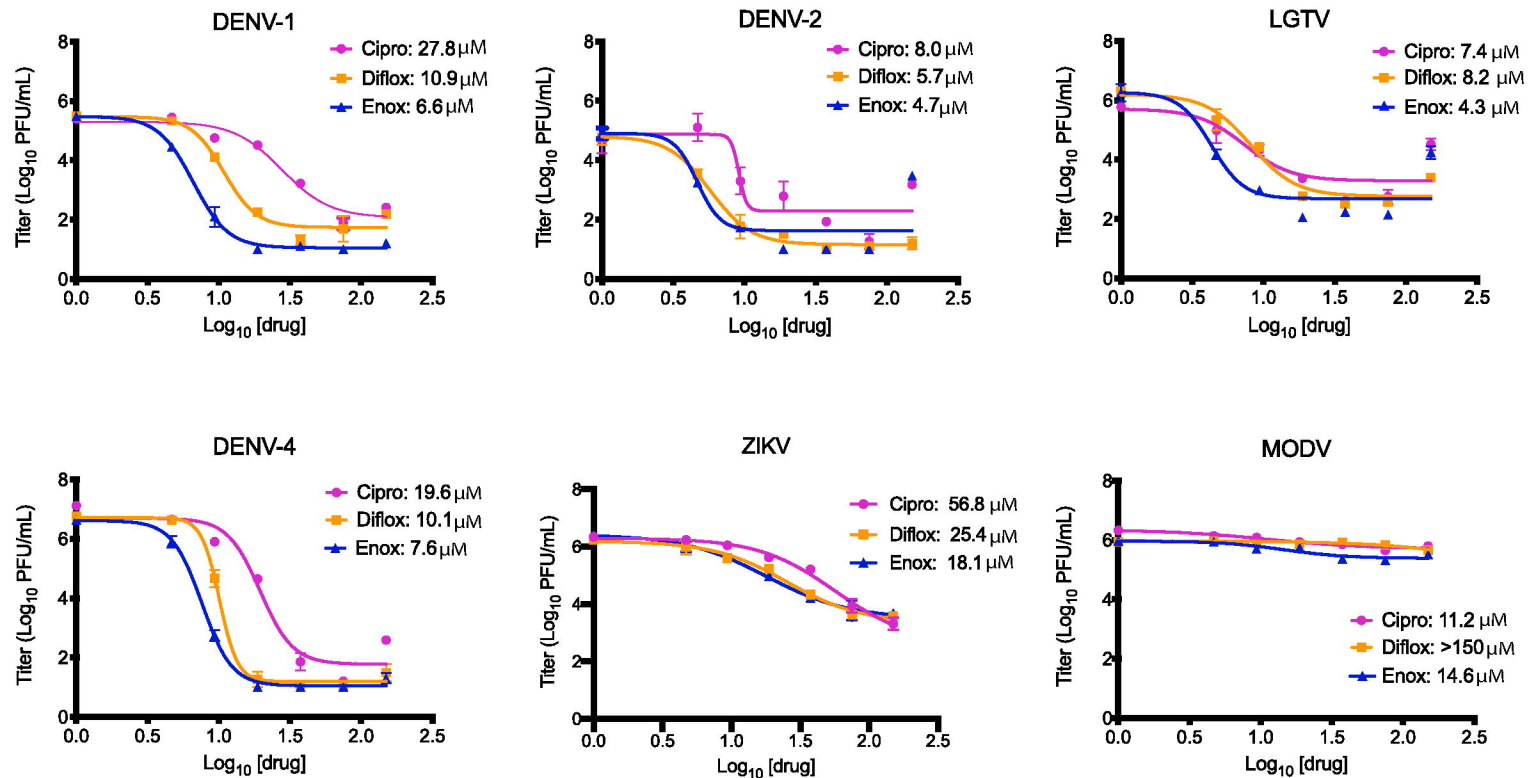1×10^5 pfu ZIKV
or PBS

100 µl
enoxacin (10 or 15mg/kg) or diluent
b.i.d

5 wko

0   1   2   3   4   5   6

collect
serum

euthanize; collect
brain and testes

monitor body condition and weight

b)

100 µl
enoxacin
(15mg/kg)
or diluent

i.p.
1×10^2 pfu ZIKV
or PBS

100 µl
enoxacin (15mg/kg) or diluent
b.i.d

5 wko

0   1   2   3   4   5   6

collect
serum

euthanize; collect
brain, testes, and liver
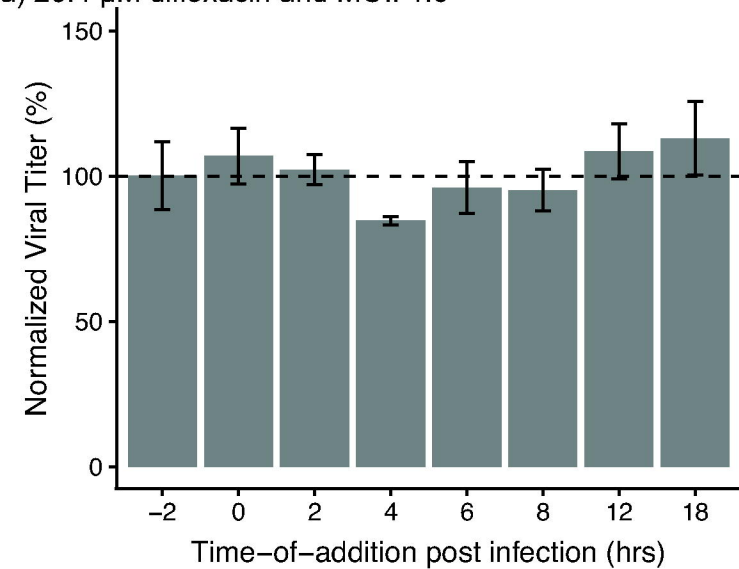
monitor body condition and weight

a)

b)
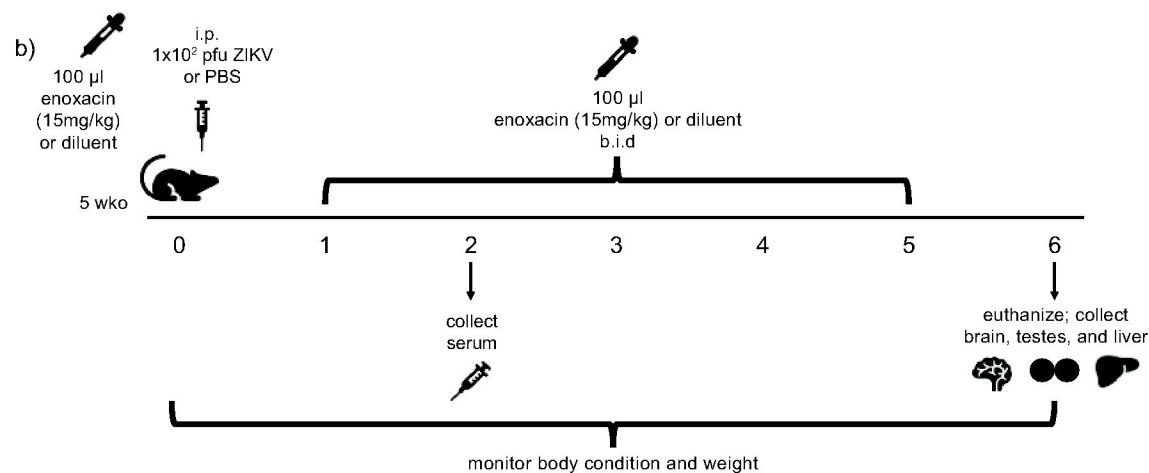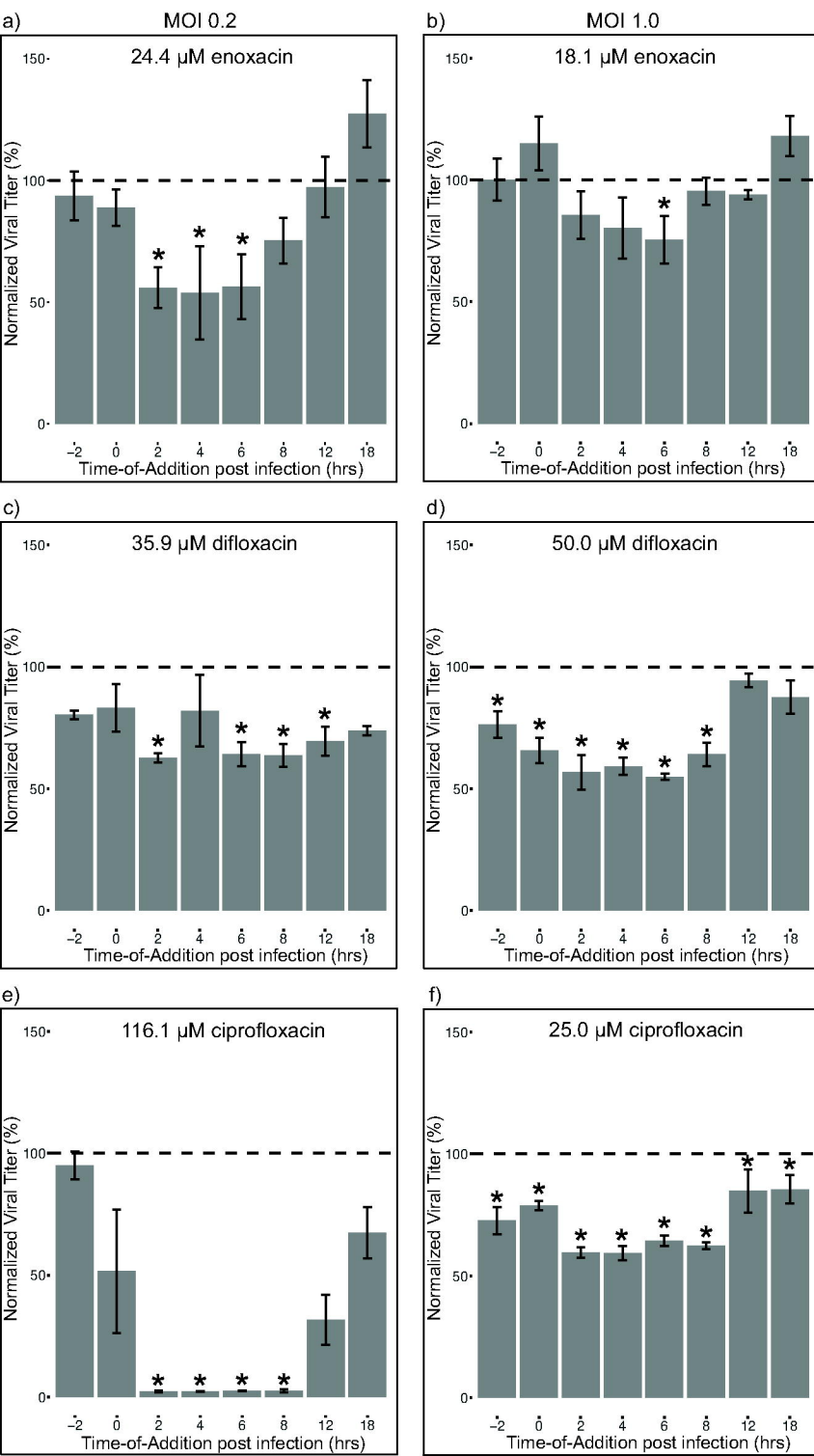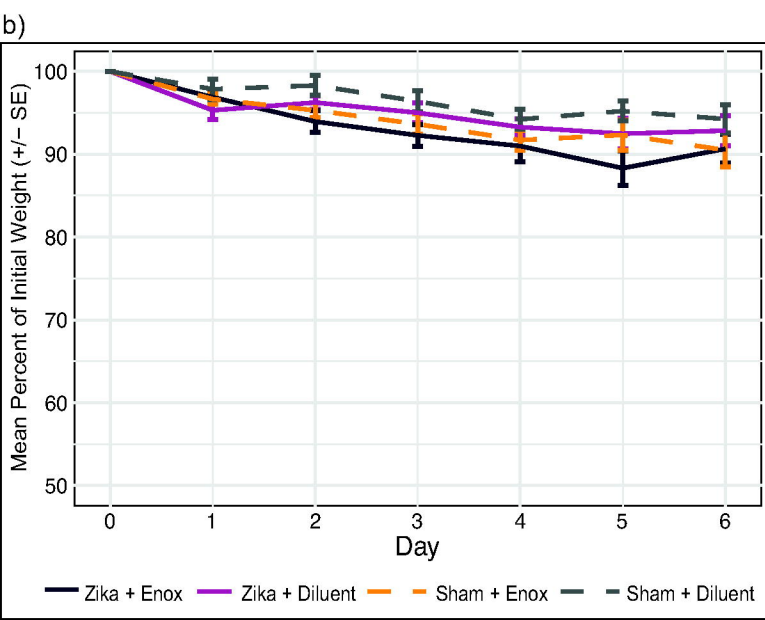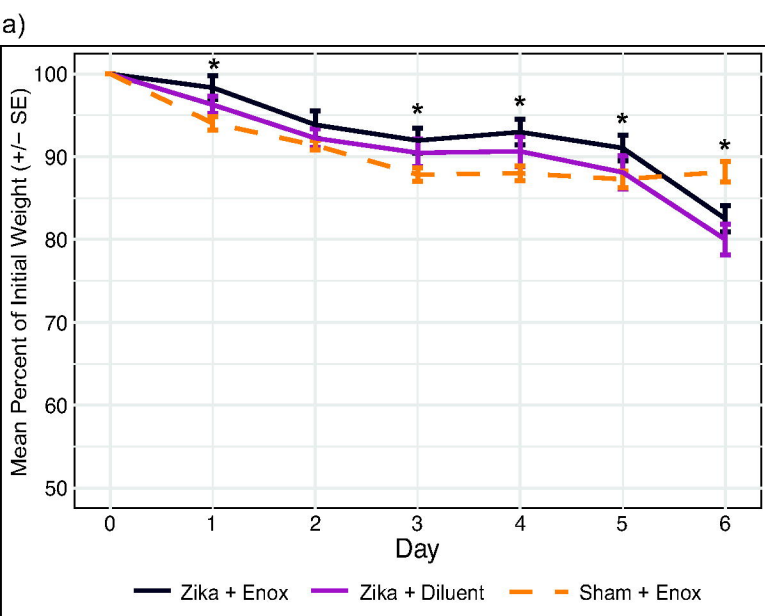
a) 25.4 μM difloxacin and MOI: 1.0

b) 56.8 μM ciprofloxacin and MOI: 1.0