

18 **ABSTRACT**

19 Obesity has been epidemiologically and empirically linked with more severe disease
20 upon influenza infection. To ameliorate severe disease, treatment with antivirals, such
21 as the neuraminidase inhibitor oseltamivir, are suggested to begin within days of
22 infection, especially in hosts at higher risk for poor outcomes. However, this treatment
23 is often poorly effective and can generate resistance variants within the treated host.
24 Here, we hypothesized that oseltamivir treatment would not be effective in genetically
25 obese mice and would generate a more diverse and drug resistant viral population. We
26 demonstrated that oseltamivir treatment does not improve viral clearance in obese
27 mice. While no traditional variants associated with oseltamivir resistance emerged, we
28 did note that drug treatment failed to quench the viral population and did lead to
29 phenotypic drug resistance *in vitro*. Mechanistically, we demonstrate the blunted
30 interferon response in obese hosts may be contributing to treatment failure, as type I
31 interferon receptor deficient mice also fail to clear influenza virus infection upon
32 oseltamivir administration. Together, these studies suggest that the unique
33 pathogenesis and immune responses in obese mice could have implications for
34 pharmaceutical interventions and the within-host dynamics of the influenza virus
35 population.

36

37 **IMPORTANCE**

38 Influenza virus infections, while typically resolving within days to weeks, can turn critical
39 especially in high-risk populations. Prompt antiviral administration is crucial to mitigating
40 these severe sequelae, yet concerns remain if antiviral treatment is effective in hosts

Obesity and oseltamivir efficacy 3

41 with obesity. Here, we show that oseltamivir does not improve viral clearance in
42 genetically obese or type I IFN receptor-deficient mice and increases the genetic
43 entropy of the within-host viral population. This suggests a blunted immune response
44 may impair oseltamivir efficacy and render a host more susceptible to severe disease.
45 This study furthers our understanding of oseltamivir treatment dynamics both
46 systemically and in the lungs of obese mice, as well as the consequences of oseltamivir
47 treatment for the within-host emergence of drug-resistant variants.

48 **INTRODUCTION**

49 Influenza viruses are a seasonal and pandemic threat to human and animal
50 health worldwide (1). Influenza A and B viruses commonly cause seasonal outbreaks in
51 humans, with control measures needed to mitigate its health and economic effects (2,
52 3). Control of influenza infection is accomplished through vaccination strategies aimed
53 to prevent disease and antiviral courses designed to mitigate symptoms, severity, and
54 spread upon infection (4). Several antivirals have entered clinical use, including
55 adamantanes targeted to the M2 ion channel, neuraminidase inhibitors (NAIs) that block
56 viral release, and endonuclease inhibitors that stall viral replication (5).

57 However, these antiviral treatment strategies are not always effective. First,
58 resistance to each class of influenza antivirals has emerged, with the adamantanes no
59 longer clinically effective due to widespread resistance (6, 7). While contemporary
60 influenza viruses are largely susceptible to oseltamivir, many NAI resistant variants
61 have been characterized (8-11). Second, poor host responses and delayed treatment
62 with antivirals can impede their efficacy. Host characteristics can promote the
63 emergence of resistant variants, as influenza infection of immunocompromised hosts
64 results in higher rates of antiviral resistant variants (12-17).

65 The obesity epidemic highlights these dual concerns. Rates of worldwide obesity
66 have nearly tripled in the past three decades (18). Obesity results in a chronic state of
67 immunosuppression that impairs the antiviral response to infection, including the type I
68 interferon response (19-22). Epidemiological data suggests antivirals such as
69 oseltamivir are protective in hosts with obesity, yet empirical studies show obese mice
70 require 10-fold higher doses to achieve complete protection (23, 24). We have

71 previously shown that the obesogenic state impacts viral population dynamics by
72 increasing viral diversity and promoting a more virulent influenza population (21, 25).
73 Thus, we questioned if oseltamivir treatment of obese mice would accelerate viral
74 clearance or lead to the acquisition of antiviral resistant variants. In these studies, we
75 treated obese mice with the NAI oseltamivir (Tamiflu), and monitored viral clearance
76 over 7 days. Oseltamivir treatment improved viral clearance in wild-type mice; however,
77 no such improvement was detected in treated, leptin-deficient obese mice. Additionally,
78 we report that this subpar protection promotes the emergence of influenza viral variants
79 with increased neuraminidase activity and greater oseltamivir resistance. Together,
80 these findings suggest study is warranted on how host characteristics can influence
81 pharmaceutical efficacy.

82

83 **RESULTS**

84 **Oseltamivir treatment does not reduce viral titers or improve viral clearance in** 85 **influenza-infected obese mice.**

86 We have previously determined that higher doses of oseltamivir are needed to
87 improve survival in obese mouse models (23, 24); however, why the standard dose
88 treatment fails in obese mice was unknown. To answer, we asked if oseltamivir
89 treatment could reduce viral titers or improve viral clearance in wild-type and obese
90 mice. Beginning 12 hours pre-infection lean and obese animals were orally gavaged
91 with 10 mg/kg oseltamivir or PBS vehicle control every 12 hours for 5 days post-
92 infection (p.i.) (23). Mice were infected with A/California/04/2009 (CA/09) H1N1
93 influenza virus and lungs excised at 0.5-, 1-, 3-, 5- and 7-days p.i. (Figure 1A). No

94 significant differences in viral titers were detected (Figure 1B), but area under the curve
95 (AUC) analysis suggests significantly accelerated viral clearance upon oseltamivir
96 treatment for wild-type mice compared to all other experimental groups (vs wild-type +
97 PBS $p=0.012$; vs obese + PBS $p=0.0007$; vs obese + oseltamivir $p=0.0014$; Figure 1C).
98 No trend in accelerated viral clearance was observed for oseltamivir-treated obese mice
99 compared to untreated obese mice in AUC analysis. The prolonged viral replication
100 coupled with oseltamivir pressure could result in the selection of potentially resistant
101 variants.

102

103 **Obese-derived viruses are more resistant to oseltamivir treatment compared to**
104 **those obtained from lean hosts.**

105 While emergence of antiviral resistant markers is relatively rare, we hypothesized
106 that this delayed viral clearance could generate resistance to the drug in
107 immunocompromised obese hosts (17, 26). To test this, we compared the
108 neuraminidase (NA) activity of lean and obese-derived viruses at different days p.i. by
109 MUNANA. Wild-type derived viruses have low NA activity regardless of days post-
110 infection in the presence of oseltamivir. In contrast, the virus isolated from obese host
111 has high NA activity by day 5 p.i. that remains high throughout the course of infection.
112 While the presence of oseltamivir initially suppresses NA activity in obese mice, without
113 impacting overall viral titers (Figure 1B). by day 7 p.i., viruses have emerged that have
114 NA activity in the presence of oseltamivir. Initially, viruses derived from all four
115 experimental groups showed similar levels of neuraminidase activity as measured by
116 fluorescence (Figure 2A). By day 5 p.i., virus derived from untreated obese-derived

117 virus showed greater relative fluorescence than other groups. After the removal of
118 oseltamivir treatment, oseltamivir-treated obese mice displayed a rebound in
119 neuraminidase activity (Figure 2A). This suggests there may be a selection of higher-NA
120 activity viral variants within obese, oseltamivir-treated mice.

121 To quantitate the difference in potential oseltamivir resistance between wild-type
122 and obese derived-viruses, the indicated viruses were titrated on MDCK cells in the
123 presence of DMSO control or increasing concentrations of oseltamivir carboxylate as
124 indicated. Viruses derived from obese, treated mice trended towards higher titers at all
125 experimental conditions compared to both treated and untreated wild-type-derived
126 viruses (Figure 2B). AUC analysis showed significantly reduced viral titers across all
127 experimental conditions in treated wild-type ($p=0.0316$) and all untreated (wild-type +
128 PBS, $p=0.0173$; obese + PBS $p=0.0425$) mice compared to treated obese mice (obese
129 + oseltamivir). The half-maximal inhibitory concentration (IC_{50}) for oseltamivir ranges
130 from 0.8 nM to greater than 35 μ M (6, 12). Within our experimental conditions, (IC_{50})
131 values for all obese-derived viruses were shifted higher compared to wild-type derived
132 viruses, with oseltamivir -treatment further shifting resistance in both groups (Figure
133 2C). Mean IC_{50} values are as follows: obese + oseltamivir, 206.4 ± 86.3 nM; obese +
134 PBS, 52.8 ± 24.4 nM, wild-type + oseltamivir, 13.7 ± 3.5 nM, wild-type + PBS 3.8 ± 1.6
135 nM. In total, obese-derived viruses show greater phenotypic resistance to oseltamivir
136 carboxylate treatment *in vitro* compared to lean-derived viruses.

137

138 **Obese-derived viruses do not have genetic changes commonly associated with**
139 **antiviral resistance.**

140 The influenza virus hemagglutinin (HA) and NA segments are genetically plastic
141 allowing for accrual of potentially resistant single nucleotide variants (SNVs). The HA
142 mutations G155E and D222G as well as the NA mutation H275Y are implicated in
143 reducing oseltamivir efficacy, with several others also suggested as modulators of
144 resistance (27, 28). To determine if these mutations were present in the obese-derived
145 viruses, NGS was performed and we quantified the overall number of single nucleotide
146 variants SNVs, entropy, and if any classically NAI-resistant mutants emerged. No
147 consensus changes associated with oseltamivir resistance were detected.

148 Viruses derived from obese mice treated with oseltamivir had significantly
149 increased numbers of unique SNVs compared to viruses derived from wild-type mice
150 treated with oseltamivir ($p=0.0053$; Figure 3A). This translated to increased overall
151 genetic diversity in viruses derived from oseltamivir-treated obese mice. Measures of
152 Shannon's entropy (H) is reduced in wild-type treated ($p=0.0035$) and wild-type
153 untreated ($p=0.0357$) compared to oseltamivir treated obese mice (Figure 3B).
154 Oseltamivir ablated overall viral diversity in lean mice. There was a non-significant trend
155 to reduced numbers of SNVs and total Shannon's entropy in treated lean mice,
156 compared to no difference in treated obese mice (Figure 3A, B).

157 More SNVs were detected in the HA segment of viruses derived from obese
158 compared to lean oseltamivir treated mice (9 and 4, respectively) translating to higher
159 average entropy of 3.27 in obese mice compared to 1.24 in wild-type mice. Oseltamivir
160 treatment decreased HA entropy in treated wild-type mice (from 1.98 to 1.24) but not in
161 obese mice, where it increased from 2.30 to 3.27 (Table 1). We detected fewer SNVs in
162 NA. Oseltamivir treatment resulted in 1 NA variant in obese and 3 NA variants in lean,

163 compared to 2 and 1 in obese and lean PBS control mice, respectively (Table 1). While
164 we noted higher genetic diversity and phenotypic resistance in both oseltamivir-treated
165 and untreated mice, no classical NAI-resistant mutants emerged in the viral populations.
166 However, there is no exhaustive list of mutations than may render oseltamivir treatment
167 less effective. Combined with the increased rate of replication and viral population
168 diversification in obese mice, this may be the ideal context for emergence of novel
169 resistance markers.

170

171 **Similar oseltamivir clearance but reduced maximal concentrations in obese**
172 **compared to lean mice**

173 Appropriate dosage and concentration at the site of infection is crucial for
174 antiviral efficacy; inappropriate levels could lead to severe complications due to
175 treatment failure or the quick emergence of resistance phenotypes. To determine if
176 obesity impacts the pharmacokinetic dynamics of oseltamivir and the metabolite
177 oseltamivir carboxylate, we dosed male, 10-week-old wild-type or obese mice with a
178 single oral gavage of oseltamivir at 10 mg/kg in 100 μ L of PBS. Whole lungs and
179 plasma were collected at 0.5-, 1-, 4-, 8-, and 16-hours post treatment and immediately
180 processed for pharmacokinetic (PK) analysis (Figure 4A). There were no practical or
181 significant differences in oseltamivir or oseltamivir carboxylate clearance in plasma and
182 lung between wild-type and obese mice (Table 2). We observed a similar half-life of
183 oseltamivir (2.12 versus 2.28 hours; Figure 4B) and its metabolite oseltamivir
184 carboxylate (2.30 versus 3.3 hours; Figure 4C) in the lungs of obese mice compared to
185 wild-type mice. Analysis of plasma revealed similar findings, with no difference in the

186 elimination half-life of oseltamivir (1.36 versus 1.98 hours, respectively; Figure 4E) or
187 oseltamivir carboxylate (1.87 versus 2.75 hours, respectively; Figure 4F) in lean versus
188 obese mice. However, the maximum concentration of oseltamivir was significantly
189 increased in both plasma and lung tissue. In lungs, maximal oseltamivir concentration
190 rose from an average of 600 ug/L in obese mice lungs to 1500 ug/L in the lungs of wild-
191 type mice ($p=0.036$; Figure 4D). Plasma again showed similar trends ($p=0.063$; Figure
192 4G). Overall, these findings suggest that clearance of oseltamivir is equal between
193 obese and lean mice and most likely has little impact on the observed failure of viral
194 clearance.

195

196 **Interferon is important for oseltamivir control of influenza virus infection.**

197 Obesity is associated with reduced type I (IFN) responses. We further
198 demonstrated that robust IFN responses restrict genetic diversity. To test the hypothesis
199 that a reduced IFN response can decrease the effectiveness of oseltamivir due to
200 increased phenotypically resistant variants, male and female IFNAR^{-/-} or WT mice were
201 treated with 10 mg/kg oseltamivir or vehicle control (PBS) and inoculated intranasally
202 with CA/09 H1N1 virus or PBS as in Figure 1A. Like obese mice (Figure 1B-C),
203 oseltamivir treatment showed little effectiveness in reducing viral load in IFNAR-null
204 mice (Figure 5A). Only, wild-type mice treated with oseltamivir had reduced viral
205 clearance. IFNAR^{-/-} mice, with and without oseltamivir treatment, had detectable
206 pulmonary viral loads at both days 3 and 7 p.i. This suggests that IFN signaling is
207 required for robust oseltamivir antiviral activity and improved viral clearance in mice,
208 even when equal dosage and pKa dynamics point towards treatment efficacy.

209

210 **DISCUSSION**

211 In these studies, we demonstrate that phenotypic resistance to oseltamivir
212 emerged by day 5 p.i. in obese mice during treatment. We hypothesize that the delayed
213 type I IFN response, in conjunction with reduced maximum concentration of oseltamivir
214 in the pulmonary environment, may result in poor control of viral replication allowing the
215 emergence of resistant viral variants. Population-based pharmacokinetic studies have
216 found that oseltamivir clearance is accelerated in obese hosts, but not at a biologically
217 significant rate spurring no need for dosage based on weight (24, 27-29). However,
218 these studies have relied on plasma concentrations and not the concentration at the
219 respiratory epithelium. No studies to our knowledge have either empirically tested
220 antiviral efficacy in humans with obesity or the local concentration of oseltamivir at the
221 site of infection.

222 Emergence of dominant antiviral resistant mutations often begin as minor
223 variants in the within-host population, especially in infected, immunocompromised hosts
224 (30, 31). Compounding these risks, oseltamivir-resistant variants in already
225 immunocompromised hosts, such as those with obesity, may complicate an already
226 high-risk medical presentation (32). Weakened immune pressures, including blunted
227 innate and adaptive immunity, coupled with extended shed and potentially higher viral
228 replication may increase the likelihood of adaptive mutations emerging in obese hosts
229 (33). While paradoxically, oseltamivir treatment is not required for the emergence of
230 oseltamivir-resistant variants, adding on this selection pressure in an already
231 compromised situation may prove a perfect storm for viral adaptation. To remedy this,

232 early and appropriate antiviral responses are crucial to control of viral replication,
233 spread and pathology. This is mirrored by the window in which oseltamivir is effective in
234 preventing the onset of severe disease (23). While those with obesity are more at-risk
235 for severe sequelae following influenza infection, prompt antiviral administration may
236 ameliorate these risks and improve overall outcomes (34). Treatment delayed as little
237 as 2 days after symptoms onset is ineffective in most hosts, mirroring the sensitive
238 timing for robust action of endogenous IFN responses (4, 35).

239 We have previously shown that IFN is crucial for controlling the emergence of a
240 diverse viral population that may harbor virulent genotypes (21). Type I IFN and
241 oseltamivir may also show synergistic benefit in treating seasonal IAV infection, and
242 oseltamivir has been shown to modulate immune responses (36-38). The blunted
243 pulmonary IFN responses coupled with reductions in maximal oseltamivir
244 concentrations in obese hosts may increase the likelihood for antiviral resistant variants
245 to emerge leading to the observed phenotypic resistance (23, 39, 40). Identifying the
246 molecular mechanisms behind the blunted IFN responses will be crucial to untangling
247 the multiple impacts the obesity epidemic has on public health.

248

249 **ACKNOWLEDGEMENTS**

250 This manuscript has benefitted from helpful discussion with Drs. Nicholas Wohlgemuth
251 and Victoria A. Meliopoulos. This work was supported by the National Institute of Allergy
252 and Infectious Diseases under HHS contract HHSN27220140006C for the St. Jude
253 Center of Excellence for Influenza Research and Surveillance funding from the National
254 Institutes of Health, NIH grant R01AI140766, and ALSAC.

255

256 **METHODS**

257 **Viruses and titer determination**

258 Eight- to 12-week old mice were inoculated with indicated doses of A/California/04/2009
259 (H1N1) virus and viral titer determined through tissue-culture infectious dose-50
260 (TCID₅₀) assays as previously reported (23).

261

262 **Animal husbandry**

263 Eight to 12-week old wild-type C57Bl/6 male (wild-type) (JAX:000664) and B6.C-
264 Lep^{ob/ob}/J genetically obese (obese) (JAX:000632) male mice were obtained from
265 Jackson Laboratory. IFNAR KO mice were obtained from Dr. Laura Knoll (University of
266 Wisconsin) and bred in house. Knockouts were confirmed by PCR using primer sets
267 (IFNAR^{-/-}) reported on the Jackson Laboratories website. All animals were housed
268 under standard conditions with food and water provided *ad libitum*. All procedures were
269 approved by the St. Jude Children's Research Hospital Institutional Animal Care and
270 Use Committee and followed the Guide for the Care and Use of Laboratory Animals.

271

272 **In vivo pharmacokinetics (PK)**

273 Plasma and lung tissue pharmacokinetic (PK) profiles of prodrug oseltamivir and active
274 metabolite oseltamivir carboxylate was evaluated in male C57BL6 and obese mice
275 (Jackson Labs), approximately 12 weeks in age. Oseltamivir phosphate was dissolved
276 in PBS at 5 mg/mL for a 10 mL/kg gavage, yielding a 10 mg/kg dose. Terminal blood
277 samples were obtained at various times up to 16 hours post-dose and immediately

278 processed to plasma. A 200 μ L aliquot of plasma was then quickly pipetted from each
279 sample and transferred into a separate 2 mL microcentrifuge tube containing 800 μ L of
280 ice cold 15 ng/mL oseltamivir-d3 carboxylate (Toronto Research Chemicals, Lot 14-
281 SBK-47-3, Purity 97%) in methanol, capped, vortexed for 30 seconds and then
282 centrifuged to pellet the precipitated protein. An 800 to 950 μ L aliquot of the supernatant
283 was then pipetted into an empty pre-labeled microcentrifuge tube, capped and stored at
284 -80 °C until analysis. Following terminal bleeds, animals were perfused with PBS to
285 flush blood from the vasculature. Lungs were then extracted, rinsed with PBS as
286 necessary, and snap frozen in liquid nitrogen. Lungs were then placed in appropriately
287 labeled microcentrifuge tubes in a cooler on dry ice and transferred to -80°C for storage.

288

289 **Bioanalysis**

290 Frozen lung samples were weighed in tared 2 mL Lysing Matrix A (MP Biomedical,
291 Santa Ana, CA) tubes and diluted with a 5:1 volume of LCMS grade methanol. The
292 lungs were then homogenized with a FastPrep-24 system (MP Biomedicals, Santa Ana,
293 CA). The homogenization consisted of three 6.0 M/S vibratory cycles of 1 min each on
294 the FastPrep-24 system. To prevent over-heating due to friction, samples were placed
295 on wet ice for 5 min between each cycle. The homogenates were then centrifuged at
296 14,000 x g and stored at -80 °C until analysis. Plasma and lung homogenate samples
297 were analyzed for oseltamivir (oseltamivir phosphate, Cayman Chemical Co., Batch
298 0458276-29, purity 98%) and oseltamivir carboxylate (Toronto Research Chemicals, Lot
299 3-SKC-52-1, Purity 98%) with a qualified LC MS/MS assay. Calibration and quality
300 control stock solutions were prepared in DMSO and serially diluted in DMSO to prepare

301 calibration/QC spiking solutions. For the analysis of plasma samples, these spiking
302 solutions were then used to prepare calibration and QC samples in methanol. Methanol
303 calibration and QC samples, 200 μ L each, were then pipetted into glass HPLC vials and
304 evaporated on a CentriVap Console (Labconco) evaporator (40 minutes at 60 °C, then
305 30 minutes at 70 °C). The residue was reconstituted in 800 μ L of ice cold 15 ng/mL
306 osetalmivir-d3 carboxylate in methanol as an internal standard. Blank male C57Bl/6
307 plasma (200 μ L) was then pipetted into each vial, immediately vortexed for 1 minute and
308 centrifuged to pellet the protein.

309 Since the lungs were simultaneously homogenized and protein precipitated in
310 methanol, the calibration and QC samples were prepared by spiking into blank male
311 C57Bl/6 lung homogenate. Aliquots (25 μ L) of the standards, QC solutions and samples
312 were then pipetted into the IS spiking solution and vortexed to mix. A 2 μ L aliquot of the
313 extracted supernatant was injected onto a Shimadzu LC-20ADXR high performance
314 liquid chromatography system via a LEAP CTC PAL autosampler. The LC separation
315 was performed using a Phenomenex Kinetex Polar C18 (2.6 μ m, 50 mm x 2.1 mm)
316 column maintained at 50 °C with gradient elution at a flow rate of 0.50 mL/min. The
317 binary mobile phase consisted of water-formic acid (100:0.1 v/v) in reservoir A and
318 acetonitrile-formic acid (100: 0.1 v/v) in reservoir B. The initial mobile phase consisted of
319 5% B with a linear increase to 55% B in 3 min. The column was then rinsed for 2 min at
320 100% B and then equilibrated at the initial conditions for 2 min for a total run time of 7
321 min. Under these conditions, oseltamivir carboxylate, IS and oseltamivir eluted at 1.22,
322 1.22 and 1.83 min, respectively.

323 Analyte and IS were detected with tandem mass spectrometry using a SCIEX
324 5500 QTRAP in the positive ESI mode and the following mass transitions were
325 monitored: oseltamivir carboxylate 285.18 → 138.10, oseltamivir-d3 carboxylate 288.20
326 → 139.20 and oseltamivir 313.20 → 166.20. The method qualification and bioanalytical
327 runs all passed acceptance criteria for non-GLP assay performance. A linear model
328 (1/X² weighting) fit the calibrators across the 1.00 to 500 ng/mL range, with a
329 correlation coefficient (R) of ≥ 0.9973. The lower limit of quantitation (LLOQ), defined as
330 a peak area signal-to-noise ratio of 5 or greater verses a matrix blank with IS, was 1.00
331 ng/mL. Sample dilution integrity was confirmed. The plasma intra-run precision and
332 accuracy was ≤ 6.63% CV and 95.8% to 109%, respectively. The lung homogenate
333 intra-run precision and accuracy was ≤ 4.48% CV and 94.9% to 111%, respectively.

334

335 **Pharmacokinetic (PK) analysis**

336 Oseltamivir plasma Ct data were grouped by matrix and nominal time point, and the
337 mean Ct values were subjected to noncompartmental analysis (NCA) using Phoenix
338 WinNonlin 8.1 (Certara USA, Inc., Princeton, NJ). The extravascular model was applied,
339 and area under the Ct curve (AUC) values were estimated using the “linear-up log-
340 down” method. The terminal phase was defined as at least three time points at the end
341 of the Ct profile, and the elimination rate constant (K_{el}) was estimated using an
342 unweighted log-linear regression of the terminal phase. The terminal elimination half-life
343 ($T_{1/2}$) was estimated as $0.693/K_{el}$, and the AUC from time 0 to infinity (AUC_{inf}) was
344 estimated as the AUC to the last time point (AUC_{last}) + C_{last} (predicted)/ K_{el} . Other
345 parameters estimated included observed maximum concentration (C_{max}), time of C_{max}

346 (T_{max}), concentration at the last observed time point (C_{last}), time of C_{last} (T_{last}), apparent
347 clearance ($CL/F = \text{Dose}/AUC_{inf}$), and apparent terminal volume of distribution (V_z/F).
348 The apparent plasma-to-lung partition coefficient ($K_{p_{inf}}$) was estimated as the ratio of
349 the AUC_{inf} in tissue to AUC_{inf} plasma, whereas $K_{p_{last}}$ was similarly estimated using
350 AUC_{last} values.

351

352 **Antiviral resistance *in vivo***

353 Oseltamivir was administered by oral gavage at 10 mg/kg free-base equivalencies twice
354 daily for 5 days. At 12 hours after the initial dose, mice were lightly anesthetized with
355 isoflurane and inoculated with 10^3 TCID₅₀ units of A/California/04/2009 (H1N1) virus in
356 25 μ L PBS. Control mice received a PBS oral gavage at the same timepoints. At 0.5-, 1-
357 , 3-, 5- and 7-days post infection, lungs were collected, homogenized in 1 mL PBS and
358 stored at -80°C for downstream viral titer determination and deep sequencing. For deep
359 sequencing statistical analysis, days 1 and 3 and days 5 and 7 are grouped as early
360 and late points in infection, respectively, due to low copies of viral RNA at very early and
361 very late periods in infection.

362

363 **Drug susceptibility assays**

364 Madin-Darby canine kidney cells (MDCK cells; RRID: CVCL_0422) were maintained in
365 minimum essential medium (MEM; Lonza) supplemented with 2 mM GlutaMAX (Gibco)
366 and 10% fetal bovine sera (FBS; Atlanta Biologicals) and grown at 37°C under 5% CO_2 .
367 MDCK cells were seeded in 12-well or 96-well cell culture treated plate. Upon
368 confluency, MDCK cells in 96-well plates were inoculated in triplicate with 10-fold serial

369 dilutions of indicated viruses in infection media (MEM, 2 mM GlutaMAX (Gibco), 1%
370 BSA, and 1 μ g/ml TPCK-treated trypsin) containing increasing concentration of
371 oseltamivir carboxylate (0, 0.1, 0.5, 1, 10, 50, 100, 500, 1000, and 10,000 nM) or
372 DMSO. Plates were maintained at 37°C under 5% CO₂ for three days, at which time
373 viral titer determined through hemagglutination of 0.5% turkey red blood cells in PBS.
374 Infectious viral titers were calculated using the Reed-Muench method (41). The dose-
375 response curve was fitted to determine the necessary concentration of oseltamivir to
376 reduce viral titers by 50% (IC₅₀). Assays were repeated three times with average IC₅₀
377 reported. For modified plaque assays in 12-well plates, MDCK cells were washed twice
378 with PBS, then inoculated at a MOI=0.01 with indicated viruses. After a 1-hour
379 adsorption, cells were washed twice with PBS and overlaid with an 1.2% agarose in
380 DMEM mixture containing TPCK-trypsin and increasing concentrations of oseltamivir
381 carboxylate as indicated. At day 3 p.i., overlay was removed, and cells stained with
382 crystal violet to visualized cytopathic effects (CPE).

383

384 **Quantifying neuraminidase activity**

385 The relative neuraminidase activity of the oseltamivir-treated and untreated obese and
386 wild-type-derived viruses was measured by using the fluorogenic substrate MUNANA
387 (Sigma-Aldrich, St Louis, MO). Two-fold dilutions of day 1, 5, and 7 p.i. lung
388 homogenates were prepared in enzyme buffer (32.5 mM of 2-(N-morpholino)
389 ethanesulfonic acid (MES), 4 mM of calcium chloride, pH 6.5) and added in duplicate to
390 a flat-bottom 96-well opaque black plate (Corning). Pre-warmed MUNANA substrate
391 was added to all wells (30 μ L/well) to achieve a final concentration of 100 μ M.

392 Immediately after adding the MUNANA substrate, the plate was incubated for one hour
393 at 37°C. After incubation, the reaction was terminated by addition of 150 μ L per well of
394 stop solution (0.014M NaOH in 83% EtOH) and the fluorescence was read at excitation
395 at 355 nm and emission at 460 nm (BioTek). Background-corrected relative
396 fluorescence units were compared to a six-point standard curve of 4-MU.

397

398 **Deep sequencing and bioinformatics**

399 Viral RNA was extracted from 50 μ L of whole lung homogenate or NHBE cell lysates
400 and supernatant on a Kingfisher Flex Magnetic Particle Processor (Thermo Fisher
401 Scientific) by using the Ambion MagMAX-96 AI/ND Viral RNA Isolation kit (Applied
402 Biosystems, cat#AM1834). RNA concentration was measured spectrophotometrically
403 (Nanodrop). Multi-segment polymerase chain reaction (MS RT-PCR) was performed
404 using SuperScript IV One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA
405 Polymerases (ThermoFisher, cat#12574-035) and influenza-specific universal set of
406 primers(44) (Opti-F1 5'-GTTACGCGCCAGCAAAGCAGG-3', Opti-F2 5'-
407 GTTACGCGCCAGCGAAAGCAGG-3', Opti-R1 5'-GTTACGCGCCAGTAGAAACAAGG-
408 3'). RNA (5 μ L) was added and placed into a thermocycler paused at 55°C. The
409 following cycling parameters were followed: 1 cycle of 55°C/2min; 1 cycle of
410 42°C/60min; 94°C/2min; 5 cycles of 94°C/30s, 44°C/30s, 68°C/3.5min; 26 cycles of
411 94°C/30s, 57°C/30s, 68°C/3.5min; 1 cycle of 68°C/10min; and then held at 4°C. 5 μ L of
412 the reaction was analyzed by 0.8% agarose gel electrophoresis to verify all genomic
413 segments are present, and the reaction purified using the Agencourt AMPure XP Kit
414 (Beckman Coulter) according to manufacturer's instructions. The concentration of the

415 purified DNA was measured spectrophotometrically prior to storage at -20°C
416 (Nanodrop). DNA amplicons were deep sequenced using Illumina MiSeq technology
417 performed by the St. Jude Children's Research Hospital Hartwell Center with DNA
418 libraries prepared using Nextera XT DNA-Seq library prep kits (Illumina, cat#FC-131-
419 1024) with 96 dual-index bar codes and sequenced on an Illumina MiSeq personal
420 genome sequencer. Single nucleotide variants (SNVs) relative to the reference
421 sequence (A/California/04/2009 (H1N1)) were determined in by mapping reads using
422 the low-variant detection method in CLC Genomics Workbench 12 (42). To determine
423 whether the variants identified have been previously detected in human surveillance
424 samples, we used the protein sequence variance analysis for HA and NA at the
425 Influenza Research Database (43). Comparison of variants was made in reference to
426 A/California/04/2009 virus, and relative variant frequencies were calculated by dividing
427 the number of the amino acid variants by the total number of sequences queried.

428

429 **Statistical analyses and data visualization**

430 Data were organized in Microsoft Excel and GraphPad Prism 8. Experiment schematics
431 in Figures 1 and 5 were created using bioRender. Specifics of statistical details for each
432 experiment can be found in the figure legends. All data are displayed as means \pm
433 standard error of the mean with stars used to denote statistical significance.
434 Significance was set at $\alpha=0.05$.

435 **REFERENCES**

- 436 1. Yoon SW, Webby RJ, Webster RG. 2014. Evolution and ecology of influenza A
437 viruses. *Curr Top Microbiol Immunol* 385:359-75.
- 438 2. Joseph U, Su YC, Vijaykrishna D, Smith GJ. 2017. The ecology and adaptive
439 evolution of influenza A interspecies transmission. *Influenza Other Respir*
440 *Viruses* 11:74-84.
- 441 3. Iuliano AD, Roguski KM, Chang HH, Muscatello DJ, Palekar R, Tempia S, Cohen C,
442 Gran JM, Schanzer D, Cowling BJ, Wu P, Kyncl J, Ang LW, Park M, Redlberger-
443 Fritz M, Yu H, Espenhain L, Krishnan A, Emukule G, van Asten L, Pereira da
444 Silva S, Aungkulanon S, Buchholz U, Widdowson MA, Bresee JS, Global
445 Seasonal Influenza-associated Mortality Collaborator N. 2018. Estimates of
446 global seasonal influenza-associated respiratory mortality: a modelling study.
447 *Lancet* 391:1285-1300.
- 448 4. Lytras T, Mouratidou E, Andreopoulou A, Bonovas S, Tsiodras S. 2019. Effect of
449 Early Oseltamivir Treatment on Mortality in Critically Ill Patients With Different
450 Types of Influenza: A Multiseason Cohort Study. *Clin Infect Dis* 69:1896-1902.
- 451 5. Giwa A, Ogedegbe C, Murphy CG. 2018. Influenza: diagnosis and management in
452 the emergency department. *Emerg Med Pract* 20:1-20.
- 453 6. Li TC, Chan MC, Lee N. 2015. Clinical Implications of Antiviral Resistance in
454 Influenza. *Viruses* 7:4929-44.
- 455 7. Hussain M, Galvin HD, Haw TY, Nutsford AN, Husain M. 2017. Drug resistance in
456 influenza A virus: the epidemiology and management. *Infect Drug Resist* 10:121-
457 134.

- 458 8. Prachanronarong KL, Ozen A, Thayer KM, Yilmaz LS, Zeldovich KB, Bolon DN,
459 Kowalik TF, Jensen JD, Finberg RW, Wang JP, Kurt-Yilmaz N, Schiffer CA.
460 2016. Molecular Basis for Differential Patterns of Drug Resistance in Influenza
461 N1 and N2 Neuraminidase. *J Chem Theory Comput* 12:6098-6108.
- 462 9. Hussain M, Galvin HD, Haw TY, Nutsford AN, Husain M. 2017. Drug resistance in
463 influenza A virus: the epidemiology and management, p 121-34, *Infect Drug*
464 *Resist*, vol 10.
- 465 10. Collins PJ, Haire LF, Lin YP, Liu J, Russell RJ, Walker PA, Martin SR, Daniels RS,
466 Gregory V, Skehel JJ, Gamblin SJ, Hay AJ. 2009. Structural basis for oseltamivir
467 resistance of influenza viruses. *Vaccine* 27:6317-23.
- 468 11. McKimm-Breschkin JL. 2013. Influenza neuraminidase inhibitors: antiviral action and
469 mechanisms of resistance. *Influenza Other Respir Viruses* 7 Suppl 1:25-36.
- 470 12. Lee N, Hurt AC. 2018. Neuraminidase inhibitor resistance in influenza: a clinical
471 perspective. *Curr Opin Infect Dis* 31:520-526.
- 472 13. Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, Hayden
473 FG, Sugaya N, Kawaoka Y. 2004. Resistant influenza A viruses in children
474 treated with oseltamivir: descriptive study. *Lancet* 364:759-65.
- 475 14. Kondrich J, Rosenthal M. 2017. Influenza in children. *Curr Opin Pediatr* 29:297-302.
- 476 15. Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG. 1998.
477 Evidence for zanamivir resistance in an immunocompromised child infected with
478 influenza B virus. *J Infect Dis* 178:1257-62.
- 479 16. Roosenhoff R, van der Vries E, van der Linden A, van Amerongen G, Stittelaar KJ,
480 Smits SL, Schutten M, Fouchier RAM. 2018. Influenza A/H3N2 virus infection in

- 481 immunocompromised ferrets and emergence of antiviral resistance. PLoS One
482 13:e0200849.
- 483 17. Chaudhry A, Bastien N, Li Y, Scott A, Pabbaraju K, Stewart D, Wong S, Drews SJ.
484 2016. Oseltamivir resistance in an influenza A (H3N2) virus isolated from an
485 immunocompromised patient during the 2014-2015 influenza season in Alberta,
486 Canada. *Influenza Other Respir Viruses* 10:532-535.
- 487 18. WHO. 2019. Obesity and overweight, *on* World Health Organization.
488 <https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight>.
489 Accessed
- 490 19. Andersen CJ, Murphy KE, Fernandez ML. 2016. Impact of Obesity and Metabolic
491 Syndrome on Immunity. *Adv Nutr* 7:66-75.
- 492 20. Honce R, Schultz-Cherry S. 2019. Impact of Obesity on Influenza A Virus
493 Pathogenesis, Immune Response, and Evolution. *Front Immunol* 10:1071.
- 494 21. Honce R, Karlsson EA, Wohlgemuth N, Estrada LD, Meliopoulos VA, Yao J,
495 Schultz-Cherry S. 2020. Obesity-Related Microenvironment Promotes
496 Emergence of Virulent Influenza Virus Strains. *mBio* 11.
- 497 22. Namkoong H, Ishii M, Fujii H, Asami T, Yagi K, Suzuki S, Azekawa S, Tasaka S,
498 Hasegawa N, Betsuyaku T. 2019. Obesity worsens the outcome of influenza
499 virus infection associated with impaired type I interferon induction in mice.
500 *Biochem Biophys Res Commun* 513:405-411.
- 501 23. O'Brien KB, Vogel P, Duan S, Govorkova EA, Webby RJ, McCullers JA, Schultz-
502 Cherry S. 2012. Impaired wound healing predisposes obese mice to severe
503 influenza virus infection. *J Infect Dis* 205:252-61.

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- 504 24. Chairat K, Jittamala P, Hanpithakpong W, Day NP, White NJ, Pukrittayakamee S,
505 Tarning J. 2016. Population pharmacokinetics of oseltamivir and oseltamivir
506 carboxylate in obese and non-obese volunteers. *Br J Clin Pharmacol* 81:1103-
507 12.
- 508 25. Beck MA, Handy J, Levander OA. 2004. Host nutritional status: the neglected
509 virulence factor. *Trends Microbiol* 12:417-23.
- 510 26. van der Vries E, Stittelaar KJ, van Amerongen G, Veldhuis Kroeze EJB, de Waal L,
511 Fraaij PLA, Meesters RJ, Luider TM, van der Nagel B, Koch B, Vulto AG,
512 Schutten M, Osterhaus A. 2013. Prolonged Influenza Virus Shedding and
513 Emergence of Antiviral Resistance in Immunocompromised Patients and Ferrets,
514 *PLoS Pathog*, vol 9.
- 515 27. Jittamala P, Pukrittayakamee S, Tarning J, Lindegardh N, Hanpithakpong W, Taylor
516 WR, Lawpoolsri S, Charunwattana P, Panapipat S, White NJ, Day NP. 2014.
517 Pharmacokinetics of orally administered oseltamivir in healthy obese and
518 nonobese Thai subjects. *Antimicrob Agents Chemother* 58:1615-21.
- 519 28. Pai MP, Lodise TP, Jr. 2011. Oseltamivir and oseltamivir carboxylate
520 pharmacokinetics in obese adults: dose modification for weight is not necessary.
521 *Antimicrob Agents Chemother* 55:5640-5.
- 522 29. Thorne-Humphrey LM, Goralski KB, Slayter KL, Hatchette TF, Johnston BL, McNeil
523 SA, Group OS. 2011. Oseltamivir pharmacokinetics in morbid obesity (OPTIMO
524 trial). *J Antimicrob Chemother* 66:2083-91.
- 525 30. Ghedin E, Laplante J, DePasse J, Wentworth DE, Santos RP, Lepow ML, Porter J,
526 Stellrecht K, Lin X, Operario D, Griesemer S, Fitch A, Halpin RA, Stockwell TB,

- 527 Spiro DJ, Holmes EC, St George K. 2011. Deep sequencing reveals mixed
528 infection with 2009 pandemic influenza A (H1N1) virus strains and the
529 emergence of oseltamivir resistance. *J Infect Dis* 203:168-74.
- 530 31. Ghedin E, Holmes EC, DePasse JV, Pinilla LT, Fitch A, Hamelin ME, Papenburg J,
531 Boivin G. 2012. Presence of oseltamivir-resistant pandemic A/H1N1 minor
532 variants before drug therapy with subsequent selection and transmission. *J Infect*
533 *Dis* 206:1504-11.
- 534 32. Renaud C, Kuypers J, Englund JA. 2011. Emerging oseltamivir resistance in
535 seasonal and pandemic influenza A/H1N1. *J Clin Virol* 52:70-8.
- 536 33. Xue KS, Moncla LH, Bedford T, Bloom JD. 2018. Within-Host Evolution of Human
537 Influenza Virus. *Trends Microbiol* 26:781-793.
- 538 34. Segaloff HE, Evans R, Arshad S, Zervos MJ, Archer C, Kaye KS, Martin ET. 2018.
539 The impact of obesity and timely antiviral administration on severe influenza
540 outcomes among hospitalized adults. *J Med Virol* 90:212-218.
- 541 35. Sidwell RW, Bailey KW, Bemis PA, Wong MH, Eisenberg EJ, Huffman JH. 1999.
542 Influence of treatment schedule and viral challenge dose on the in vivo influenza
543 virus-inhibitory effects of the orally administered neuraminidase inhibitor GS
544 4104. *Antivir Chem Chemother* 10:187-93.
- 545 36. Ilyushina NA, Donnelly RP. 2014. In vitro anti-influenza A activity of interferon (IFN)-
546 lambda1 combined with IFN-beta or oseltamivir carboxylate. *Antiviral Res*
547 111:112-20.
- 548 37. Jefferson T, Jones MA, Doshi P, Del Mar CB, Hama R, Thompson MJ, Spencer EA,
549 Onakpoya I, Mahtani KR, Nunan D, Howick J, Heneghan CJ. 2014.

- 550 Neuraminidase inhibitors for preventing and treating influenza in healthy adults
551 and children. *Cochrane Database Syst Rev*
552 doi:10.1002/14651858.CD008965.pub4:CD008965.
- 553 38. Takahashi E, Kataoka K, Fujii K, Chida J, Mizuno D, Fukui M, Hiro OI, Fujihashi K,
554 Kido H. 2010. Attenuation of inducible respiratory immune responses by
555 oseltamivir treatment in mice infected with influenza A virus. *Microbes Infect*
556 12:778-83.
- 557 39. Meliopoulos V, Livingston B, Van de Velde LA, Honce R, Schultz-Cherry S. 2019.
558 Absence of beta6 Integrin Reduces Influenza Disease Severity in Highly
559 Susceptible Obese Mice. *J Virol* 93.
- 560 40. Green WD, Beck MA. 2017. Obesity Impairs the Adaptive Immune Response to
561 Influenza Virus. *Ann Am Thorac Soc* 14:S406-S409.
- 562 41. Reed LJ, Muench H. 1938. A Simple Method of Estimating Fifty Per Cent
563 Endpoints¹². *American Journal of Epidemiology* 27:493-497.
- 564 42. Van den Hoecke S, Verhelst J, Vuylsteke M, Saelens X. 2015. Analysis of the
565 genetic diversity of influenza A viruses using next-generation DNA sequencing.
566 *BMC Genomics* 16:79.
- 567 43. Zhang Y, Aevermann BD, Anderson TK, Burke DF, Dauphin G, Gu Z, He S, Kumar
568 S, Larsen CN, Lee AJ, Li X, Macken C, Mahaffey C, Pickett BE, Reardon B,
569 Smith T, Stewart L, Suloway C, Sun G, Tong L, Vincent AL, Walters B, Zaremba
570 S, Zhao H, Zhou L, Zmasek C, Klem EB, Scheuermann RH. 2017. Influenza
571 Research Database: An integrated bioinformatics resource for influenza virus
572 research. *Nucleic Acids Res* 45:D466-D474.

573 **FIGURE LEGENDS**

574 **Figure 1. Oseltamivir treatment improves viral clearance in wild-type, but not**

575 **obese, mice.** (A) Male WT and OB mice were treated with 10 mg/kg oseltamivir or PBS

576 vehicle control every 12 hours starting 12 hours pre-infection with CA/09 virus. (B)

577 Oseltamivir treatment has no impacts on overall viral load but increased viral clearance

578 is observed in WT mice treated with oseltamivir. (C) Area-under-the-curve analysis for

579 viral titers in (B). Data represented as means \pm SEM. Data in (B,C) represented as

580 means \pm SEM and analyzed via (B) repeated measures one-way ANOVA with Tukey's

581 multiple comparisons test and in (C) with ordinary one-way ANOVA with Tukey's

582 multiple comparisons test with $\alpha=0.05$. Yellow shading indicates active treatment.

583 OSV=oseltamivir phosphate.

584

585 **Figure 2. Obese-derived viruses are more resistant to oseltamivir carboxylate.** (A)

586 Relative neuraminidase activity is higher in obese-derived viruses. (B-C) Indicated

587 viruses were titrated in the presence of increasing concentrations of oseltamivir

588 carboxylate, with (B) viral titers determined through TCID₅₀ and average non-linear

589 curve fits of viral loads compared to maximum at no treatment, and (C) inhibitory

590 concentrations of oseltamivir carboxylate needed to reduce viral load by 50% compared

591 to no treatment. Data presented as (A,B) means \pm SEM and analyzed in (B) through

592 area-under the curve analysis via one-way ANOVA with Tukey's multiple comparisons

593 test and non-linear fits with a non-linear fit curve to determine IC₅₀s reported in (C).

594 OSV=oseltamivir phosphate.

595

596 **Figure 3. Oseltamivir treatment ablates viral diversity in wild-type, but not obese,**
597 **mice.** (A-B) Viral RNA was extracted from lungs of mice treated with oseltamivir as
598 described in Figure 2A. Total unique SNVs are significantly higher in oseltamivir treated
599 OB mice compared to treated WT mice ($p=0.0053$). (B) Oseltamivir treated OB mice
600 harbor a more diverse viral population compared to WT Oseltamivir treated ($p=0.0035$)
601 and PBS-control treated ($p=0.0357$) mice. Statistical comparisons made via two-way
602 ANOVA with Tukey's multiple comparisons test. Significance was set at $\alpha=0.05$. Data
603 represented as means \pm SEM. OSV=oseltamivir phosphate.

604
605 **Figure 4. Similar oseltamivir pharmacokinetic parameters in lean and obese mice.**
606 (A) Naïve male, WT or OB mice were dosed once with 10 mg/kg of oseltamivir. Plasma
607 and lungs were collected at indicated time points for bioanalysis of (B) oseltamivir and
608 (C) oseltamivir carboxylate in lungs. (D) Significantly increased maximum concentration
609 of oseltamivir ($p=0.0364$) and trends towards increased oseltamivir carboxylate levels
610 was observed in the lungs of WT compared to OB mice. (E-F) After one dose of
611 oseltamivir, bioanalysis of (E) oseltamivir and (F) oseltamivir carboxylate in plasma of
612 WT and OB mice. (D) Maximum concentration of oseltamivir and oseltamivir
613 carboxylate in plasma of WT and OB mice. Non-significant trend towards increased
614 concentration of oseltamivir and oseltamivir carboxylate in WT compared to OB plasma.
615 Data analyzed in (B, C, E, F) with ordinary two-way ANOVA and in (D, G) with two-way
616 ANOVA and Sidak's post hoc with $\alpha=0.05$. Data represented as means \pm SEM.
617

618 **Figure 5. Interferon-deficient animals are not responsive to oseltamivir treatment.**

619 WT and IFNAR^{-/-} mice treated with OSV or vehicle (PBS) and inoculated with CA/09 or

620 mock (PBS) were monitored for 7 days. (A) Viral load in lungs at days 3 and 7 p.i. with

621 CA/09 virus. Data in represented as means ± SEM and analyzed via ordinary one-way

622 ANOVA with Tukey's multiple comparisons with $\alpha=0.05$. OSV=oseltamivir phosphate.

623 **Table 1. Percentage of detected minor variants in obese and wild-type mice**
 624 **treated with oseltamivir.**

| Segment | SNV | Mutation | Obese ^a | | Wild-type ^b | | Natural Prevalence ^c |
|---------|--------|----------|--------------------|------|------------------------|------|---------------------------------|
| | | | OSV | PBS | OSV | PBS | |
| HA | T28C | I10T | 6.1 | | | | 80.8 |
| | T333A | D111E | 30.6 | | | | >0.1 |
| | A399G | I133M | | | | 5.9 | 0.7 |
| | C432A | D144E | | 11.0 | | 36.4 | >0.1 |
| | A515G | E172G | 5.3 | 8.1 | 28.1 | 13.4 | 78.2 |
| | A517G | N173D | | 7.1 | | | 0.3 |
| | C598T | P200S | | 5.0 | | | 67.6 |
| | T853A | S285T | 5.9 | | | | >0.1 |
| | T935C | I312T | 22.3 | | | | >0.1 |
| | A957T | K319N | | | 5.4 | | >0.1 |
| | G1168A | D390N | | 7.8 | | | 13.7 |
| | T1187C | V396A | | | | 8.2 | >0.1 |
| | C1193T | S398F | | | | 31.8 | >0.1 |
| | G1228A | V410I | | 16.4 | | | >0.1 |
| | T1298A | L433Q | | | 6.0 | | >0.1 |
| | C1421T | A474V | 19.1 | | | | >0.1 |
| | A1573G | T525A | 10.0 | | | | >0.1 |
| | A1579G | I527V | | | 6.7 | | 13.7 |
| | T1610C | V537A | 5.8 | | | | 4.8 |
| | T1654C | F552L | 43.6 | | | | >0.1 |
| NA | G1688A | R563K | | 19.2 | | | 0.3 |
| | A129T | Q43H | 11.6 | | | | >0.1 |
| | A149G | N50S | | | | 5.0 | 0.1 |
| | C377T | P126L | | | 7.5 | | 0.1 |
| | T614C | V205A | | 7.5 | | | >0.1 |
| | G700A | V234I | | | 5.4 | | 0.4 |
| | G1139A | W380* | | 9.7 | | | >0.1 |
| | G1197A | W399* | | | 6.7 | | >0.1 |

625 ^aShannon's entropy (H) of HA of obese non-treated versus treated mice: 2.30 to 3.27,
 626 NA: 0.60 to 0.36.

627 ^bShannon's entropy (H) of HA in wild-type non-treated versus treated mice: 1.98 vs
 628 1.25, NA: 0.22 to 0.77.

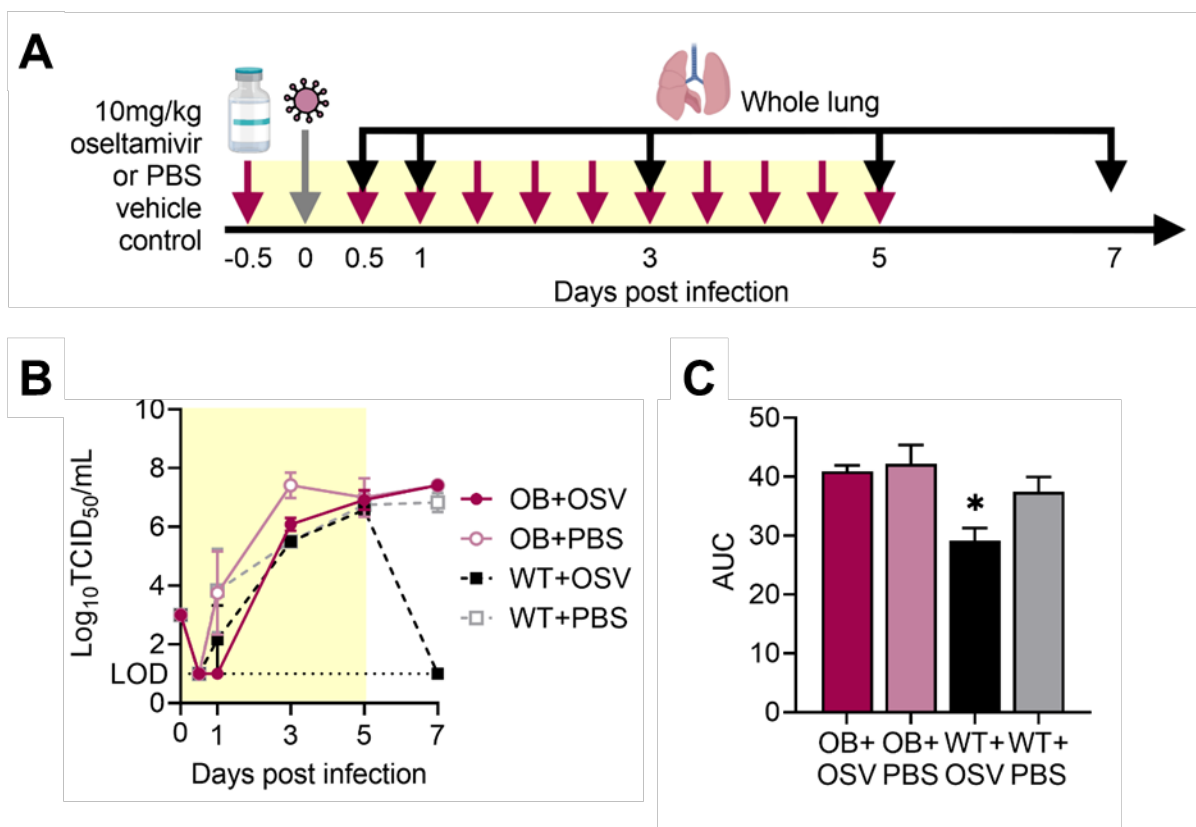
629 ^cPrevalence of minor variants in human surveillance samples on the Influenza Research
 630 Database. Queried on 28 March 2021.

631 **Table 2. Pharmacokinetic parameters of oseltamivir and oseltamivir carboxylate**
 632 **in plasma and lungs of obese and wild-type mice.**

| Parameter ¹ | unit | Oseltamivir | | | | Oseltamivir carboxylate | | | |
|------------------------|----------|-------------|-------|-----------|-------|-------------------------|-------|-----------|-------|
| | | Obese | | Wild-type | | Obese | | Wild-type | |
| | | Plasma | Lung | Plasma | Lung | Plasma | Lung | Plasma | Lung |
| C_{max} | µg/L | 428 | 839 | 950 | 1570 | 1800 | 162 | 2960 | 565 |
| t_{max} | hr | 0.5 | 1 | 0.5 | 0.5 | 1 | 0.5 | 0.5 | 1 |
| AUC_{last} | hr*µg/L | 1110 | 3290 | 1080 | 3350 | 4810 | 439 | 6150 | 637 |
| AUC_{inf} | hr*µg /L | 1110 | 3310 | 1100 | 3630 | 4860 | 486 | 6170 | 735 |
| Kp_{last} | - | 0.3374 | | 0.3224 | | 10.96 | | 9.655 | |
| Kp_{inf} | - | 0.3353 | | 0.3030 | | 10.00 | | 8.395 | |
| K_{el} | 1/hr | 0.35 | 0.328 | 0.509 | 0.304 | 0.252 | 0.301 | 0.372 | 0.208 |
| $t_{1/2}$ | hr | 1.98 | 2.12 | 1.36 | 2.28 | 2.75 | 2.3 | 1.87 | 3.33 |
| CL/F | L/hr/kg | 8.99 | 3.02 | 9.09 | 2.75 | 2.06 | 20.6 | 1.62 | 13.6 |
| V_z/F | vol/kg | 25.7 | 9.22 | 17.9 | 9.05 | 8.16 | 68.2 | 4.36 | 65.2 |
| C_{last} | µg /L | 1.99 | 7.02 | 9.32 | 69.1 | 14.1 | 15.9 | 6.8 | 16.2 |
| t_{last} | hr | 16 | 16 | 8 | 8 | 16 | 8 | 16 | 8 |

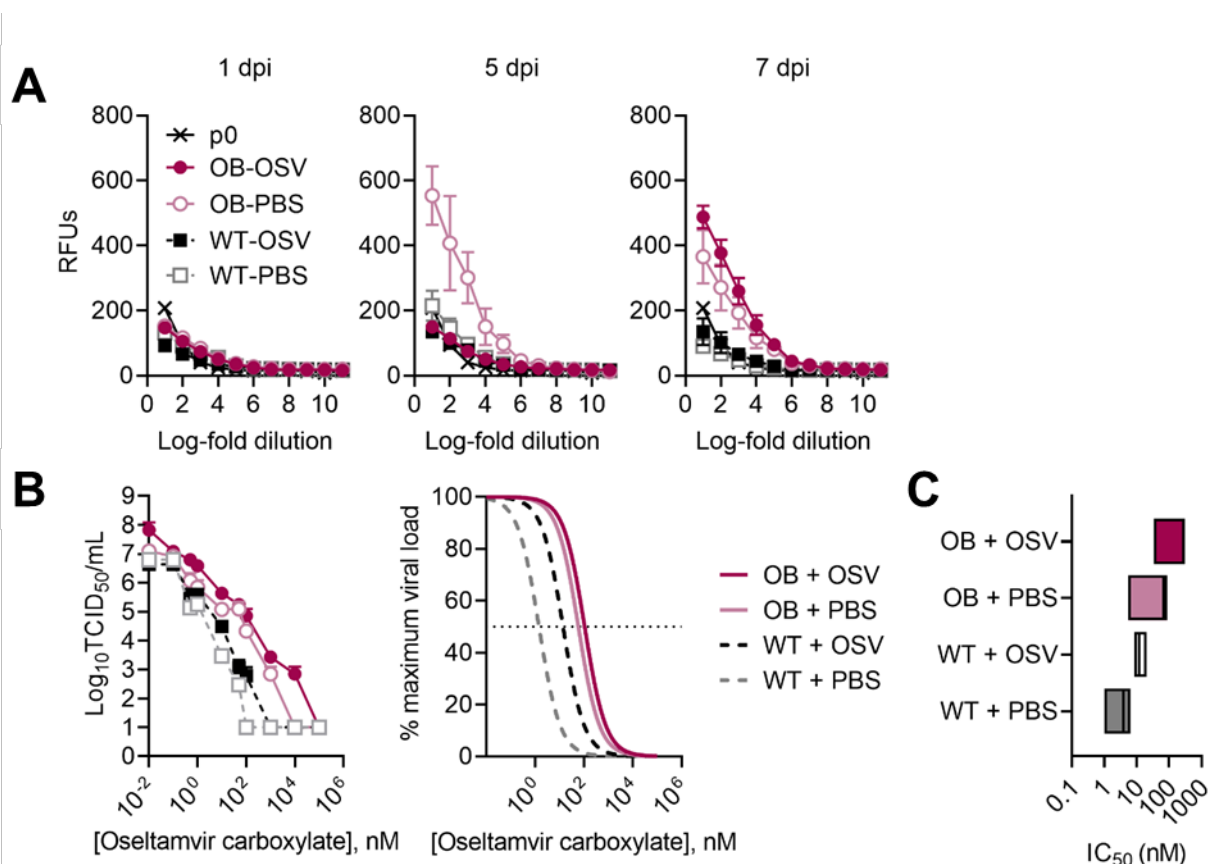
633 ¹parameters measured in units reported in parenthesis with C_{max} =maximum
 634 concentration of analyte; t_{max} =time to reach maximum concentration of analyte;
 635 AUC_{last} =area under the concentration-time curve from time zero to time of last
 636 measurement concentration; AUC_{inf} =area under the concentration-time curve from time
 637 zero to infinity; Kp_{last} =apparent plasma-to-lung partition coefficient ratio of AUC_{last} in
 638 plasma to lungs; Kp_{inf} =apparent plasma-to-lung partition coefficient ratio of AUC_{inf} in
 639 plasma to lungs; K_{el} =rate constant; $t_{1/2}$ =elimination half-life; CL/F=time of apparent total
 640 clearance of the drug; V_z/F =apparent volume of distribution during terminal phase;
 641 C_{last} =concentration of analyte at last measured time; t_{last} =time at which concentration of
 642 analyte was above the lower limit of detection.

643 **Figures and Figure Legends**



644

645 **Figure 1. Oseltamivir treatment improves viral clearance in wild-type, but not**
646 **obese, mice.** (A) Male WT and OB mice were treated with 10 mg/kg oseltamivir or PBS
647 vehicle control every 12 hours starting 12 hours pre-infection with CA/09 virus. (B)
648 Oseltamivir treatment has no impacts on overall viral load but increased viral clearance
649 is observed in WT mice treated with oseltamivir. (C) Area-under-the-curve analysis for
650 viral titers in (B). Data represented as means \pm SEM. Data in (B,C) represented as
651 means \pm SEM and analyzed via (B) repeated measures one-way ANOVA with Tukey's
652 multiple comparisons test and in (C) with ordinary one-way ANOVA with Tukey's
653 multiple comparisons test with $\alpha=0.05$. Yellow shading indicates active treatment.
654 OSV=oseltamivir phosphate.



655

656 **Figure 2. Obese-derived viruses are more resistant to oseltamivir carboxylate.** (A)

657 Relative neuraminidase activity is higher in obese-derived viruses. (B-C) Indicated

658 viruses were titrated in the presence of increasing concentrations of oseltamivir

659 carboxylate, with (B) viral titers determined through TCID₅₀ and average non-linear

660 curve fits of viral loads compared to maximum at no treatment, and (C) inhibitory

661 concentrations of oseltamivir carboxylate needed to reduce viral load by 50% compared

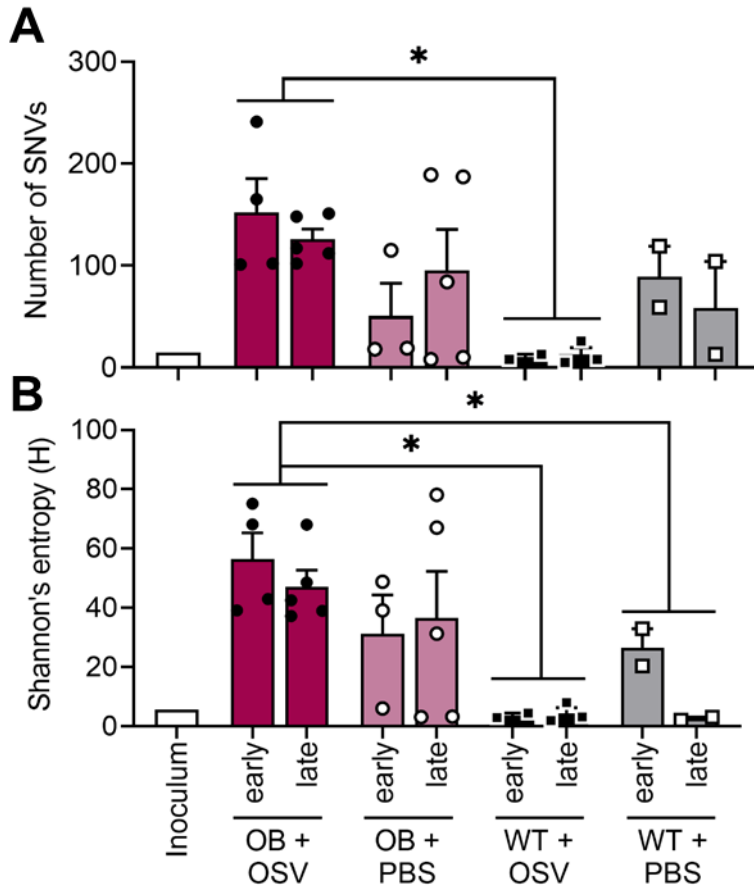
662 to no treatment. Data presented as (A,B) means ± SEM and analyzed in (B) through

663 area-under the curve analysis via one-way ANOVA with Tukey's multiple comparisons

664 test and non-linear fits with a non-linear fit curve to determine IC₅₀s reported in (C).

665 OSV=oseltamivir phosphate.

666



667

668 **Figure 3. Oseltamivir treatment ablates viral diversity in wild-type, but not obese,**
669 **mice.** (A-B) Viral RNA was extracted from lungs of mice treated with oseltamivir as
670 described in Figure 2A. Total unique SNVs are significantly higher in oseltamivir treated
671 OB mice compared to treated WT mice ($p=0.0053$). (B) Oseltamivir treated OB mice
672 harbor a more diverse viral population compared to WT Oseltamivir treated ($p=0.0035$)
673 and PBS-control treated ($p=0.0357$) mice. Statistical comparisons made via two-way
674 ANOVA with Tukey's multiple comparisons test. Significance was set at $\alpha=0.05$. Data
675 represented as means \pm SEM. OSV=oseltamivir phosphate.

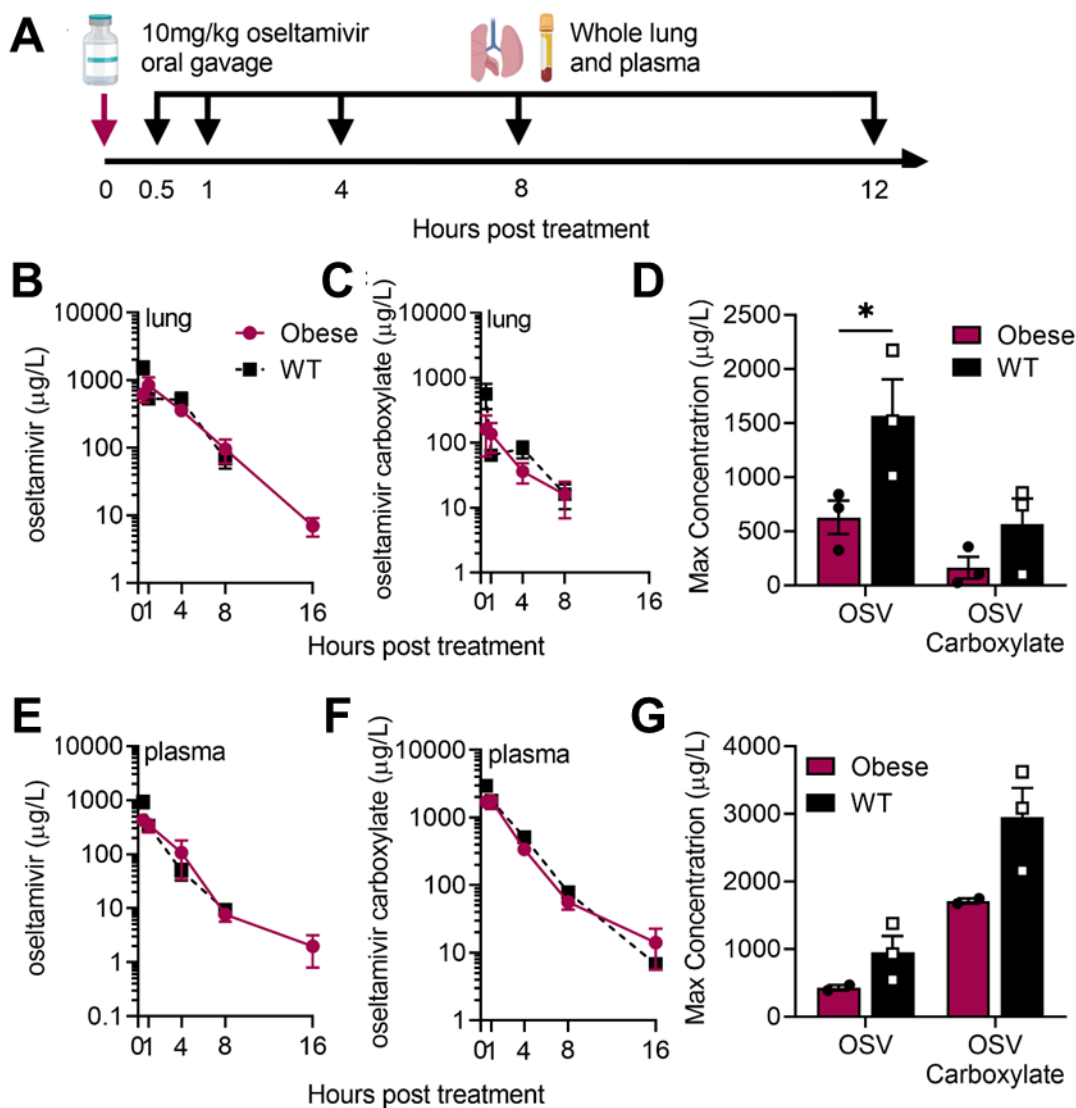
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|---------|--------|----------|--------------------|------|------------------------|------|---------------------------------|------|
| | | | OSV | PBS | OSV | PBS | | |
| HA | T28C | I10T | 6.1 | | | | 80.8 | |
| | T333A | D111E | 30.6 | | | | >0.1 | |
| | A399G | I133M | | | | 5.9 | 0.7 | |
| | C432A | D144E | | 11.0 | | 36.4 | >0.1 | |
| | A515G | E172G | 5.3 | 8.1 | 28.1 | 13.4 | 78.2 | |
| | A517G | N173D | | 7.1 | | | 0.3 | |
| | C598T | P200S | | 5.0 | | | 67.6 | |
| | T853A | S285T | 5.9 | | | | >0.1 | |
| | T935C | I312T | 22.3 | | | | >0.1 | |
| | A957T | K319N | | | 5.4 | | >0.1 | |
| | G1168A | D390N | | 7.8 | | | 13.7 | |
| | T1187C | V396A | | | | 8.2 | >0.1 | |
| | C1193T | S398F | | | | 31.8 | >0.1 | |
| | G1228A | V410I | | 16.4 | | | >0.1 | |
| | T1298A | L433Q | | | 6.0 | | >0.1 | |
| | C1421T | A474V | 19.1 | | | | >0.1 | |
| | A1573G | T525A | 10.0 | | | | >0.1 | |
| | A1579G | I527V | | | 6.7 | | 13.7 | |
| | T1610C | V537A | 5.8 | | | | 4.8 | |
| | T1654C | F552L | 43.6 | | | | >0.1 | |
| | G1688A | R563K | | 19.2 | | | 0.3 | |
| | NA | A129T | Q43H | 11.6 | | | | >0.1 |
| | | A149G | N50S | | | | 5.0 | 0.1 |
| C377T | | P126L | | | 7.5 | | 0.1 | |
| T614C | | V205A | | 7.5 | | | >0.1 | |
| G700A | | V234I | | | 5.4 | | 0.4 | |
| G1139A | | W380* | | 9.7 | | | >0.1 | |
| G1197A | | W399* | | | 6.7 | | >0.1 | |

678 ^aShannon's entropy (H) of HA of obese non-treated versus treated mice: 2.30 to 3.27,
 679 NA: 0.60 to 0.36.

680 ^bShannon's entropy (H) of HA in wild-type non-treated versus treated mice: 1.98 vs
 681 1.25, NA: 0.22 to 0.77.

682 ^cPrevalence of minor variants in human surveillance samples on the Influenza Research
 683 Database. Queried on 28 March 2021.



684

685 **Figure 4. Similar oseltamivir pharmacokinetic parameters in lean and obese mice.**

686 (A) Naïve male, WT or OB mice were dosed once with 10 mg/kg of oseltamivir. Plasma

687 and lungs were collected at indicated time points for bioanalysis of (B) oseltamivir and

688 (C) oseltamivir carboxylate in lungs. (D) Significantly increased maximum concentration

689 of oseltamivir ($p=0.0364$) and trends towards increased oseltamivir carboxylate levels

690 was observed in the lungs of WT compared to OB mice. (E-F) After one dose of

691 oseltamivir, bioanalysis of (E) oseltamivir and (F) oseltamivir carboxylate in plasma of

692 WT and OB mice. (D) Maximum concentration of oseltamivir and oseltamivir

693 carboxylate in plasma of WT and OB mice. Non-significant trend towards increased

694 concentration of oseltamivir and oseltamivir carboxylate in WT compared to OB plasma.

695 Data analyzed in (B, C, E, F) with ordinary two-way ANOVA and in (D, G) with two-way

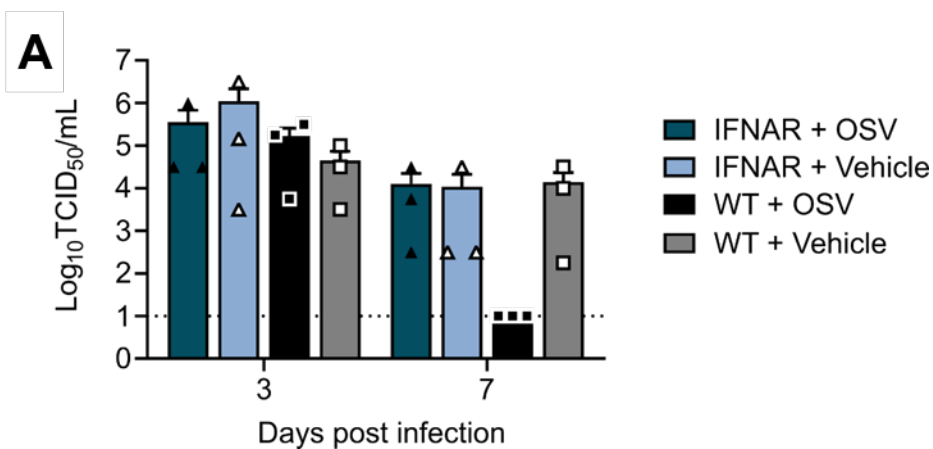
696 ANOVA and Sidak's post hoc with $\alpha=0.05$. Data represented as means \pm SEM.

697

698 **Table 2. Pharmacokinetic parameters of oseltamivir and oseltamivir carboxylate**
 699 **in plasma and lungs of obese and wild-type mice.**

| Parameter ^a | unit | Oseltamivir | | | | Oseltamivir carboxylate | | | |
|------------------------|----------|-------------|-------|-----------|-------|-------------------------|-------|-----------|-------|
| | | Obese | | Wild-type | | Obese | | Wild-type | |
| | | Plasma | Lung | Plasma | Lung | Plasma | Lung | Plasma | Lung |
| C_{max} | µg/L | 428 | 839 | 950 | 1570 | 1800 | 162 | 2960 | 565 |
| t_{max} | hr | 0.5 | 1 | 0.5 | 0.5 | 1 | 0.5 | 0.5 | 1 |
| AUC_{last} | hr*µg/L | 1110 | 3290 | 1080 | 3350 | 4810 | 439 | 6150 | 637 |
| AUC_{inf} | hr*µg /L | 1110 | 3310 | 1100 | 3630 | 4860 | 486 | 6170 | 735 |
| $K_{p_{last}}$ | - | 0.3374 | | 0.3224 | | 10.96 | | 9.655 | |
| $K_{p_{inf}}$ | - | 0.3353 | | 0.3030 | | 10.00 | | 8.395 | |
| K_{el} | 1/hr | 0.35 | 0.328 | 0.509 | 0.304 | 0.252 | 0.301 | 0.372 | 0.208 |
| $t_{1/2}$ | hr | 1.98 | 2.12 | 1.36 | 2.28 | 2.75 | 2.3 | 1.87 | 3.33 |
| CL/F | L/hr/kg | 8.99 | 3.02 | 9.09 | 2.75 | 2.06 | 20.6 | 1.62 | 13.6 |
| V_z/F | vol/kg | 25.7 | 9.22 | 17.9 | 9.05 | 8.16 | 68.2 | 4.36 | 65.2 |
| C_{last} | µg /L | 1.99 | 7.02 | 9.32 | 69.1 | 14.1 | 15.9 | 6.8 | 16.2 |
| t_{last} | hr | 16 | 16 | 8 | 8 | 16 | 8 | 16 | 8 |

700 ^aparameters measured in units reported in parenthesis with C_{max} =maximum
 701 concentration of analyte; t_{max} =time to reach maximum concentration of analyte;
 702 AUC_{last} =area under the concentration-time curve from time zero to time of last
 703 measurement concentration; AUC_{inf} =area under the concentration-time curve from time
 704 zero to infinity; $K_{p_{last}}$ =apparent plasma-to-lung partition coefficient ratio of AUC_{last} in
 705 plasma to lungs; $K_{p_{inf}}$ =apparent plasma-to-lung partition coefficient ratio of AUC_{inf} in
 706 plasma to lungs; K_{el} =rate constant; $t_{1/2}$ =elimination half-life; CL/F=time of apparent total
 707 clearance of the drug; V_z/F =apparent volume of distribution during terminal phase;
 708 C_{last} =concentration of analyte at last measured time; t_{last} =time at which concentration of
 709 analyte was above the lower limit of detection.



710

711 **Figure 5. Interferon-deficient animals are not responsive to oseltamivir treatment.**

712 WT and IFNAR^{-/-} mice treated with OSV or vehicle (PBS) and inoculated with CA/09 or

713 mock (PBS) were monitored for 7 days. (A) Viral load in lungs at days 3 and 7 p.i. with

714 CA/09 virus. Data in represented as means ± SEM and analyzed via ordinary one-way

715 ANOVA with Tukey's multiple comparisons with $\alpha=0.05$. OSV=oseltamivir phosphate.