Identification of a novel multiple kinase inhibitor with potent antiviral activity against influenza virus by reducing viral polymerase activity

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18 **ABSTRACT**

19 Neuraminidase inhibitors are the only currently available influenza treatment, although
20 resistant viruses to these drugs have already been reported. Thus, new antiviral drugs
21 with novel mechanisms of action are urgently required. In this study, we identified a
22 novel antiviral compound, WV970, through cell-based screening of a 50,000 compound
23 library and subsequent lead optimization. This compound exhibited potent antiviral
24 activity with nanomolar IC₅₀ values against both influenza A and B viruses but not
25 non-influenza RNA viruses. Time-of-addition and indirect immunofluorescence assays
26 indicated that WV970 acted at an early stage of the influenza life cycle, but likely after
27 nuclear entry of viral ribonucleoprotein (vRNP). Further analyses of viral RNA
28 expression and viral polymerase activity indicated that WV970 inhibited
29 vRNP-mediated viral genome replication and transcription. Finally, structure-based
30 virtual screening and comprehensive human kinome screening were used to demonstrate
31 that WV970 acts as a multiple kinase inhibitor, many of which are associated with
32 influenza virus replication. Collectively, these results strongly suggest that WV970 is a
33 promising anti-influenza drug candidate and that several kinases associated with viral
34 replication are promising drug targets.

35 **1. Introduction**

36 Seasonal influenza infection causes large scale economic burdens, and pandemics
37 such as those caused by the highly pathogenic avian influenza A subtypes, including
38 H5N1 and H7N7 could result in millions of deaths [1,2]. Thus, anti-influenza drugs
39 have been developed for prevention and treatment of influenza infections. Two classes
40 of anti-influenza drugs are currently approved for clinical use, M2 channel blockers and
41 neuraminidase (NA) inhibitors [3]. However, the clinical uses of M2 channel blockers
42 are limited since all currently circulating influenza A virus strains are resistant to these
43 drugs [4]. Moreover, the emergence of NA inhibitor-resistant viruses has also been
44 reported [4,5]. If NA inhibitor-resistant viruses become more widespread then there will
45 be few effective means of treating influenza infections. Therefore, new antivirals based
46 on novel mechanisms of action are urgently needed.

47 One potential approach is to target host factors involved in influenza replication,
48 which may provide a novel antiviral strategy to counteract viral drug resistance. Recent
49 reports revealed that a large number of host factors are involved in influenza replication
50 and that the majority of these are kinases [6]. Indeed, several kinase inhibitors including
51 Ca²⁺/calmodulin-dependent protein kinase (CAMK), protein kinase C (PKC),
52 phosphoinositide 3-kinase (PI3K), receptor tyrosine kinases (RTKs) and MAPK/ERK

53 kinase (MEK) exhibit potent anti-influenza activity [7].

54 Structure-based virtual screening is one of the most convenient and promising
55 approaches for identifying potential binding partners for test compounds, and can
56 provide targets that have a high likelihood of binding to the query compound [8]. In
57 addition, a comprehensive human kinome screening system (KINOMEScan) was used
58 to provide quantitative data on binding selectivity between test compounds and more
59 than 450 human kinases [9]. In this study, we identified a novel antiviral compound,
60 WV970, using a cell-based influenza A infection assay and optimized the compound by
61 assessing structure-activity relationships (SAR). The mechanism of action was further
62 characterized, wherein WV970 behaved as a multiple kinase inhibitor.

63

64 **2. Materials and Methods**

65 *2.1. Compounds*

66 The chemical library for cell-based screening was purchased from ChemBridge, and
67 hit compound RK188 was purchased from Sundia MediTech. WV635 and WV970 were
68 synthesized by Wakunaga Pharmaceuticals and a stock solution of 10 mM was prepared
69 in dimethyl sulfoxide (DMSO). Oseltamivir calboxylate and nucleozin were purchased
70 from Kemprotec and Shanghai Haoyuan Chemexpress, respectively.

71

72 *2.2. Cells and viruses*

73 Madin-Darby canine kidney (MDCK) cells, Vero cells and HEK293T cells were
74 grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing
75 penicillin, streptomycin, and glutamine (PSG, GIBCO Industries Inc.) and 10% fetal
76 bovine serum (Sigma-Aldrich). Influenza A viruses, including A/WSN/1933 (H1N1),
77 A/Udorn/307/1972 (H3N2), A/ck/Yamaguchi/7/2004 (H5N1), and A/Anhui/1/2013
78 (H7N9), and influenza B viruses, including B/Yamagata/16/88 and B/Nagasaki/1/87,
79 were propagated in MDCK cells. Japanese encephalitis virus (JEV) (Mie/41/2002
80 strain) and dengue virus type I (NIID 02-20) were propagated in Vero cells.

81

82 *2.3. Random screening of compounds with MDCK cell-based influenza A infection* 83 *assays*

84 MDCK cells (3×10^4 cells/well) were seeded in 96-well plates and incubated at 37°C
85 in 5% CO₂ overnight with DMEM containing 10 µM library compounds, and then
86 inoculated with influenza A virus (A/WSN/1933) at a multiplicity of infection (MOI) of
87 0.005 with 1 µg/ml of tosylsulfonyl henylalanyl chloromethyl ketone-treated trypsin
88 (Trypsin-TPCK, Worthington Biochemical Corporation). After a 48 h incubation, the

89 cells were washed twice with phosphate-buffered saline (PBS), and 10 μ l of WST-1
90 reagent (Takara Bio) was added into each well. The plate was incubated at 37°C for 1 h,
91 and absorbance was measured at 450 nm. Inhibition (%) of virus-induced cytopathic
92 effects (CPEs) was evaluated by comparing test compound-treated cells to
93 DMSO-treated cells.

94

95 *2.4. Plaque assays of antiviral activity*

96 Plaque assays for influenza viruses were performed as previously described [10]. For
97 plaque assays with JEV and dengue virus, Vero cells (6×10^5 cells/well) were seeded in
98 6-well plates and incubated at 37°C in 5% CO₂ for 24 h. Cells were washed once with
99 PBS and inoculated with approximately 200 plaque forming units (PFU) of JEV or
100 dengue virus. After incubating for 1 h at 37°C in 5% CO₂, 2 ml of DMEM containing
101 1% methylcellulose was overlaid onto each well. Cells were incubated at 37°C in 5%
102 CO₂ for 5 days after JEV inoculation or 7 days after dengue virus inoculation. After the
103 removal of DMEM containing methylcellulose, cells were fixed with 3.7%
104 formaldehyde and stained with 0.1% crystal violet. The 50% inhibitory concentration
105 (IC₅₀) was then evaluated by counting the number of plaques.

106

107 *2.5. Cell toxicity*

108 Cell toxicity was measured as previously described [10] using a WST-1 assay. The
109 50% cytotoxic concentration (CC₅₀) was evaluated by comparing test compound-treated
110 cells to DMSO-treated cells.

111

112 *2.6. Indirect immunofluorescence*

113 Indirect immunofluorescence was performed as described previously [11] with a
114 primary anti-NP monoclonal antibody (MAb) (Santa Cruz Biotechnology) and
115 subsequent secondary Alexa Fluor 488-conjugated rabbit anti-mouse IgG (Invitrogen).
116 Samples were then incubated with PBS containing Hoechst 33342 (ImmunoChemistry
117 Technologies LLC) for nuclear staining. Prepared samples were observed under a
118 confocal laser-scanning microscope (FV 1000, Olympus).

119

120 *2.7. RNA fluorescence in situ hybridization (FISH) analysis*

121 RNA FISH was performed according to published protocols [12]. Probes targeting the
122 PB2 segment of the influenza virus were designed by Stellaris RNA FISH Probe
123 Designer and purchased from Biosearch Technologies. Prepared samples were observed
124 under a confocal laser-scanning microscope (FV 1000, Olympus).

125

126 *2.8. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)*

127 RNA was extracted from MDCK cells (1.2×10^6 cells/well) infected with

128 A/WSN/1933 at an MOI of 10 using TRIzol® LS Reagent (Invitrogen).

129 RT-PCR was performed using SuperScript™ III First-Strand Synthesis System

130 (Invitrogen) according to the manufacturer's instructions and specific primers for the

131 following three targets using a previously described protocol [13]: viral genome RNA

132 (vRNA) NP (F),

133 5'-GGCCGTCATGGTGGCGAATGAATGGACGGAGAACAAGGATTGC-3'; viral

134 mRNA NP (R),

135 5'-CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTCTTTAATTGTC-3'; and

136 mRNA MDCK actin (R), 5' -GGAACCGCTCGTTGCCAATG-3'.

137 For qPCR, each cDNA was used with 2×SYBR GreenER qPCR SuperMix

138 (Invitrogen) and ABI PRISM, according to the manufacturer's instructions. The specific

139 primers used are as follows: vRNA NP (F), 5'-GGCCGTCATGGTGGCGAAT-3'; vRNA

140 NP (R), 5'-CTCAATATGAGTGCAGACCGTGCT-3'; viral mRNA NP (F),

141 5'-CGATCGTGCCCTCCTTTG-3'; viral mRNA NP (R),

142 5'-CCAGATCGTTCGAGTCGT-3'; mRNA MDCK actin (F),

143 5'-CGTGCGTGACATCAAGGAAGAAG- 3'; and mRNA MDCK actin (R),
144 5'-GGAACCGCTCGTTGCCAATG-3'. Data were analyzed using the $\Delta\Delta CT$ method.
145 MDCK actin was used as a control.

146

147 2.9. *Mini-genome assays*

148 Mini-genome assays were performed using the expression plasmids PB1/pCAGGS,
149 PB2/pCAGGS, PA/pCAGGS, NP/pCAGGS and vNP-luc/pHH21, as described
150 previously [10].

151

152 2.10. *Western blot analysis*

153 Western blot analysis was performed with an anti-WSN polyclonal antibody (Ab) and
154 HRP-conjugated goat anti-rabbit IgG (Amersham Bioscience) or anti- β -actin MAb
155 (Sigma-Aldrich) and HRP-conjugated goat anti-mouse IgG (Amersham Bioscience), as
156 described previously [11].

157

158 2.11. *LASSO (Ligand Activity by Surface Similarity Order) screening with the* 159 *ChemSpider database*

160 Target molecule searches using the structure-based virtual screening database,

161 ChemSpider (Royal Society of Chemistry), were conducted to identify potential binding
162 partners for test compounds. LASSO scores ranges from 0 to 1.0, where a score of 0
163 represents a likely absence of binding and 1.0 represents the highest likelihood of
164 binding to the target molecule [8].

165

166 2.12. KINOMEScan

167 KINOMEScan (DiscoverX) employs a competition binding assay to quantitatively
168 measure interactions between a test compound and more than 450 human kinases [9].

169 After the competition reaction, the amount of kinase bound to the immobilized ligand is
170 determined using quantitative PCR of a DNA tag conjugated to the kinase. Data are
171 represented as a percentage of control (% Ctrl) where 100% indicates that the test
172 compound did not inhibit kinase activity and lower numbers indicate stronger hits.

173 KINOMEScan technology was used to screen WV970 (50 nM) against 456 kinases
174 according to the protocol above.

175

176 3. Results

177 3.1. Screening of antiviral compounds and identification of WV970

178 To identify novel compounds targeting the influenza virus, we screened 50,000
179 compounds using an MDCK cell-based influenza infection assay. This assay identifies

180 compounds according to their ability to protect against influenza virus-induced CPEs.

181 Forty seven primary hits were identified. Plaque assays were then used as a secondary

182 screen to evaluate the IC_{50} of these compounds. Four hit compounds showed an $IC_{50} \leq$

183 $3 \mu M$ in the plaque assays. Notably, RK188 was a racemic mixture (with an IC_{50} of 2.2

184 $\pm 0.25 \mu M$) resulting from two chiral carbons at the C_7 and C_{14} positions (Fig. 1A). Next,

185 we evaluated four compounds with different configurations ('*R*' or '*S*') that were

186 stereoselectively synthesized at the C_7 and C_{14} positions of RK188. Among the four

187 stereoisomers, WV635, which has the '*S*' configuration at both the C_7 and C_{14} positions,

188 exhibited the most potent antiviral activity with an IC_{50} of $0.53 \pm 0.05 \mu M$ against the

189 A/WSN/1933 strain. Based on a SAR study of the lead compound WV635, we obtained

190 WV970. This compound was the most potent (with an IC_{50} of $0.015 \pm 0.060 \mu M$) of

191 approximately 160 derivatives that were evaluated against the A/WSN/1933 strain. Its

192 antiviral activity was similar to that of oseltamivir carboxylate ($IC_{50} = 0.012 \pm 0.004$

193 μM), which is a currently available antiviral drug, and its cytotoxic effects were lower

194 than its antiviral effects ($CC_{50} = 3.8 \pm 4.5 \mu M$, $SI = 253.3$). Moreover, WV970 exhibited

195 potent antiviral activity against seasonal viral strains, including influenza A strain,

196 A/Udorn/307/1972 (H3N2) and influenza B strains, B/Yamagata/16/88 and

197 B/Nagasaki/1/87, in addition to the highly pathogenic avian influenza viral strains

198 A/ck/Yamaguchi/7/2004 (H5N1) and A/Anhui/1/2013 (H7N9) (Fig. 1B). Interestingly,
199 WV970 had no antiviral effects against non-influenza RNA viruses, such as JEV and
200 dengue virus (Fig.1 B), suggesting that WV970 may target an influenza virus-specific
201 replication mechanism.

202

203 *3.2. WV970 acts at an early stage of the viral life cycle*

204 To determine the mechanisms by which WV970 acts during the influenza virus life
205 cycle, a time-of-addition experiment was conducted following the scheme illustrated in
206 Fig. 2A upper panel. WV970 inhibited viral production by up to approximately 20–50%
207 when added to infected cells at an early time point (up to 4 h post-infection; Fig. 2A,
208 lower panel). By contrast, WV970 had no inhibitory effects on viral production when
209 added 4 h post-infection or later. This indicates that WV970 acts at an early stage of
210 influenza virus life cycle unlike the oseltamivir carboxylate that inhibits the late step of
211 the life cycle by blocking viral NA activity.

212 To further examine this hypothesis, we first explored the effect of WV970 on the
213 nuclear entry of NP, which is the major component of viral ribonucleoprotein (vRNP:
214 the complex with one copy of vRNA and a trimeric RNA polymerase (PB1, PB2 and
215 PA) and multiple copies of NP) and can be detected in the nucleus of infected cells

216 within 1 h post-infection [14]. At 1.5 h post-infection, NP accumulated in the nucleus
217 and no differences were observed in DMSO- and WV970-treated cells, indicating that
218 WV970 might act at a later stage than nuclear entry (Fig. 2B, upper panel). Next, we
219 examined NP localization at 5 h post-infection when viral replication and transcription
220 has begun and the vRNP reconstructed by newly synthesized viral proteins is exported
221 to cytoplasm from the nucleus [15]. In DMSO-treated cells, NP was localized
222 predominantly in the cytoplasm, with lesser amounts in the nucleus. Interestingly, in
223 WV970-treated cells, NP was observed in the cytoplasm but expression levels decreased
224 dose-dependently (Fig. 2B, lower panel). These results suggest that WV970 might
225 inhibit vRNP activity, which regulates viral genome replication and transcription.

226

227 *3.3 WV970-mediated inhibition of the viral genome replication, transcription and* 228 *protein expression*

229 To examine the effects of WV970 on vRNP activity, we performed FISH analysis
230 using Cy5-labeled DNA oligos targeted against vRNA encoding the PB2 protein (Fig.
231 3A). In DMSO-treated cells, vRNA expression increased in the nucleus and cytoplasm
232 of infected cells in a time-dependent manner. By contrast, a significant decrease in
233 vRNA expression was observed in WV970-treated cells.

234 To examine whether WV970 also affects viral genome transcription, we assessed
235 vRNA and viral mRNA expression levels by qRT-PCR analysis. In WV970-treated cells,
236 vRNA and mRNA expression levels were significantly lower (at both 5 h and 10 h
237 post-infection) than those of DMSO-treated cells. Similar results were obtained with
238 nucleozin-treated cells, as nucleozin is an NP inhibitor used as a positive control for
239 blocking of viral transcription and replication [16]. By contrast, oseltamivir carboxylate
240 had no significant effect (Fig. 3B).

241 To further investigate whether WV970 inhibits vRNP activity, we conducted a
242 mini-genome assay using four viral protein expression plasmids (pCAGGS) encoding
243 PB1, PB2, PA and NP, and the viral-like genome expression plasmid NP-luc/pPoll
244 encoding firefly luciferase. WV970, but not oseltamivir carboxylate or DMSO,
245 decreased luciferase activity in a dose-dependent manner (Fig. 3C).

246 Finally, we examined the effect of WV970 on the subsequent viral protein expression
247 (Fig. 3D). Western blot analysis showed that WV970 inhibited expression of HA, NP,
248 NA and M1 when compared to that of oseltamivir carboxylate and DMSO.

249 Collectively, these results indicate that WV970 inhibits vRNP-mediated viral genome
250 replication and transcription.

251

252 4.4 Target protein searches and comprehensive kinome screening for WV970

253 Since we could not isolate WV970 resistant viruses after several passages of viral
254 propagation under selection pressure for WV970 (data not shown), we hypothesized that
255 WV970 may be involved in the inhibition of an essential host factor for virus replication
256 and not viral components per se. Thus, we conducted a target search with a
257 structure-based virtual screening database [8] and several kinase molecules were
258 identified with high LASSO scores when the chemical structure of WV635 was used as
259 a query. These results suggest that WV970, which is a derivative compound of WV635,
260 may also target a kinase even though no target molecules were identified with WV970
261 (Fig. 4A). To confirm this hypothesis, we examined the effect of WV970 on kinase
262 inhibition. It is well known that most kinase inhibitors interact with a wide range of
263 kinases with different activities [17]. To examine the binding affinity of WV970 against
264 a large number of kinases, we conducted KINOMEScan screening, which is based on an
265 ATP site competition binding assay [9]. WV970 was screened at a concentration of 50
266 nM against 456 kinases, covering more than 80% of human protein kinases. WV970
267 exhibited inhibitory effects (lower than 50% of Ctrl, which indicates a greater than 50%
268 displacement from an immobilized target kinase) against only 15 kinases. These results
269 are consistent with the hypothesis above (based on the structure-based virtual screening)

270 that WV970 may target a kinase and indicate that WV970 acts as a multiple kinase
271 inhibitor with a potent antiviral activity.

272

273 **4. Discussion**

274 In this study, we identified a novel anti-influenza compound, WV970, that inhibited
275 RNP-mediated viral genome replication and transcription, thereby inhibiting viral
276 replication of several influenza A and B virus strains, including the highly pathogenic
277 avian influenza virus strains, A/ck/Yamaguchi/7/2004 (H5N1) and A/Anhui/1/2013
278 (H7N9). Further analyses, using structure-based virtual screening and comprehensive
279 human kinome screening, revealed that WV970 selectively binds to 15 kinases. Several
280 genome-wide RNAi screens have indicated that numerous host factors are required for
281 influenza A virus replication and that the majority of these are kinases [6]. Previous
282 reports demonstrate that various kinase inhibitors can suppress influenza A virus
283 replication [7]. For example, PI3K inhibitors reduce influenza A virus replication by
284 blocking vRNP activity [7]. In regard to our results, nine of the KINOMEscan hits have
285 been reported to have the possibility of regulating influenza A virus replication [6].
286 Notably, five of these, DCLK1, EPHB2, JAK2, OXSR1 and HIPK2, have been reported
287 to regulate the early stages of the influenza A virus life cycle, including viral genome

288 replication and transcription [6]. In addition, some kinase inhibitors, such as MEK and
289 PKC inhibitors interfere with both influenza A and B viruses [7]. Interestingly, WV970
290 did not exhibit antiviral effect against non-influenza RNA viruses. These findings
291 indicate that WV970 may specifically inhibit viral genome replication and transcription
292 of both influenza A and B viruses via inhibition of these kinase activities. However, to
293 confirm this hypothesis, further investigations are required to define the detailed
294 mechanisms by which these kinases regulate viral replication. Although the remaining
295 six kinases identified, DSTYK, MAP3K1, SBK1, ULK1, IRAK4 and INSR, have not
296 been reported to play a role in influenza replication [6], they may still influence this
297 process, although further studies are required to confirm this possibility.

298 In addition to host factor effects, vRNP polymerase activity is regulated by its
299 component viral proteins, including PB1, PB2, PA and NP. Apart from their
300 fundamental protein functions such as cap-snatching and endonuclease activity [18],
301 phosphoproteome analysis has demonstrated that phosphorylation of these proteins may
302 also regulate viral replication, although the functional roles of this phosphorylation are
303 not fully understood [19]. Notably, NP contains a number of potential phosphorylation
304 sites, and phosphorylation of S165 is essential for genome replication and
305 transcriptional activity [19], which indicates the importance of viral protein

306 phosphorylation for vRNP activity. These results also strongly suggest that kinases play
307 an essential role in influenza replication and that kinase inhibition is a promising drug
308 target.

309 When targeting host factors as drug targets, drug-induced cytotoxic effects are a
310 major concern because host functions may also be affected. However, viral polymerase
311 activity was far more sensitive to WV970 than host factor activity, which thereby results
312 in limited cytotoxicity. Thus, WV970 is a novel antiviral drug candidate with potential
313 for further development.

314 In conclusion, WV970 acts as a multiple kinase inhibitor and exhibits potent antiviral
315 effects against influenza virus through vRNP inhibition. Further studies are required to
316 investigate the mechanisms of WV970-mediated inhibition of viral replication.
317 Moreover, WV970 shows promise as a novel antiviral drug.

318

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330

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386

387 **Figure legends**

388

389 **Fig. 1.** The effect of WV970 on viral replication and cell viability. (A) Chemical
390 structures of hit, lead and optimized compounds and their 50% inhibitory concentration
391 (IC_{50}) against influenza A/WSN/1933 (H1N1) virus and 50% cytotoxic concentration
392 (CC_{50}) against MDCK cells. (B) IC_{50} and CC_{50} values of WV970 and oseltamivir
393 carboxylate against the indicated influenza A and B virus strains, non-influenza viruses
394 or cell line. The IC_{50} of each compound was evaluated by plaque assay and the CC_{50} of
395 each compound was evaluated by WST-1 assay in the absence or presence of the test
396 compound (0–30 μ M). Oseltamivir carboxylate was used as a positive control. Values
397 represent the mean $\pm$ SD of three independent experiments. A Selectivity Index (SI) was
398 calculated from the CC_{50} to IC_{50} ratio. (-) not tested.

399

400 **Fig. 2.** Stage of the viral life cycle targeted by WV970. (A) WV970 was added at the
401 indicated time points into MDCK cells preliminarily inoculated with A/WSN/1933
402 viruses at a multiplicity of infection (MOI) of 1 and cell supernatants were collected 11
403 h post-infection. The viral titer of collected supernatants was estimated by the
404 hemagglutinin (HA) assay. (B) MDCK cells were infected with A/WSN/1933 at an MOI
405 of 10 for 1.5 h, or MOI of 5 for 5 h in the absence or presence of WV970.

406 Immunofluorescence staining was performed with an anti-NP monoclonal antibody
407 (MAb) followed by anti-mouse Alexa Fluor 488 and Hoechst 333342. Cells were then
408 observed under a confocal laser-scanning microscope.

409

410 **Fig. 3.** Effects of WV970 on viral transcription, replication and protein expression. (A)

411 MDCK cells were infected with A/WSN/1933 virus at an MOI of 5 in the absence or

412 presence of 0.1 μ M WV970, and fixed at 0, 2, 4 and 6 h post-infection. Cells were

413 probed for PB2 vRNA using Cy5-labeled probes targeting the PB2 segment and then

414 observed under a confocal laser-scanning microscope. (B) MDCK cells were infected

415 with A/WSN/33 virus at an MOI of 10 in the absence or presence of test compounds. Os

416 and Nuc represent oseltamivir carboxylate and nucleozin, respectively. After 5 h or 10 h

417 post-infection, cell lysates were harvested and qRT-PCR was performed to detect NP

418 vRNA and NP mRNA. The ratio of gene expression to its respective internal control

419 (actin) was normalized to the control (DMSO) sample. Values represent the mean $\pm$ SD

420 of three independent experiments. (C) HEK-293T cells were transfected with pCAGGS

421 encoding PB1, PB2, PA and NP, and the viral-like genome expression plasmid,

422 NP-luc/pol, in the absence or presence of test compounds. After 48 h, relative luciferase

423 activity was measured. Values represent the mean $\pm$ SD of three independent

424 experiments. (D) MDCK cells were infected with A/WSN/1933 virus at a MOI of 10 in
425 the absence or presence of test compounds. Cell lysates were harvested 18 h
426 post-infection, and Western blot analysis was performed to detect viral protein
427 expression using an anti-WSN polyclonal antibody (Ab) and an anti- β -actin MAb. NP,
428 HA, M1, and M2 proteins are shown. β -actin was used as a loading control.

429

430 **Fig. 4.** Target search for WV635 and the inhibitory effects of WV970 in a kinase library.

431 (A) A target search was performed with the structure-based virtual screening database,
432 ChemSpider, using the structure of the lead compound WV635 as a query. LASSO
433 scores assessing the likelihood of the target (category and PDB code are shown) binding
434 to the query compound are shown in the left panel. (B) Dendrogram (left panel) and
435 table (right panel) of the human kinome demonstrating a kinase selectivity of 50 nM for
436 WV970 against a panel of 456 human kinases (KINOMEScan gene symbol and enzyme
437 gene symbol are listed in the table) including eight assay groups. Targets hit are colored
438 blue in the dendrogram. The dendrogram was generated using TREEspot software with
439 50% control (% Ctrl) used as a cutoff.

440

Figure 1

A

Hit compound
(racemic mixture)

RK188

$IC_{50}=2.2\pm0.25\ \mu M$

$CC_{50}>30\ \mu M$

Lead compound

WV635

$IC_{50}=0.53\pm0.05\ \mu M$

$CC_{50}=9.8\pm0.28\ \mu M$

Optimized compound

WV970

$IC_{50}=0.015\pm0.060\ \mu M$

$CC_{50}=3.8+4.50\ \mu M$

B

Host cell or Virus strain	WV970			Oseltamivir carboxylate	
	$CC_{50}\ (\mu M)$	$IC_{50}\ (\mu M)$	SI	$CC_{50}\ (\mu M)$	$IC_{50}\ (\mu M)$
MDCK	3.8 ± 4.5	-	-	>30	-
A/WSN/1933(H1N1)	-	0.015 ± 0.060	253.3	-	0.012 ± 0.004
A/Udorn/307/1972 (H3N2)	-	0.024 ± 0.031	158.3	-	-
A/Ck/yamaguchi /7/2004(H5N1)	-	0.240 ± 0.100	15.8	-	0.570 ± 0.040
A/Anhui/1/2013(H7N9)	-	0.230 ± 0.180	16.5	-	0.600 ± 0.120
B/Yamagata/16/88	-	0.058 ± 0.014	65.5	-	0.098 ± 0.003
B/Nagasaki/1/87	-	0.020 ± 0.030	190.0	-	0.019 ± 0.004
Japanese encephalitis virus Mie/41/2002 strain	-	>30	-	-	-
Dengue virus type 1 NID 02-20 strain	-	>30	-	-	-

Figure 2

Figure 3

Figure 4

A

1-[(7,8-Dimethyl-2-oxo-1,2-dihydro-3-quinolinyl)methyl]-3-(1-phenylethyl)-1-(tetrahydro-2-furanylmethyl)thiourea

Category	Target	PDB Code	LASSO Score
Kinases	SRC, tyrosine kinase SRC	2src	0.81
Kinases	PDGFrb, platelet derived growth factor receptor kinase	N/A	0.7
Nuclear Hormone Receptors	GR, glucocorticoid receptor	1m2z	0.6
Kinases	FGFr1, fibroblast growth factor receptor kinase	1agw	0.39
Kinases	CDK2, cyclindependent kinase 2	1ckp	0.21

B

KINOMEScan Gene Symbol	Entrez Gene Symbol	Assay Group	%Ctrl
RSK2(Kin.Dom.2-C-terminal)	RPS6KA3	CAMK	22
RIPK5	DSTYK	OTHER	29
DCAMKL1	DCLK1	CAMK	30
EPHB2	EPHB2	TK	36
JAK2(JH1domain-catalytic)	JAK2	TK	39
LYN	LYN	TK	40
OSR1	OXSRI	STE	40
MAP3K1	MAP3K1	STE	42
NDR1	STK38	AGC	43
SBK1	SBK1	OTHER	43
ULK1	ULK1	OTHER	43
IRAK4	IRAK4	TKL	47
INSR	INSR	TK	48
TYK2(JH1domain-catalytic)	TYK2	TK	48
HIPK2	HIPK2	CMGC	49