

1 **Structural insight into the mechanism of neuraminidase inhibitor-resistant mutations in**
2 **human-infecting H10N8 Influenza A virus**

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29 **Abstract**

30 The emergence of drug resistance in avian influenza virus (AIV) is a serious concern for public
31 health. Neuraminidase (NA) isolated from a fatal case of avian-origin H10N8 influenza virus
32 infection was found to carry a drug-resistant mutation, NA-Arg292Lys (291 in N8 numbering). In
33 order to understand the full potential of H10N8 drug resistance, the virus was first passaged in the
34 presence of the most commonly used neuraminidase inhibitors (NAIs), oseltamivir and zanamivir.
35 As expected, the Arg292Lys substitution was detected after oseltamivir treatment, however a novel
36 Val116Asp substitution (114 in N8 numbering) was selected by zanamivir treatment. Next
37 generation sequencing (NGS) confirmed that the mutations arose early (after passages 1-3) and
38 became dominant in the presence of the NAI inhibitors. Extensive crystallographic studies
39 revealed that N8-Arg292Lys resistance results mainly from loss of interactions with the inhibitor
40 carboxylate, while rotation of Glu276 was not impaired as observed in the N9-Arg292Lys, a group
41 2 NA structure. In the case of Val116Asp, the binding mode between oseltamivir and zanamivir is
42 different. Asp151 forms stabilized hydrogen bond to guanidine group of zanamivir, which may
43 compensate the resistance caused by Val116Asp. By contrast, the amino group of oseltamivir is
44 too short to maintain this hydrogen bond, which result in resistant. Moreover, the oseltamivir-
45 zanamivir hybrid inhibitor MS-257 displays higher effectiveness to Val116Asp than oseltamivir,
46 which support this notion.

47 **Author Summary**

48 Aside from vaccination, NAIs are currently the only alternative for the clinical treatment and
49 prophylaxis of influenza. Understanding the mechanisms of resistance is critical to guide in drug
50 development. In this study, two drug-resistant NA substitutions, Val116Asp and Arg292Lys, were
51 discovered from oseltamivir and zanamivir treatment of H10N8 virus. Crystal structural analyses
52 revealed two distinct mechanisms of these two resistant mutations and provide the explanation for
53 the difference in susceptibility of different NAIs. Zanamivir and laninamivir were more effective
54 against the resistant variants than oseltamivir, and Arg292Lys results in more serious oseltamivir
55 resistance in N9 than N8 subtype. This study is well-correlated to influenza pandemic/epidemic
56 pre-warning, as the discovery of inhibitor resistant viruses will help for new drug preparedness.

57 **Introduction**

58 Four NAIs, oseltamivir, zanamivir, peramivir and laninamivir, are currently available for the
59 clinical treatment of influenza virus infections [1-4]. Oseltamivir has been extensively used due to
60 its high oral bioavailability [5], however resistance to both oseltamivir and peramivir is prevalent.
61 On the other hand, although zanamivir and laninamivir offer advantages in terms of drug
62 resistance, both inhibitors are highly polar in their active forms and therefore have lower
63 bioavailability. Furthermore, there are still many known substitutions that lead to zanamivir and
64 laninamivir resistance including Glu119Gly/Ala/Asp, Gln136Lys, Asp151Ala/Asn/Gly/Val,
65 Arg152Lys, Ile222Arg, Asp198Asn, Arg292Lys and Arg371Lys (N2 numbering) [6-10].

66 Resistance to NAIs usually results from substitutions of highly conserved amino acid residues that
67 form the NA active site. The influenza NA active site contains 8 conserved catalytic residues, and
68 an additional frame of 11 residues that provides structural support to the active site [11, 12].
69 Substitution of non/semi-conserved residues has also been shown to lead to NAI resistance [13].
70 Semi-conserved influenza NA residues such as Ile117 and Lys150 have been observed to confer
71 NAI resistance in N1 subtype NA[14, 15], while Gln136 has been observed in both N1 [16] and
72 N2 [17] NA subtypes. However, the mechanisms of drug resistance related to semi-conserved
73 residues are poorly understood [6, 18, 19].

74 In recent years, some new AIVs, either highly pathogenic (HPAIV) or low pathogen AIV
75 (LPAIV), have emerged with ability to infect humans in addition to H5N1 HPAIV[20-24], due to
76 extensive poultry transportation and wild bird migration [25]. LPAIV H10N8 human-infecting
77 cases were identified in Jiangxi Province, China in 2013 [24, 26]. In December 2013, A/Jiangxi-
78 Donghu/346/2013/H10N8 with the NA-Arg292Lys (N2 numbering, 291 in N8 numbering)

79 mutation was discovered in the trachea aspirate of a 73-year-old patient who died 3 days following
80 oseltamivir treatment [27]. Therefore, H10N8 and other avian-origin influenza A viruses possess
81 a high potential for human pathogenicity and hence pose public health concern.

82 There are two phylogenetic groups of influenza A NAs (N1, N4, N5 and N8 belong to group 1,
83 while N2, N3, N6, N7 and N9 belong to group 2) [28]. Arg292Lys has been widely reported in
84 group 2 NAs (N2 and N9), while the report of Arg292Lys in a group 1 NAs has never been
85 observed in nature. Structural analysis of N9-Arg292Lys has been shown to result in unfavorable
86 Glu276 conformation for oseltamivir binding [10]. However, the mechanism of Arg292Lys
87 resistance in a group 1 N8 is not well understood due to lack of crystal structures (apo and holo).

88 In this study, we explored the potential drug-resistant substitutions of A/Jiangxi-
89 Donghu/346/2013/H10N8 by passaging the virus in the presence of zanamivir and oseltamivir,
90 respectively. Besides the clinical Arg292Lys substitution which was identified after *in vitro*
91 oseltamivir treatment, a novel Val116Asp substitution was also identified after zanamivir
92 treatment. The residue 116 (residue 114 in N8 numbering) does not play a direct role in active site
93 framework or drug binding and therefore the mechanism underlying Val116Asp resistance is
94 perplexing. We have determined the binding capability (K_m) of N8 wildtype and two substitutions.
95 The data indicated that Val116Asp and Arg292Lys mutations reduce the affinity between the
96 enzyme and substrate, which results from the conformational shift of the key residues in the active
97 site. Moreover, the inhibition assay demonstrated that the oseltamivir resistance (1,000-fold)
98 caused by Arg292Lys in N8 is much less severe than that of H7N9 (100,000-fold). We have also
99 determined the *apo* crystal structures of N8 wild type and the two mutants and the *holo* structures
100 in complex with oseltamivir, zanamivir, peramivir and laninamivir. These crystal structures reveal

101 the underlying mechanism by which Arg292Lys and Val116Asp mutations develop resistance
102 towards NAIs.

103 **Results**

104 ***In vitro* NAI Selective Pressure**

105 H10N8 virus was subjected to 2-fold increases in the concentration of oseltamivir (80-10240 μ M)
106 and zanamivir (20-2560 μ M) over 8 passages. Prior quantification by plaque assays showed that
107 the virus was resistant to oseltamivir at 13.3 μ M and zanamivir at 1.33 μ M. After each passage,
108 the hemagglutinin (HA) titer was determined, recorded in triplicate and used to estimate the
109 concentration of virus to be seeded in the next passage. A negative HA titer was observed in the
110 last passage indicating the absence of virus (S1 Table).

111 **Substitutions under NAI Pressure**

112 Viruses from each passage under inhibitor treatment were subject to high-throughput sequencing
113 for whole genome substitution and intra-cell substitution analysis. The result showed that as
114 expected, the drug-resistant Arg292Lys substitution in NA was detected after oseltamivir
115 treatment; however, a novel NA Val116Asp substitution arose after zanamivir treatment,
116 suggesting a direct role in zanamivir-resistance (S1 Fig). The Arg292Lys mutation under
117 oseltamivir treatment and the Val116Asp mutation under zanamivir treatment arose and became
118 dominant quickly (1-3 passages). Unexpectedly, the pre-exist frequency of Arg292Lys was found
119 to be at 28 % in the non-drug treatment control. However, just after 1 passage under oseltamivir
120 treatment, it increased to 92% and levelled off at 92% throughout the next 6 oseltamivir treatment
121 passages (Fig 1A and 2). For zanamivir treatment, the pre-exist frequency of Val116Asp was found

122 to be at 2%; after 1 passage, it increased quickly to 34%, and became absolute dominant (90%)
123 (Fig 1B and 2).

124 In addition to these two NA substitutions, several substitutions occurred on the HA and other
125 certain internal genes. Two HA substitutions, Lys167Glu and Ile413Thr, were pre-existing at the
126 frequency of 49% and 48% respectively, and both became dominant under oseltamivir/ zanamivir
127 treatment and kept steady along cell passages with the increase of drug concentration. Importantly,
128 Val139Ile in Polymerase basic protein 2 (PB2) arose and became dominant in the first passage of
129 zanamivir treatment, with the frequency of 81%. However, this was not observed in oseltamivir
130 treatment passages. One common substitution Cys95Phe in Polymerase acidic protein (PA)
131 occurred in both treatment passages. PA Cys95Phe also pre-existed in the drug-free treatment with
132 frequency of 12%, which showed a slow increasing trend and became dominant at passage 7 in
133 oseltamivir treatment. Meanwhile, in zanamivir treatment passages, it became absolute dominant
134 within 3 passages and levelled off. Another PA substitution Val91Leu was only observed in
135 oseltamivir treatment. This mutation pre-existed in the drug-free treatment control at a frequency
136 of 10% and showed a fluctuating trend along passages. Other two substitutions, HA Gly89Arg and
137 M1 Gln158Arg also showed fluctuating trends along oseltamivir treatment passages. Fluctuating
138 trends for HA Gly89Arg mutation was also observed in zanamivir treatment passages (S1 Fig and
139 Fig 2).

140 Collectively, we can find that most of these substitutions are pre-existing in the original NAI-free
141 cultures. These pre-existing substitutions might be the natural selection pools when under drug
142 treatment. Correlation analysis of these substitutions using their frequencies values found several
143 strong links: one negative link between HA 179Arg and M1 Gln158Arg, one negative link between
144 HA 179Arg and PA Cye95Phe along oseltamivir treatment; one positive link between NA

145 V116Asp and 292Arg. These results suggest that synergy might exist along the dynamic changes
146 of these substitutions, which will be further studied in the future.

147 **Reduced Substrate Affinity and Inhibition of N8-Arg292Lys and N8-Val116Asp**

148 NA was prepared according to the previously reported methods [29-31]. Lower substrate affinity
149 was observed for N8-Arg292Lys (30-fold) and Val116Asp (6-fold) mutants when compared to the
150 wildtype N8. The recombinant Val116Asp and Arg292Lys variants also exhibited reduced
151 sensitivity to the four clinically used NAIs (oseltamivir, zanamivir, peramivir and laninamivir)
152 relative to the wildtype. The Arg292Lys mutant exhibited higher resistance to all of the four NAIs
153 compared to the Val116Asp mutant. Both mutants showed high resistance to oseltamivir, with a
154 926-fold and 128-fold increase in mean IC_{50} for Arg292Lys and Val116Asp, respectively.
155 Val116Asp was moderately resistant to zanamivir (40-fold) while Arg292Lys displays much
156 higher zanamivir resistance (704-fold). The resistance pattern of Val116Asp and Arg292Lys to
157 laninamivir is similar to that of zanamivir, with 10-fold and 90-fold lower potencies, respectively.
158 On the other hand, Arg292Lys showed much higher resistance to peramivir with a 3,169-fold
159 increase in mean IC_{50} value, while Val116Asp exhibited a moderate 20-fold reduced sensitivity
160 (Table 1). Interestingly, the oseltamivir-zanamivir hybrid inhibitor, MS-257 was found to be the
161 least affected inhibitor by these mutations when compared to all four clinically used NAIs. The
162 increase in the mean IC_{50} values of MS-257 against Arg292Lys and Val116Asp mutants were 19-
163 fold and 7-fold, respectively.

164 **Val116Asp and Arg292Lys Result in Subtle N8 Active Site Changes**

165 The crystal structures of native N8, Val116Asp and Arg292Lys were determined at resolutions of
166 1.9 Å, 1.9 Å and 1.6 Å, respectively (S2 Table). The apo structures of N8 wildtype, Val116Asp

167 and Arg292Lys showed similar overall active site arrangements, with some differences observed
168 regarding Gln136, Thr148, Glu276 and Tyr406 (Fig 3). Specifically, the conformations of 150-
169 loop between N8 wildtype and Val116Asp show slightly different, because of the conformational
170 change of Thr148 and Gln136. The conformations of 430-loop between N8 wildtype and
171 Arg292Lys display variable configurations. Moreover, the side chain of Gln136, Arg118, Tyr406
172 and Glu276 between these two native structures exhibit different conformations. All these
173 observed conformational differences explain why the two N8 mutants have lower affinity to the
174 substrate compared to N8 wildtype.

175 **A Distinct Mechanism of Arg292Lys Drug Resistance in Group 1 N8**

176 To understand the mechanisms of N8 drug resistance, inhibitor complex structures with wildtype
177 or mutant N8 were compared. The crystal structures of wildtype N8 complexed with zanamivir,
178 oseltamivir, laninamivir and peramivir were determined at resolutions of 1.8 Å, 2.1 Å, 1.8 Å and
179 2.0 Å, respectively (S3 Table). N8-Arg292Lys complexes were determined at resolutions of 1.9
180 Å, 1.8 Å, 2.0 Å and 1.8 Å for zanamivir, oseltamivir, laninamivir and peramivir, respectively (S4
181 Table). Binding of oseltamivir, zanamivir, peramivir and laninamivir to wildtype H10N8 NA
182 resembles that of typical group 1 NA binding (Fig 4A, C, E and G), with differences observed
183 regarding Tyr347 in both oseltamivir and laninamivir complex structures. In the wildtype N8
184 complexes with zanamivir, oseltamivir and laninamivir, as well as the Arg292Lys complex with
185 zanamivir, Tyr347 hydrogen bonds with Arg371 and the inhibitor carboxylate. In the wildtype
186 peramivir complex, as well as the Arg292Lys complexes with laninamivir, oseltamivir and
187 peramivir, Tyr347 points away from the active site where it can no longer interact with the
188 inhibitor.

189 Arg292 is part of the NA tri-arginine cluster that forms strong ionic interactions with the first-
190 generation NAI carboxylates. In the structures of N8-Arg292Lys complexed with the four NAIs,
191 Lys292 interacts with the carboxylate group of NAIs by a bridging water molecule (Fig 4A, C, E
192 and G). The Glu276 adopts similar conformation in N8-Arg292Lys-zanamivir and laninamivir
193 complexes (Fig 4C and G), but slightly differ in oseltamivir and peramivir-complexes (Fig 4A and
194 E).

195 The binding of oseltamivir to N8 (group 1 NA) and N9 (group 2 NA) was carefully compared (Fig
196 4B, D, F and H). The flexibility of residue Glu276 is observed in the N9-Arg292Lys oseltamivir
197 complex structure, but only a slightly shift in the N8-Arg292Lys oseltamivir complex structure.
198 Specifically, in N8-Arg292Lys, Glu276 rotates towards Arg224 for optimal oseltamivir binding,
199 which results in a mild shift of the oseltamivir hydrophobic group (1.11 Å). In contrast, the side
200 chain of Glu276 in N9-Arg292Lys is oriented toward the oseltamivir carboxylate hydrophobic
201 pentyloxy group, which is pushed 2.98 Å away from the active site. Furthermore, Arg292Lys
202 substitution in N8 has no effect on the hydrogen bonds between Glu276 and Arg224, while, in N9,
203 this substitution results in loss of one hydrogen bond between Glu276 and Arg224 (Fig 4B). These
204 differences help to explain the observation that the effect of N8-Arg292Lysoseltamivir resistance
205 is 1000-fold whereas the effect of N9-Arg292Lys resistance is 100,000-fold [10].

206 Notably, in the structure of N8-Arg292Lys-zanamivir, the orientation of Tyr347 is the same as N8
207 wildtype, which forms hydrogen bond with the side chain of Arg371 (Fig 4C). However, the
208 residue 347 in N9 is Aspartic acid, which side chain is not enough to form hydrogen bond with
209 Arg371 (Fig 4D). Therefore, zanamivir shows better inhibition to N8 than N9 with Arg292Lys
210 substitution (Table 1).

211 **The guanidine group of zanamivir compensates the Val116Asp resistant N8 substitution**

212 In order to understand the mechanism of Val116Asp resulting in more severe resistance to
213 oseltamivir than zanamivir, the zanamivir and oseltamivir complex structures of N8-Val116Asp
214 were determined at resolutions of 1.9 Å and 2.1 Å, respectively (S5 Table). The 150-loop of
215 inhibitor bound-N8 (Val116Asp) complexes exhibited closed conformation, in which the Asp151
216 interacts with inhibitors. When compared the oseltamivir complex-N8 wild type with that of the
217 Val116Asp mutant, it was found that the side chain of Asp151 in N8 wildtype hydrogen bonds to
218 C4-amino group of oseltamivir with a distance of 2.70 Å, while the corresponding distance in N8-
219 Val116Asp was found to be 3.34 Å. In the case of zanamivir complexed structures, oxygen atom
220 on the main chain of Asp151 makes hydrogen bond interactions with the guanidine group of
221 zanamivir, with distance of 2.88 Å and 3.03 Å in N8 wildtype and N8-Val116Asp mutant,
222 respectively. The hydrogen bond interactions of zanamivir guanidine group with Asp151 is less
223 affected by the Val116Asp substitution than that of oseltamivir amino group. Moreover, Tyr347
224 in these two Val116Asp complex structures are shifted away from both inhibitors, while Tyr347
225 hydrogen bonds with the zanamivir and oseltamivir carboxylates groups in wildtype N8 complex
226 structures (Fig 5). Interestingly, the oseltamivir-zanamivir hybrid inhibitor MS-257 showed better
227 inhibition to N8-Val116Asp than oseltamivir (Table1).

228 **Discussion**

229 Although the crystal structures of all 9 influenza A NA subtypes have been solved to date [10, 28,
230 30-37], the influenza A virus is constantly adapting and many new variations are being discovered,
231 especially those with drug resistance. For example, the crystal structure analysis of Arg292Lys
232 mutant of group 2 N9 was completed in 1998 [38], yet the corresponding group 1 N8 structure

233 reported here contains unique features which are clearly observed during oseltamivir binding.
234 These structural differences are also reflected in the K_m values which increased 30-fold for N8-
235 Arg292Lys and 88-fold for H7N9 N9-Arg292Lys [10], relative to the corresponding wildtype
236 NAs.

237 The Val116Lys mutation was the most intriguing N8 substitution. It is challenging to predict a
238 precise mechanism for NAI resistance, because the site of mutation is distal from the active site
239 frame work. Prior to solving the crystal structures, we anticipated that the Val116Asp substitution
240 might affect the interaction between Arg118 and the carboxylate group of the inhibitor, however
241 contrary to expectation, the N8-Val116Lys complex structures clearly suggested that this is not
242 the case and the loss of Tyr347-inhibitor interaction as the resistance mechanism. Tyr347 is found
243 only in group 1 NAs which should contain the 150-loop cavity [28]. This residue was also observed
244 to be a key factor in explaining the slightly higher NAI inhibition observed in N5 (typical group 1
245 NA) [36] relative to pandemic 09N1 (atypical group 1 NA) [39] and 57N2 (group 2 NA) [31].
246 Thus, we previously speculated that Tyr347 might compensate for the open 150-loop in regards to
247 substrate binding [31].

248 Val116Lys also resulted in an altered conformation of the 150-loop residue Thr148 (Fig 3). It is
249 possible that the further changes observed in the loop residues 146-148 upon oseltamivir binding
250 might affect the ionic interaction of Asp151 with the oseltamivir amino group (Fig 5). Despite the
251 moderate effect on NAI inhibition, the Val116Asp substitution also resulted in a 6-fold K_m increase
252 relative to the wildtype (Table 1). This indicates that Val116Asp also interferes with substrate
253 binding.

254 Zanamivir and laninamivir are more similar to the human sialic acid, *N*-acetylneuraminic acid,
255 than oseltamivir and peramivir, and therefore should be less susceptible to drug-resistance. Of the
256 4 clinical NAIs, zanamivir bound to both mutants in the most optimal conformation, whereas
257 oseltamivir binding was the least optimal. Resistance of both N8 variants to zanamivir and
258 laninamivir was also lower than that of oseltamivir and peramivir.

259 Some questions remain from the present analysis, including why peramivir was the most potent
260 inhibitor of wildtype N8 despite lacking any Tyr347 interactions. Moreover, binding of the prodrug
261 laninamivir octanoate (CS-8958) to N8 was distinct from group 1 09N1 and similar to group 2 N2,
262 which indicates that NAI binding is not always group specific.

263 In summary, our current study revealed the 4-clinical available NAI resistant substitutions for N8
264 and the underlying mechanisms have also been structurally delineated, which will help for next-
265 generation drug development guide for drug usage in future prewarning of H10N8 AIV human
266 infections.

267 **Materials and Methods**

268 **Cells, Virus and NAIs.** A549 cells were obtained from China Infrastructure of Cell Line
269 Resources, Beijing and were grown in Dulbecco Modified Eagle's Medium (DMEM) (Gibco by
270 Life Technologies IncorporationTM, Grand Island, New York, USA) containing 5% fetal bovine
271 sera (FBS) (Irvine Scientific). The human influenza A/Jiangxi-Donghu/346/2013(H10N8) virus of
272 avian origin was propagated in the allantoic cavity of 10-day-old fertilized specific-pathogen-free
273 (SPF) hen eggs (Beijing Vital River Laboratory Animal Technology Co., Ltd.) for 96 hours at
274 37°C. A hemagglutination assay was carried out on the harvested infected allantoic fluid in 96-
275 well plates. After adding 25 μ L of PBS to each well, 25 μ L of virus suspension was added to the

276 first well, followed by a series of 2-fold dilutions and gentle mixing. Next, about 25 μ L of freshly
277 prepared 1% chicken RBC was added to each well and left for 30 minutes at room temperature.
278 Clear hemagglutination of the allantoic fluid containing the virus with chicken RBC indicates viral
279 growth and confirms the presence of the virus. Influenza neuraminidase inhibitors oseltamivir acid
280 (GS 4071) and zanamivir were synthesized at MedChem Express LLC, NJ, USA. MS-257 was
281 provided by cooperator Prof. Mario Pinto.

282 **Isolation of H10N8 Variants with Decreased Susceptibility to Oseltamivir and Zanamivir.**

283 24-hour-old A549 cells were grown in 24 well tissue culture plates and infected with egg-grown
284 virus at a low multiplicity of infection (MOI 0.001 PFU per cell) in maintenance medium. The
285 virus was allowed to adsorb for only 15 minutes at 37°C, after which the inoculum was removed,
286 and the cells were washed twice with pre-warmed PBS followed by addition of 1 ml of each drug
287 preparation to the seeded infected cells. The plates were incubated at 37°C for 72 hours. The virus
288 titer in each culture supernatant was determined by hemagglutination of chicken RBC. The culture
289 supernatant that contained the minimal dose drug that still resulted in cytopathy and detectable HA
290 titer was used as inoculum to infect new cell monolayers at a low MOI. The virus from that sample
291 was then allowed to grow in the presence of a series of 2-fold oseltamivir and zanamivir dilutions.
292 The virus concentrations used in the selection protocol varied, depending on the HA titer in the
293 preceding passage and about 1 ml aliquot of viral stock culture supernatant from the preceding
294 passage was used to infect the fresh cell monolayer cells. The HA titer was determined after each
295 passage to estimate the amount of virus needed for infection. This selection was carried out for a
296 total of 8 passages for both inhibitors in triplicates. Drug concentration ranged from 80 μ M to
297 10.24 mM for oseltamivir and 20 μ M to 2.56 mM for zanamivir. All conditions were carried out
298 in triplicates.

299 **Virus RNA Extraction and Next-Generation Sequencing (NGS).** RNA was extracted from
300 virus isolates using a QIAamp viral RNA Mini Kit from QIAGEN, Germany (Cat. No. 52904).
301 Complete influenza A genomes were prepared from the RNA using the Takara PrimeScript™
302 One step RT-PCR Kit Ver. 2 (TAKARA BIO INC. Cat. #RR055A v201309Da, Japan) with slight
303 modifications. Each RNA preparation was mixed with the enzyme mix in a 50 µL reaction system.
304 The thermal cycle parameters were 50°C for 30 mins, 94°C for 2 mins, and then 35 cycles at 94°C
305 for 30 secs, 58°C for 30 secs, and 72°C for 3 mins 20 secs. Primers used were 20 µM MBTuni-12
306 (5'-ACGCGTGATCAGCAAAGCAGG) and MBTuni-13 (5'-
307 ACGCGTGATCAGTAGAAACAAGG) that correspond to the 5' and 3' conserved sequences of
308 all eight influenza A segments (26). NGS was used to determine the whole genomes of treated
309 H10N8 samples. The sequencing libraries were prepared from H10N8 whole genome PCR
310 products by end-repairing, dA-tailing, adapter ligation and PCR amplification, according to the
311 manufacturer instructions (Life technologies). The libraries were sequenced on an Ion Proton™
312 System, and sequencing depth was 1 G base per sample. After sequencing, raw NGS short reads
313 were processed by filtering out low-quality reads, adaptor-contaminated reads (with >15 bp
314 matched to the adapter sequence), poly-Ns (with 8Ns) (SOAP2 (v2.21), <5 mismatches). Clean
315 short reads were then mapped onto the eight reference genome segments (A/Jiangxi-
316 Donghu/346/2013(H10N8)) using TMAP (v3.4.1)
317 (<https://github.com/iontorrent/TS/tree/master/Analysis/TMAP>) with a match rate larger than 0.90,
318 producing the short-read-reference-genome mapping file (SRRG file), and the dominant base on
319 each site was called to obtain the consensus genome. Consensus sequence alignments were done
320 using MUSCLE [40] to identify the variable sites. The consensus nucleotide sites, which have
321 changed in more than any one sample comparing with the original genome (A/Jiangxi-

322 Donghu/346/2013 (H10N8)), are designated as variable sites. Intra-host single nucleotide
323 variations (iSNV) and their correlations were analyzed based on the SRRG file.

324 **Expression and Purification of Influenza Virus NA.** Recombinant NA protein was prepared
325 using the established baculovirus expression system (19, 20, 22). The ectodomain (residues 81 to
326 469 in N8 numbering) of A/JD/346/2013/H10N8 was cloned into pFastBac1 baculovirus transfer
327 vector (Invitrogen) and the Val114Asp and Arg291Lys substitutions were constructed by site-
328 directed mutagenesis PCR based on N8 wildtype and expressed in a baculovirus system for
329 structural and functional analysis. A GP67 signal peptide was added at the N terminus to facilitate
330 secretion of the recombinant protein, followed by a His tag, a tetramerizing sequence, and a
331 thrombin cleavage site. Recombinant pFastBac1 plasmid was used to transform DH₁₀Bac™
332 *Escherichia coli* (Invitrogen). The recombinant baculovirus was obtained following the
333 manufacturer's protocol, and Hi5 cell suspension cultures were infected with high-titer
334 recombinant baculovirus. After growth of the infected Hi5 suspension cultures for 2 days,
335 centrifuged media were applied to a 5-mL HisTrap FF column (GE Healthcare), which was washed
336 with 20 mM imidazole. The NA was thereafter eluted using 300 mM imidazole. After dialysis and
337 thrombin digestion (3 U/mg NA; BD Biosciences) overnight at 4°C, gel filtration chromatography
338 was performed with a Superdex® 200 10/300 GL column (GE Healthcare) using 20 mM Tris-HCl
339 and 50 mM NaCl (pH 8.0) buffer or PBS buffer for crystallization or functional assay, respectively.
340 Pure NA fractions were selected and further concentrated using a 10 kDa (Millipore) membrane
341 concentrator.

342 **Crystallization, Drug-Soaking and Crystal Structure Determination.** wildtype and mutant N8
343 crystals were obtained using the sitting-drop vapor diffusion method. The NA proteins [1 μL
344 of 10mg/mL protein in 20mM Tris and 50mM NaCl (pH 8.0)] were mixed with 1 μL of reservoir

345 solution. N8 wildtype crystals were obtained in the condition of 0.1 M sodium acetate trihydrate
346 pH 4.7 and 5% w/v Polyethylene glycol 10,000. N8-Val116Asp crystals were obtained in the
347 condition of 0.1M DL-malic acid pH 7.0 and 12% Polyethylene glycol 3,350. N8-Arg292Lys
348 crystals were obtained in the condition of 0.1 M potassium phosphate monobasic/sodium
349 phosphate dibasic pH 6.2 and 10% Polyethylene glycol, 3,000.

350 All NA crystals were cryoprotected in mother liquor with the addition of 20% (vol/vol) glycerol
351 before being flash-cooled at 100 K for obtaining apo structures. The crystals were then incubated
352 in the mother liquor containing 20 mM inhibitors (oseltamivir acid, zanamivir, peramivir, and
353 laninamivir) and then flash-cooled at 100 K.

354 Diffraction data were collected at Shanghai Synchrotron Radiation Facility beamline BL17U. The
355 collected intensities were indexed, integrated, corrected for absorption, and scaled and merged
356 using HKL-2000 (27). The structure of N8 was solved by molecular replacement using Phaser (28)
357 from the CCP4 program suite (29), with the structure of N8 (PDB ID code 2HT7) as a search
358 model. N8-Val116Asp and Arg292Lys were equally solved using the native N8 as the search
359 model. The initial model was refined by rigid body refinement using REFMAC5 (30), and
360 extensive model building was performed using COOT (31). Further rounds of refinement were
361 performed using the phenix.refine program implemented in the PHENIX package (32) with energy
362 minimization, isotropic ADP refinement and bulk solvent modeling. The stereochemical quality
363 of the final model was assessed with PROCHECK (33). Structures were aligned and analyzed
364 using PyMOL.

365 **Fluorescent NA Activity Assay.** A 4-methylumbelliferyl-Neu5ac-(MUNANA)-based
366 fluorometric NA assay (34) was used for measuring the NA activity and inhibition. The

367 appropriate protein concentrations were chosen after several rounds of preliminary tests. The km
368 values for the active NAs were determined by mixing 10 μ L of each recombinant protein with 10
369 μ L of buffer MES-CaCl₂ buffer (pH 6.0) in each 96-well plate and serial dilutions (9.76 μ M –
370 5mM) of MUNANA (30 μ L) were added to each well. The fluorescence intensity of the released
371 product was measured every 30 seconds for 1 hour at 37°C on the SpectraMax M5 (Molecular
372 Devices), with excitation and emission wavelengths of 355 nm and 460 nm, respectively. For
373 inhibition assays, 10 μ L of recombinant protein was mixed with 10 μ L of PBS or inhibitor in 96-
374 well standard opaque plate and 30 μ L of 167 μ M MUNANA (Sigma, USA) in 33 mM MES and 4
375 mM CaCl₂ (pH 6.0) for a final substrate concentration of 100 μ M. The inhibitors (in different
376 concentrations) were pre-incubated with the NA protein for 30 minutes at 37°C before adding
377 MUNANA, and then loaded on the SpectraMax[®] M5 Molecular devices. Fluorescence was
378 monitored immediately after substrate addition at 1-minute intervals for 30 minutes. All assays
379 were performed in triplicates and IC₅₀ values for each inhibitor were calculated with Graphpad
380 Prism 5.0 as the concentration of inhibitor resulting in a 50% reduction in fluorescence units (FU)
381 compared with the control.

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538

539 **Supporting Information Legends**

540 **Table 1.** K_m and IC_{50} values for N8 wildtype and mutant proteins. The IC_{50} values and 95%
541 confidence intervals (CIs) are provided.

542 **S1 Fig.** Consensus sequence variations of H10N8 virus under drug treatment. The first row
543 represents the structural proteins of H10N8 and vertical numbers represent different amino acid
544 sites. The consensus amino sites list default amino acids, dots represent no substitutions and
545 question marks represents unknown amino acids due to sites that were not covered by short reads.
546 Osel: oseltamivir, Zan: zanamivir, and the numeral to the right of Osel and Zan are the passage
547 numbers.

548 **S1 Table.** Inhibitor concentration and HA titer of passaged viruses. The inhibitor concentration
549 and HA titer for eight passages are listed in the table. “-” means no hemagglutination.

550 **S2 Table.** Crystallographic data collection and refinement statistics of native N8 and N8
551 mutations.

552 **S3 Table.** Crystallographic data collection and refinement statistics of N8-inhibitor complexes.

553 **S4 Table.** Crystallographic data collection and refinement statistics of N8-Arg292Lys-inhibitor
554 complexes.

555 **S5 Table.** Crystallographic data collection and refinement statistics of N8-Val116Asp–inhibitor
556 complexes

557

558 **Figure Legends**

559 **Fig 1. Substitution frequency of Val116Asp and Arg292Lys in N8 under NAI pressure.** (A)

560 Mutation frequency of Arg292Lys increased sharply and throughout the passage period during
561 oseltamivir treatment. (B) A similar trend was observed for Val116Asp during zanamivir
562 treatment, although there was a reduction in the mutation rate at passages 5 and 7.

563 **Fig 2. Heatmap and correlation network of oseltamivir treated samples.** (A) Heatmap of

564 amino acid residues under oseltamivir treatment from the 1st to 7th passage. (B) Heatmap of amino

565 acid residues under zanamivir treatment from the 1st to 7th passage. The values in each cell

566 represent the substitution frequencies. A sudden change in the default frequency will lead to a

567 change in the amino acid site. The ratio of the depth sequencing site or threshold of total depth is

568 10. Mutation frequency is based on color change. Each column represents the amino acid sites

569 separated by “-” while each row represents the drug passage including positive control. POS:

570 positive control, N/A: not available data to give a decisive result, OS: oseltamivir, Zan: zanamivir,

571 and the numerals next to each inhibitor represent the passage numbers. (C) Correlation networks

572 of different amino acid sites. (D) Correlation networks of different amino acid sites show a positive

573 correlation between NA Asp116 and NA Arg292. To avoid error, smooth changing sites were used

574 for correlation analysis which could be judged easily from the heatmap. Amino acid sites are

575 indicated as different colors depending on the encoded protein. 2 or more amino acids connected

576 together indicates a correlation larger than 0.8 ($r > 0.8$). Correlations were determined using

577 Pearson's rank correlation method. P-values of all correlations are less than 0.05. Positive

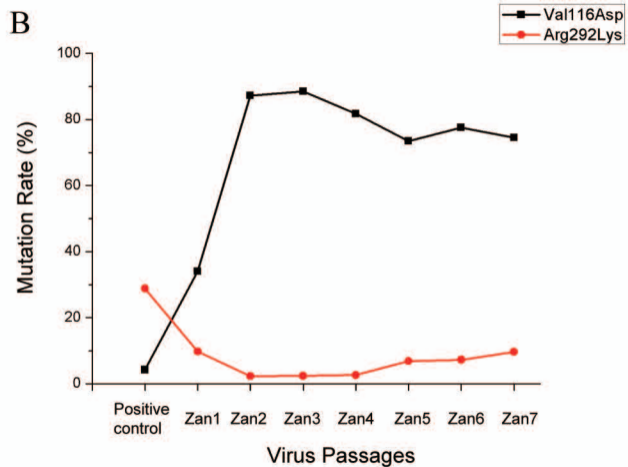
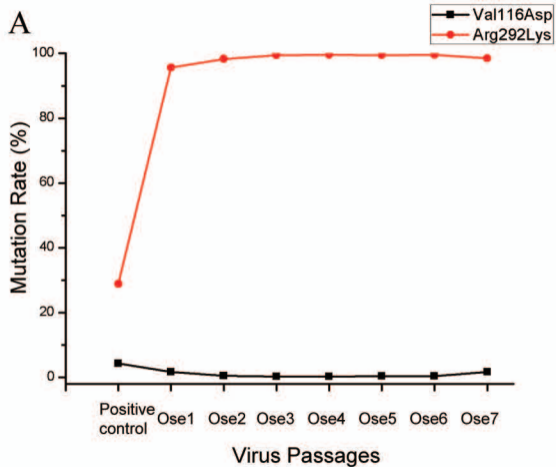
578 correlation is indicated by a red line connecting amino acids while negative correlation is indicated
579 by a green line.

580 **Fig 3. Active site comparison of N8 wildtype (green), Val116Asp (cyan) and Arg292Lys**
581 **(magenta).** (A) The comparison of the key residues conformation between N8 wildtype and N8-
582 Val116Asp. (B) Comparison of the key residues in the active site between N8 wildtype and N8-
583 Arg292Lys. Key residues are labeled in sticks.

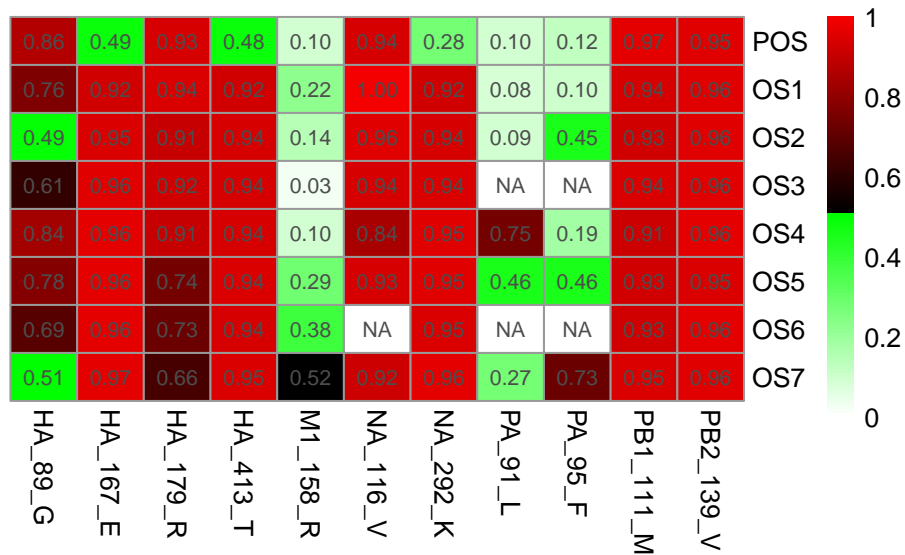
584 **Fig 4. Comparison of wildtype N8, N8-Arg292Lys and N9Arg292Lys binding to four NAIs.**
585 (A and B) oseltamivir; (C and D) zanamivir; (E and F) peramivir and (G and H) laninamivir. All
586 the inhibitors and the key residues are displayed in sticks. The hydrogen bonds are indicated in
587 dash. The PDB code of N9-Arg292Lys-oseltamivir, N9-Arg292Lys-zanamivir, N9-Arg292Lys-
588 peramivir and N9-Arg292Lys-laninamivir are 4MWW, 4MWX, 4MX0 and 4MWY, respectively.

589 **Fig 5. Comparison of the binding mode between N8 wildtype and N8-Val116Asp with two**
590 **inhibitors.** (A) The binding mode of N8 wildtype-oseltamivir (cyan) and N8-Val116Asp-
591 zanamivir (salmon). (B) The binding mode of N8 wildtype-zanamivir (bright yellow) and N8-
592 Val116Asp-zanamivir (orange).

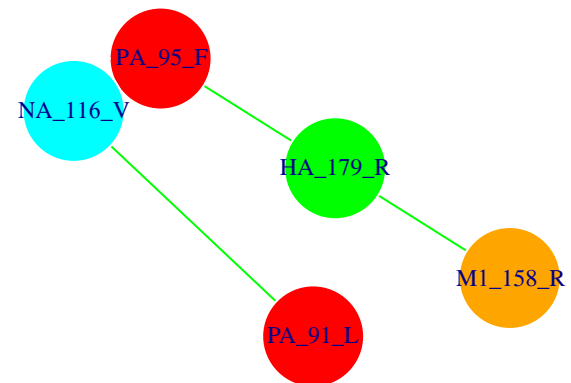
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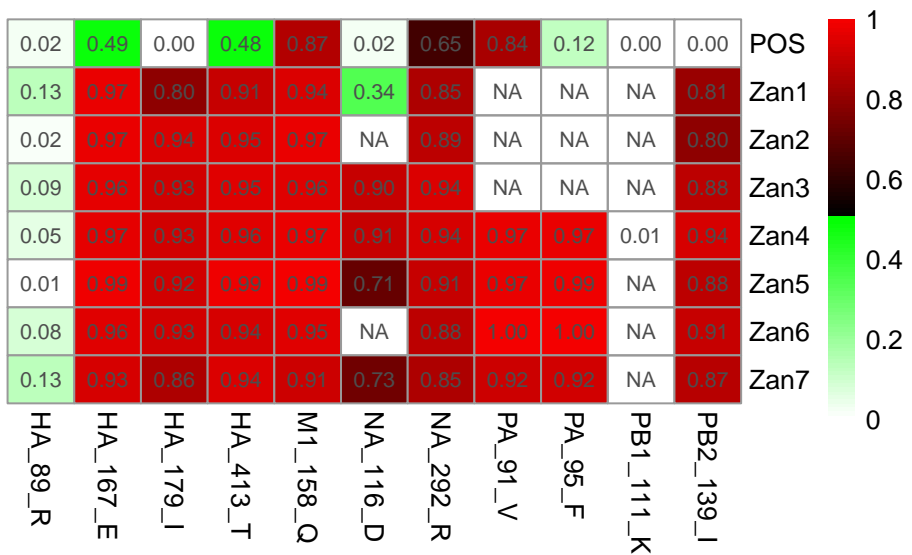
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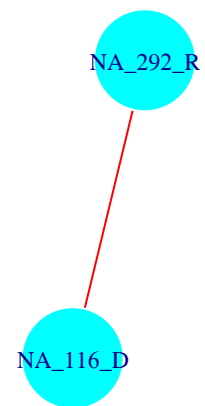
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C

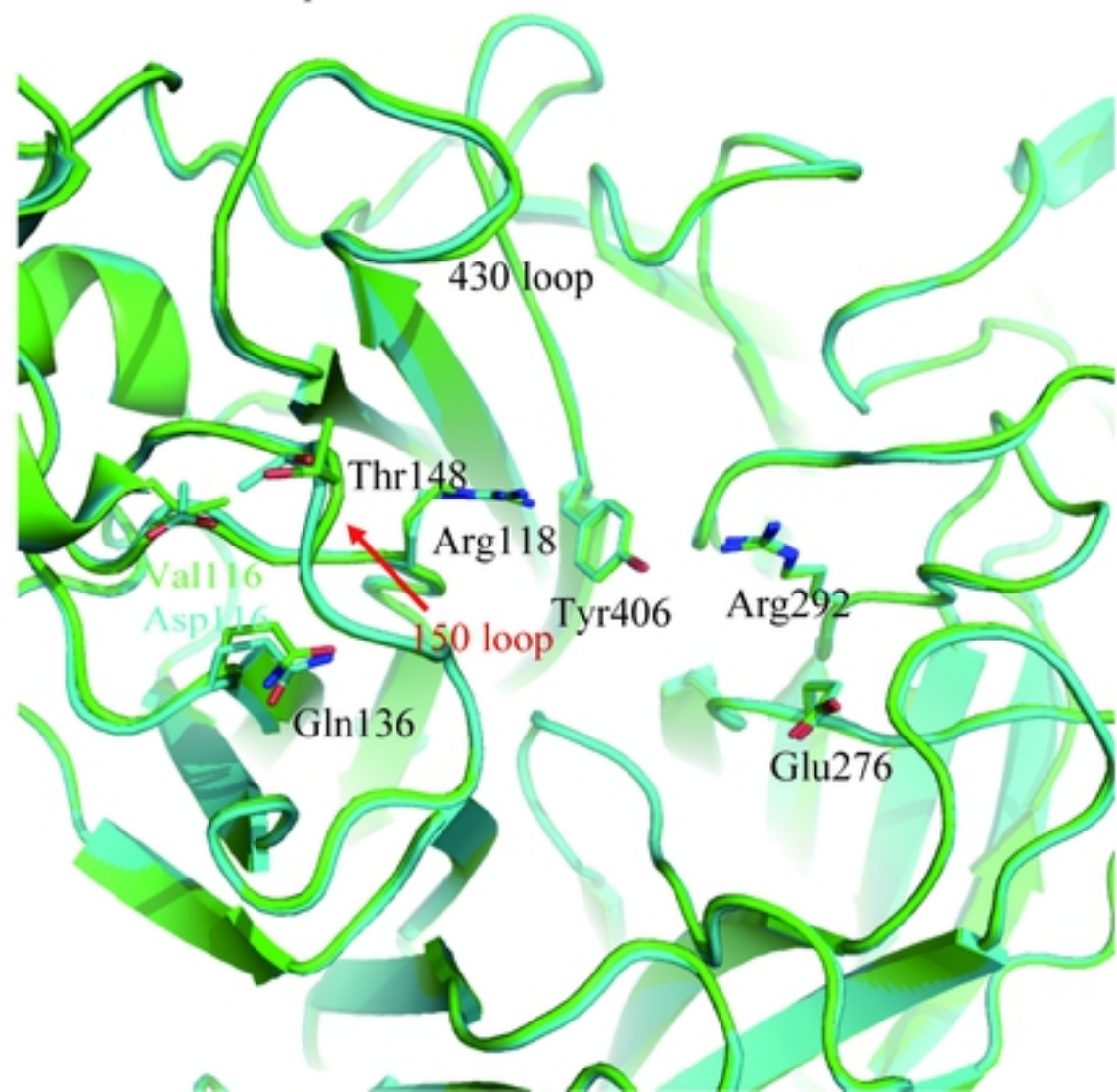


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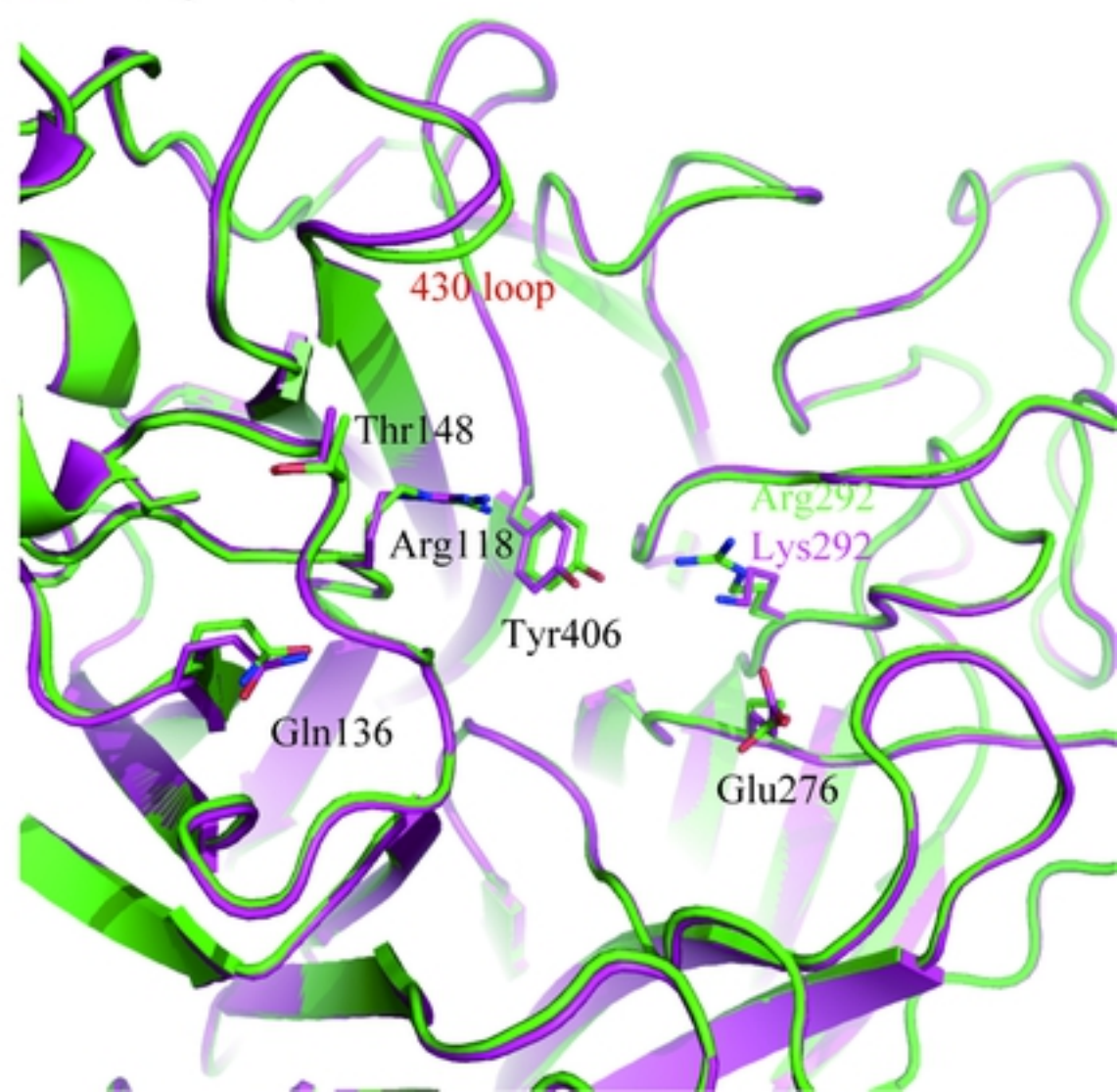
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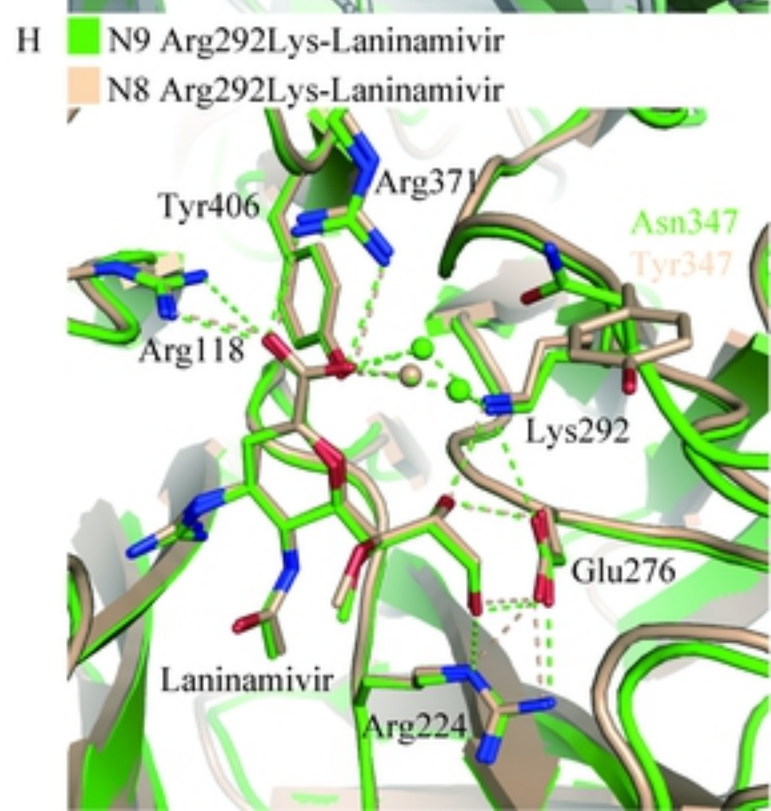
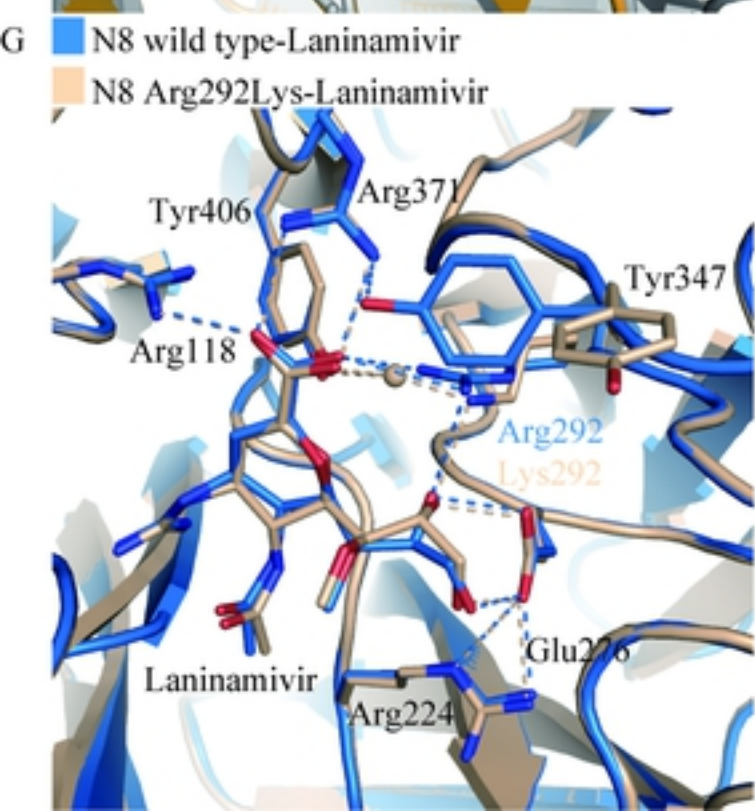
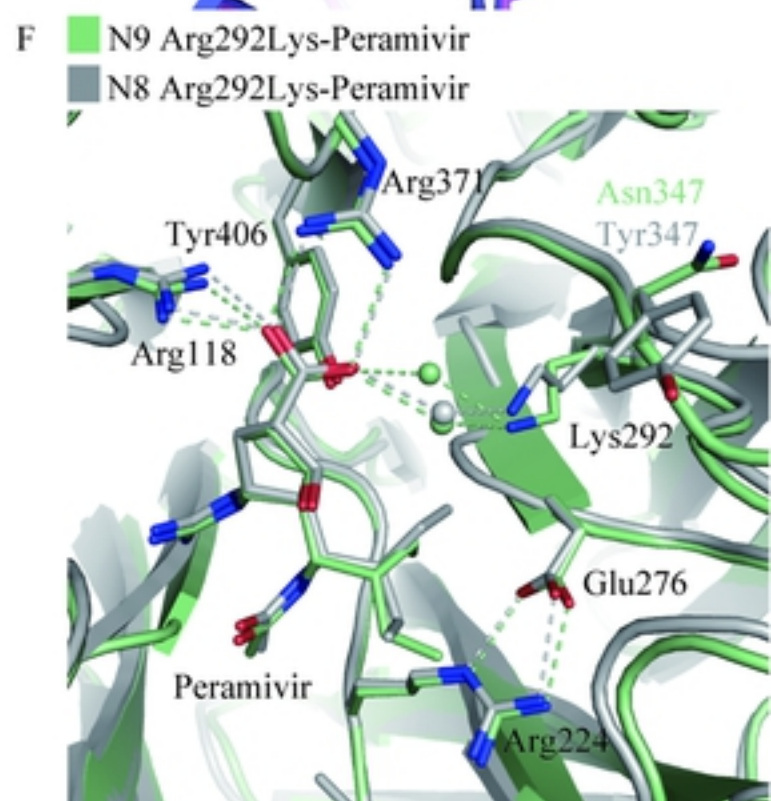
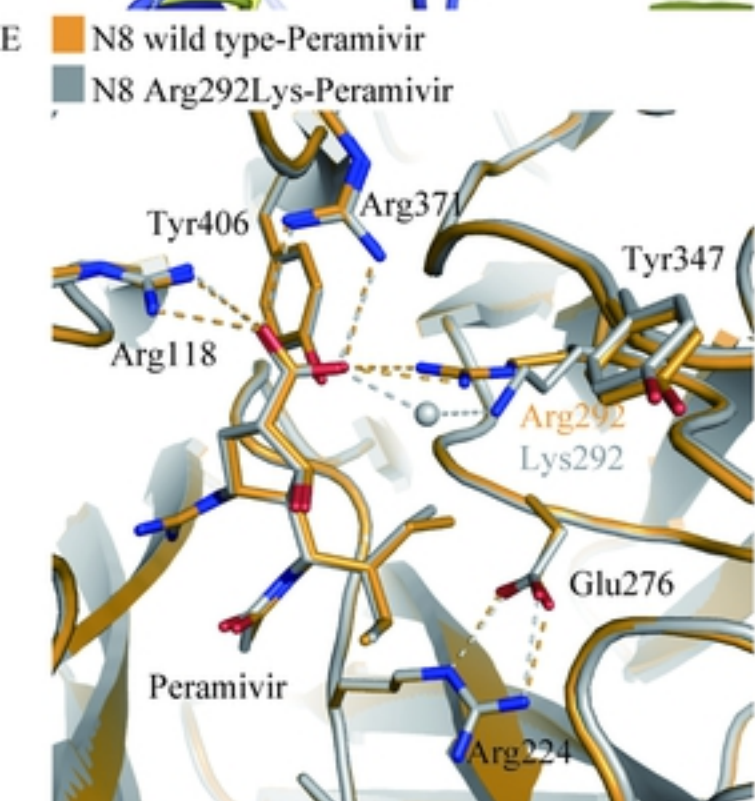
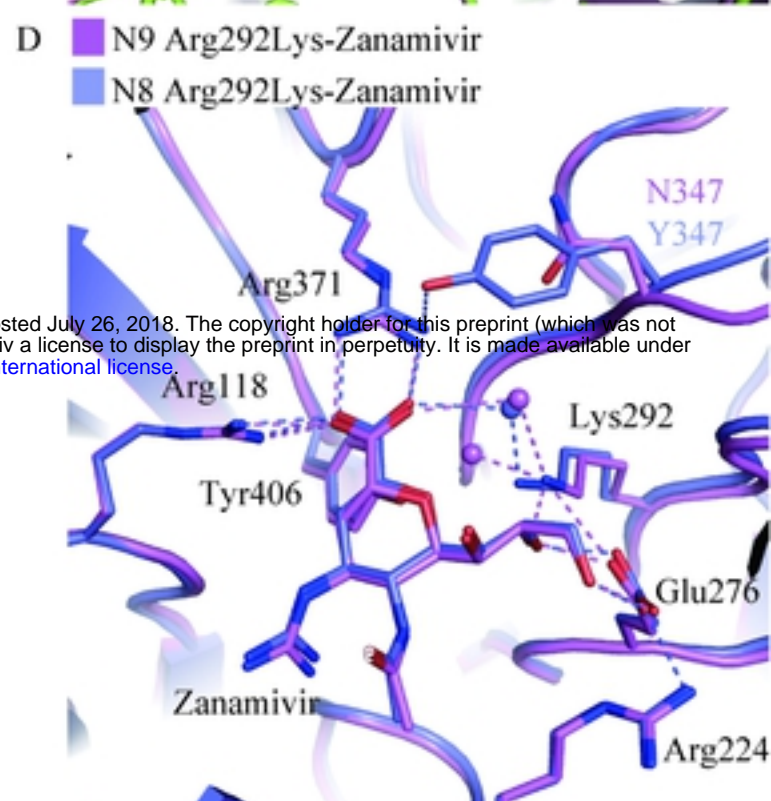
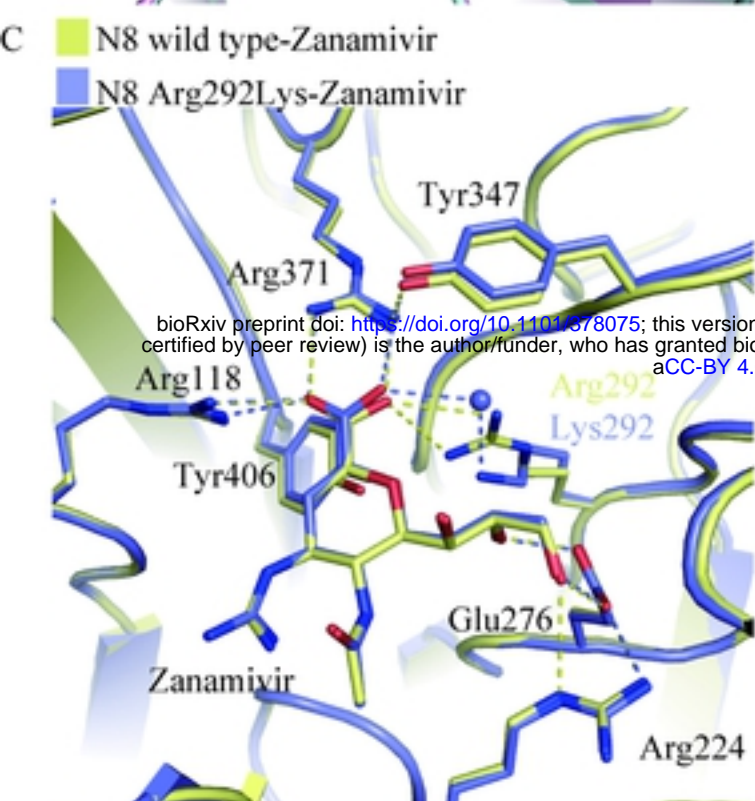
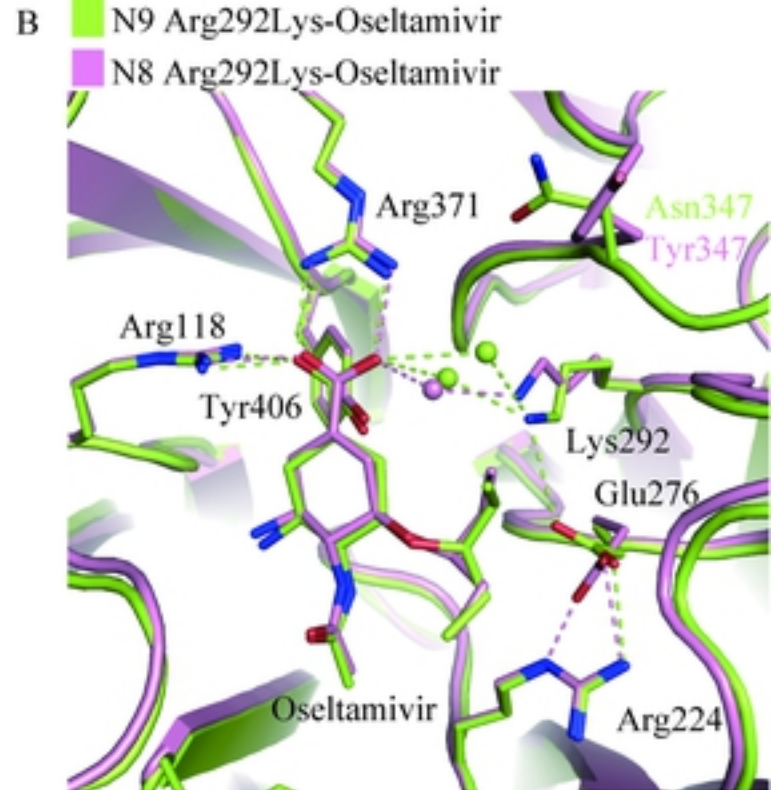
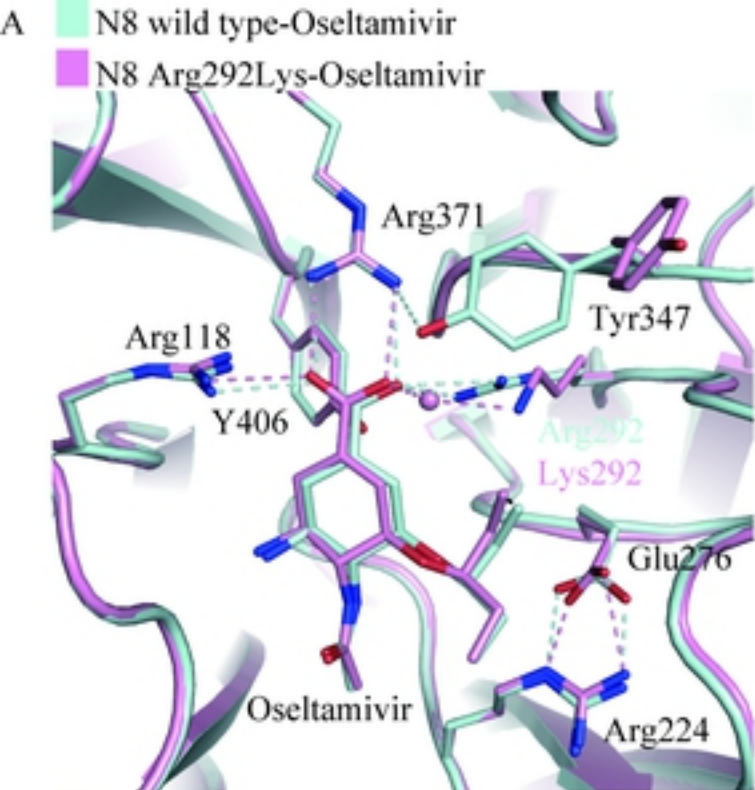
- N8 wild type
- N8 Val116Asp



B

- N8 wild type
- N8 Arg292Lys





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