1   *Ex Vivo* Validation of Six FDA-Approved Non-Receptor Tyrosine Kinase

2   Inhibitors (NRTKIs) as Antivirals to Pandemic and Seasonal Influenza A

3   Viruses

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17   **Running Title: NRTKIs are effective influenza antivirals**

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ABSTRACT

Influenza viruses are important respiratory pathogens that cause substantial morbidity and mortality annually. In addition to seasonal influenza outbreaks, new emerging influenza A viruses (IAV) can cause pandemic influenza outbreaks. Apart from effective vaccines, there is a need for better treatment options to combat infections with these viruses when vaccines are not available or show reduced efficacy (e.g., in immmunocompromised patients). The limited range of licensed antiviral drugs and emergence of drug-resistance mutations highlight the need for novel intervention strategies like host-targeted antivirals. Repurposing FDA-approved kinase inhibitors may offer a fast-track for a new generation of host-targeted antivirals. Small molecule kinase inhibitors (SMKIs) can inhibit replication of viruses and improve survival *in vivo*; however, no SMKI has been approved for clinical use against IAV infections. In the present study, we tested eight non-receptor tyrosine kinase-inhibitors (NRTKIs) used to treat cancer and autoimmune diseases for their antiviral potential. Six of those potently inhibited virus replication ($\geq$1,000-fold) in A549 cells infected with either A(H1N1)pdm09 or seasonal A(H3N2) strains. These compounds were validated in a biologically relevant *ex vivo* model of human precision-cut lung slices (hPCLS) to provide proof of principle and show efficacy against contemporary seasonal and pandemic IAVs. We identified the steps of the virus infection cycle affected by these inhibitors and assessed the effect of these NRTKIs on the host response. Considering their established safety profiles, our studies show that the use of these NRTKI shows promise and warrants further development as an alternative strategy to treat influenza virus infections.

INTRODUCTION

Influenza viruses are an important cause of respiratory tract infections in humans and are responsible for substantial annual morbidity and mortality, especially in individuals at high risk, like older adults or immunocompromised patients. The most important preventive measure to protect humans from influenza virus infections is vaccination. Currently used vaccines mainly aim at the induction of virus neutralizing antibodies to the viral hemagglutinin (HA). For vaccines to be effective they need to antigenically match the circulating strains. [1-3]. Due to a lack of proof-reading activity of their RNA-dependent RNA polymerase (RdRp), influenza viruses can accumulate mutations that lead to antigenic drift and allow them to evade recognition by virus-neutralizing (VN) antibodies induced by previous infections or vaccinations. These antigenic changes also necessitate annual update of influenza vaccines against seasonal influenza [4]. Moreover, emergence of novel influenza viruses that arise through genetic reassortment after interspecies transmission. Emergence of these viruses in human populations that largely lack VN antibodies can result in pandemic outbreaks. At the early stages of a pandemic, effective vaccines are not readily available, as was the case during the pandemic of 2009 caused by swine-origin A(H1N1) influenza A viruses (IAVs).

In the absence of efficacious vaccines, virus-targeted antivirals can offer some protection if administered within the therapeutic window. Until recently, influenza antivirals were comprised of two classes, Adamantanes that target the viral M2 ion channel protein and neuraminidase inhibitors (NAI). Due to almost ubiquitous resistance found in currently circulating strains, Adamantanes have been rendered ineffective and are no longer used in clinical practice [5]. The levels of currently circulating viruses that carry resistance

70   mutations to NAIs (such as oseltamivir) isolated from otherwise healthy individuals is only

71   ~4-5% [6]. A more recent strategy has been to target the influenza polymerase proteins,

72   with Favipiravir (T705), Baloxavir and Pimodivir targeting the viral PB1, PA and PB2

73   polymerase subunit proteins, respectively being the most widely used antivirals. All seem

74   to inhibit viruses that are resistant to Adamantanes and NAIs [7,8]. In 2018, Baloxavir was

75   approved for the treatment of acute and "uncomplicated" influenza infections in the United

76   States[9,10]. Surprisingly, Uehra et al. found that even in otherwise healthy adults and

77   adolescents the emergence of strains carrying Baloxavir resistance mutations was as

78   high as ~10% and could potentially be higher in immunocompromised patients [11,12].

79   The sustained circulation of virus variants resistant to current antivirals and the low

80   genetic barrier to achieve resistance highlight the need for host-targeted therapeutics;

81   that do not suffer from these limitations. Given that all viruses rely on host-cellular

82   machinery for replication at every step of their life cycle, several host proteins have been

83   shown to be required for efficient viral replication and pathogenesis [13-17]. Host kinases

84   link a myriad of signaling pathways used by viruses; and as such, they offer attractive

85   targets for potential host-directed therapeutics against infections by several viruses

86   including influenza viruses [13,14,18]. Host kinases catalyse phosphorylation of lipids or

87   proteins, at either tyrosine or serine/threonine residues that not only serves to relay and

88   amplify cell signaling, but phosphorylation can also lead to conformational changes in

89   proteins that facilitate protein-protein interactions [19,20].

90   Although the human kinome consists of more than 550 kinases, currently available

91   kinase inhibitors only target ~10% of kinases and are primarily used to treat cancers [21-

92   25]. To date, only 62 small-molecule kinase inhibitors (SMKIs) have been FDA-approved

93   and the majority of these target tyrosine kinases [26]. Non-receptor tyrosine kinases

94   (NRTKs) are cytoplasmic or membrane-anchored kinases that closely associate with

95   cellular receptors or receptor complexes to mediate outside-in signaling [26]. NRTKs like

96   most kinases contain a catalytic kinase domain, as well as several protein-protein

97   interaction motifs (e.g., SH2, SH3, PH domains, etc.) necessary to relay cell signals.

98   NRTKs include Abl, FAK, JAK, Src and BTK, all of which have been reported to play a

99   role in IAV infections. Previous studies have demonstrated that NRTK inhibitors (NRTKIs)

100   can modulate pro- and anti-viral signaling *in vitro* and result in reduced viral pathogenesis

101   and increased survival *in vivo* [13,16,27-32]. However, no NRTKIs or SMKIs have been

102   approved for clinical use against influenza. In the present study, we characterized the

103   effect of eight currently available and FDA-approved NRTKIs on IAV replication, six of

104   which were validated using a biologically relevant *ex vivo* model of human precision-cut

105   lung slices (hPCLS). hPCLS maintain near-native structural integrity of lung tissues that

106   allow complex and 3D cell-cell interactions of epithelial cells, mesenchymal tissue as well

107   as the vascular compartment; thereby, offering an advantage over cultured epithelial

108   monolayers [33-35]. We also identify which steps of the virus infection cycle are affected by

109   specific NRTKIs; thereby providing insights into potential mechanisms of action in the

110   context of influenza virus infections.

111

112   **RESULTS**

113   **NTRKI treatment inhibits IAV replication *in vitro***

114   To identify non-toxic concentrations of our inhibitors, we used the CellTiter-Glo (CTG)

115   assay in which cell-viability is based on ATP content in healthy cells. We identified

116   concentrations that resulted in cell viability ≥90% relative to mock-treated cells (cells

117   treated with 0.1% DMSO). The highest concentration with ≥90% relative viability was

118   defined as the 1x concentration ($[1x]_{max}$) (**Fig. 1/Table 1**).

119   Next, we determined the effect of eight NRTKIs on influenza A virus replication. A549

120   cells were infected with either pandemic A(H1N1)pdm09 strain A/Netherlands/602/09

121   (NL09) or seasonal A(H3N2) strain A/Netherlands/241/11 (NL11) at a multiplicity of

122   infection (MOI) of 1 in the presence or absence of $[1x, 0.5x$ and $0.25x]_{max}$ concentrations

123   of the respective inhibitors following inoculation. Culture supernatants were collected at

124   2, 24, 48, and 72 hours post infection (hpi) and virus titers were determined by median

125   tissue culture infectious dose ($TCID_{50}$) assay. We observed a dose-dependent reduction

126   of viral titers by six of the eight inhibitors with at least one of the concentrations ranging

127   from 2- to 1,000-fold (**Fig. 2A**) reduction of virus titers. As visualized by the heatmap (**Fig.**

128   **2B),** there was variability in both magnitude and duration of the reduction of virus titers,

129   and in general, the effect was more pronounced in NL11 (H3N2) infected cells than NL09

130   (pH1N1). Although we observed only a transient reduction at 24 hpi with Tofacitinib (TF)

131   (JAK1/2/3 inhibitor)[36], we did not observe any significant reduction with Ruxolitinib (RX)

132   (JAK1/2 inhibitor)[37]. The highest and most sustained level of reduction was observed with

133   Nilotinib (NI) (Abl/PDGFRa inhibitor)[38,39]: >1,000-fold (3-log_{10}) reduction. Bosutinib (BO)

134   (Abl/Src/Btk inhibitor)[40,41] and Saracatinib (SA) (Src inhibitor)[42] also showed marked

135   inhibition (SA ~ 5- to 25-fold; BO ~10- to 1,000-fold). Acalabrutinib (AC) (Btk inhibitor)[43,44]

136   had very little effect on NL09 replication but showed a greater and more sustained

137   inhibition (5- to 25-fold) of NL11 replication at the higher concentrations (0.25 and 0.5

138   μM). Similarly, Ibrutinib (IB) (Btk/EGFR inhibitor)[45,46] had little to no effect on NL09

6

139   replication but a more appreciable and sustained (5- to 100-fold) reduction, especially at

140   the highest concentration used (0.5 µM) of NL11 replication. Defactinib (DF) (FAK/Pyk2

141   inhibitor)[47] treatment resulted in robust reduction in viral titers (10- to 1,000-fold) in both

142   NL09 and NL11 at the higher concentrations (2.5 and 5.0 µM). In NL09 infected cells, the

143   reduction was more robust at earlier time-points (24 and 48 hpi), but in NL11 infected

144   cells a larger reduction was observed at 24 and 72 hpi than at 48 hpi.

145

146   **NRTKIs affect IAV infectivity and cell viability during IAV infection**

147   Host-targeted inhibitors can influence virus yield by affecting infectivity through effects

148   on viral entry, RNA replication, increased host antiviral responses as well as impacting

149   the viability of infected cells. To assess the effect of NRTKIs on viral infectivity and cellular

150   viability, we used immunofluorescence microscopy to determine the number of infected

151   cells as well as the total number of cells. A549 cells were infected with either NL09 or

152   NL11 (MOI=1) in the presence of NRTKIs $[0.5x]_{max}$, fixed at 48 hpi and stained to detect

153   virus and nuclei (**Fig. 3A**). Surprisingly, treatment with most inhibitors resulted in a

154   significant increase in infectivity despite the reduction in viral titers observed in **Fig. 2**. We

155   observed robust increases in relative infectivity in cells treated with TF (NL09=217%,

156   NL11=112%), RX (NL09=203%, NL11=116%) and DF (NL09=180%, NL11=139%). We

157   only observed a marginal increase in relative infectivity (~105%) in cells treated with either

158   BO or NI in both NL09- and NL11-infected cells. We observed a marked decrease in

159   relative infectivity in cells treated with AC (NL09=78%, NL11=74%) and SA (~90% for

160   both NL09 and NL11). Interestingly, we observed opposite effects on NL09 (increased)-

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161 and NL11 (decreased)-infected cells following treatment with IB (NL09=116% vs

162 NL11=91%) (**Fig. 3B**).

163   Next, we determined whether the reduction in titers was the result of reduced cell

164 viability using the CellTiter Glo assay described above. Although at the NRTKI

165 concentrations used $[0.5x]_{max}$ we observed less than 5% cytotoxicity in mock-infected

166 cells, IAV-infection led to synergistic cytotoxicity and decreased relative viability when

167 treated with TF (NL09=40%, NL11=63%), RX (NL09=66%, NL11=89%) or DF

168 (NL09=84%, NL11=92%). However, cell viability was increased by treatment of IAV-

169 infected cells with AC (NL09=112%, NL11=109%), IB (NL09=102%, NL11=109%) BO

170 (NL09=111%, NL11=114%), NI (NL09=110%, NL11=110%) or SA (NL09=150%,

171 NL11=127%) (**Fig. 3B**). Taken together, our data suggest that decreased infectivity or

172 cell viability alone do not account for the NRTKI induced reduction in viral titers we

173 observed.

174

175 **The antiviral effect of NRTKIs is MOI-independent**

176   Given the viability data and the limited reduction in viral titers above, we excluded RX

177 and TF from further analyses and focused on the remaining six NRTKIs for further

178 investigation. We assessed whether the inhibitory effects of NRTKIs we observed were

179 dependent on the infectious dose used. We infected A549 cells with either a high MOI

180 (MOI=3) or a low MOI (MOI=0.01) in the presence or absence of NRTKIs $[0.5x]_{max}$.

181 Culture supernatants were collected at 2, 24, 48, and 72 hpi and virus titers were

182 determined by $TCID_{50}$ assay. As we previously observed, the effect on the seasonal H3N2

183 (NL11) strain was more pronounced compared to that on the pandemic H1N1 (NL09)

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184 strain; presumably due to the faster growth kinetics of NL11 compared to NL09. However,

185 similarly to cells infected at MOI=1 (**Fig. 2**), NRTKI treatment of cells infected at either

186 high (3) or low (0.01) MOI resulted in viral titer reductions of at least 10-fold (1-log$_{10}$) (**Fig.**

187 **4**). Treatment with either DF, BO or NI had a larger impact on early (24 hpi) viral

188 replication, especially in NL11 infected cells. And although peak titers of untreated cells

189 infected with NL11 were similar at either MOI, cells infected at MOI=0.1 exhibited the

190 greatest reduction of ~1,000-fold (3-log$_{10}$). Although AC, IB and SA treatment had less of

191 an impact on viral titers than DF, BO or NI, viral titer reductions were still up to 100-fold

192 (2-log10) (**Fig. 4**).

193

194 **Human PCLS support robust IAV infection and confirm inhibitory effect of NRTKI**

195 **treatment**

196   We utilized human precision-cut lung slices (hPCLS) as a biologically relevant *ex vivo*

197 model that more faithfully represents lung tissues than either 2D monolayer cultures or

198 3D air-liquid-interface (ALI) cultures [33,34]. Following hPCLS preparation, they were in

199 culture for up to 4 weeks; no gross alterations in cell type or morphology were observed

200 and cilial beating was observed in all used hPCLS (8 donors; n=24). We first sought to

201 identify an infectious dose to synchronize peak titers in hPCLS infected with either NL09

202 or NL11 as these two strains have different replication kinetics *in vitro*. hPCLS were

203 infected with 10^4, 10^5, or 10^6 TCID$_{50}$ / 200 µL with either NL09 or NL11. To limit donor-

204 heterogeneity effects, we used hPCLS from 8 donors (n=24/virus). Culture supernatants

205 were collected and replenished at 2, 16, 24, 48, 72, 96, and 144 hpi and virus titers were

206 determined by TCID$_{50}$ assay using the collected supernatants. Interestingly, the 10^6 dose

207   yielded maximal titers that were lower than the other doses and were achieved by 24 hpi

208   with either strain. However, the highest peak titers were achieved at 48 hpi following

209   infection with either 10^4 or 10^5 doses of NL11, but only in the 10^5 dose of NL09 (**Fig. 5A**).

210   Based on these results the 10^5 dose was used in all our subsequent hPCLS infections.

211   Next, we determined the tolerability of hPCLS to our NRTKIs candidates. hPCLS were

212   treated with either the [1x or 10x]$_{max}$ (1x is highest non-toxic NRTKI concentration defined

213   using A549 cells). Culture supernatants were collected and replenished at 24, 48, 72, 96,

214   and 144 h. Lactate dehydrogenase (LDH) release into the culture supernatant due to loss

215   of plasma membrane integrity is an indicator for cytoxicity. Using a bioluminescence

216   based LDH detection assay (LDH-Glo Cytotoxicity Assay), we quantified LDH released

217   into the collected supernatants following NRTKI treatment. As a positive control for

218   cytotoxicity, hPCLS were treated with 0.1% Triton-X 100; DMSO-treated hPCLS were

219   used as a vehicle control (**Fig. 5B**). Our cytotoxicity cut-off was 20% of the positive control

220   treatment; none of the NRTKIs surpassed this cut-off at [1x]max. However, [10x]$_{max}$

221   concentrations of DF (50 μM), BO (50 μM) and SA (1.25 μM) showed significantly higher

222   cytotoxicity that was above the relative 20%-cutoff; therefore, only the 1x concentrations

223   of these NRTKIs were used in subsequent hPCLS experiments.

224

225   **NRTKIs inhibit *ex vivo* IAV infections**

226   Based on our *in vitro* data, we tested six NRTKIs (AC, BO, DF, IB, NI and SA) for their

227   antiviral potential. hPCLS from 3 donors (n=6/virus/condition) were infected with 10^5

228   TCID$_{50}$ of either NL09 or NL11 and then treated with NRTKIs (DF 5μM; AC 5μM; IB 5μM;

229   BO 5μM; NI 10μM; SA 0.125μM). Culture supernatants were collected and replenished

230   from the same wells at 2, 12, 24, 48, 72, and 120 hpi and virus titers were determined by

231   TCID$_{50}$ assay. We observed a significant and robust reduction in viral titers of at least 10-

232   fold or 1-log$_{10}$ (DF treatment) to more than 1,000-fold or 3-log10 (IB and NI treatments)

233   using all the NRTKIs (**Fig. 5C**). Moreover, unlike what we observed in A549 cells, NRTKI-

234   mediated IAV inhibition was observed as early as 12 hpi and maintained at 120 hpi;

235   beyond the times when peak titers were achieved (48-72 hpi) (**Fig. 5C**).

236   Next, we assessed the effect of NRTKI treatment on viral spread and associated

237   damage to the epithelium. Considering we did not observe significant differences in titer

238   reductions between NL09 and NL11, only data for NL11 is shown (**Fig. 5D**). At 120 hpi,

239   mock- and IAV-infected hPCLS (n=3/virus/condition) were fixed and paraffin-embedded.

240   Tissue sections (2 μm thick) were cut, stained and immunoprobed. H&E staining indicated

241   that no gross alterations in cell composition or the epithelium were observed in mock-

242   infected cells indicating viability of hPCLS. Typical morphological changes associated

243   with IAV infections were observed. Using consecutive sections from those H&E stained,

244   we detected the viral spread using antibodies to IAV-NP (**Fig. 5D**). We observed specific

245   staining of viral antigen in all observed cell-types including type I/II pneumocytes and

246   endothelial cells. Additionally, staining intensity and quantity was reduced in NRTKI

247   treated hPCLS compared to untreated hPCLS.

248   We carried out semiquantitative analysis of the acquired images to compare the effect

249   of the inhibitors on viral spread in hPCLS with variable tissue density. IAV-NP signal was

250   normalized to H&E staining of the respective regions in consecutive tissue section.

251   Treatment with DF, AC, BO and SA significantly reduced the infectivity by > 50%, whereas

252   treatment with IB and NI had limited effects on infectivity (**Fig. 5E**).

253

Stability of NRTKI inhibition

254

255   Host-directed antivirals/therapeutics are thought to have a higher barrier of resistance

256   than their virus-targeted counterparts. To determine the stability of the antiviral effect of

257   our NRTKIs, we phenotypically assessed the possibility of the emergence of resistant

258   escape variants [48,49]. We passaged both NL09 and NL11 viruses (MOI=0.001) in the

259   presence of NRTKIs $[1x]_{max}$ in MDCK cells for 5 passages. Untreated virus stocks were

260   also passaged as a control. At each passage, virus supernatant was quantified and used

261   to inoculate the next passage at MOI=0.001 again. In untreated passages, the virus titers

262   for both NL09 and NL11 was similar from passages 1 to 5 but were significantly lower in

263   all treated passages (**Fig. 6A**); the reduction was comparable to what we originally

264   observed in A549 cells. The viral titers were stable at all passages indicating that no

265   resistance mutations were acquired. To rule out the possibility that the NRTKIs directly

266   interact with the virion and inhibit its attachment or entry, we pre-incubated virus stocks

267   with NRTKIs $[1x]_{max}$ for 2 h then diluted the virus stocks 1:1000 to minimize the effects on

268   host cells, and infected A549 cells. At 72 hpi, culture supernatants were collected and

269   virus titrated by $TCID_{50}$ assay. As expected, pre-treatment of virus with NRTKIs had no

270   effect on viral titers, indicating that the observed effects are due to host-cell effects (**Fig.**

271   **6B**).

272

Selected NRTKIs inhibit viral entry

273

274   Kinases regulate every step of the infection cycle and a single kinase can affect

275   multiple steps [28,29]. We first assessed whether our NRTKIs impaired viral entry. A549 cells

276　were pretreated for 2 h, then chilled on ice for 15 min and infected at a high MOI (MOI=10)

277　on ice for 30 min to synchronize the infection and allow binding of the virus to cell surface

278　receptors but not trafficking of virions. Unbound and non-internalized virus was washed

279　away with room temperature PBS. Cells were then incubated with prewarmed infection

280　media in the presence or absence of NRTKIs. At 0.5 hpi, cells were fixed, stained to detect

281　viral NP, F-actin and nuclei and analyzed by confocal microscopy. We observed

282　significant retention at the membrane and periphery of the cell following DF and IB

283　treatment (**Fig. 7**). Surprisingly, no virus was detected in response to BO treatment and

284　the F-actin network was not detectable. Given the sustained viability of BO-treated cells,

285　it is likely that the altered actin dynamics are well tolerated. No significant changes were

286　detectable in AC, NI or SA treated cells suggesting these inhibitors did not affect viral

287　entry under our tested conditions (**Fig. 7**).

288

289　**NRTKIs exert differential effects on IAV polymerase activity**

290　Next, we assessed the effect of our NRTKIs on viral RNA replication using the pPOLI-

291　358-FFLuc reporter plasmid, which encodes a firefly luciferase gene under control of the

292　viral nucleoprotein (NP) promoter. In this system, luciferase activity is a surrogate for viral

293　polymerase activity [50-52]. A549 cells were transfected with pPOLI-358-FFluc and

294　pmaxGFP plasmids (transfection control). At 24 hpt, cells were either infected with NL09

295　or NL11 at MOI=1 in the presence or absence of indicated NRTKIs at either [0.5x or

296　1x]$_{max}$. At 48 h post-transfection (hpt) (~24 hpi), luciferase activity was measured,

297　normalized to GFP expression (MFI) and represented as relative of untreated infected

298　cells. We observed a significant reduction in polymerase reporter activity in response to

299   AC (NL11 only), IB (NL09 only), BO, NI and SA (NL11 only) (**Fig. 8A, left panel**). Although

300   the magnitude of reduction was higher in NL09-infected than NL11-infected cells, a

301   significant reduction was more readily observed in NL11 infected cells at a lower NRTKI

302   concentration compared to NL09 infected cells. DF also reduced reporter activity

303   (NL09=20%, NL11=13%), however this reduction was not statistically significant. At 24

304   hpi, a 3-fold increase in reporter activity could be observed in untreated NL11-infected

305   cells over untreated NL09-infected cells; this is in-line with faster replication kinetics of

306   NL11 compared to NL09 (**Fig. 8A, right panel**).

307   To better dissect the direct effect on viral RNA replication in the absence of

308   confounding factors due to NRTKI influences on viral entry and host responses, we

309   utilized an established minigenome system. A549 cells were transfected with pPOLI-358-

310   Ffluc and pmaxGFP plasmids and co-transfected with either NL09 or NL03-minigenome

311   plasmids that encode the viral NP, PA, PB1 and PB2 replication complex proteins. At 6

312   hpt, the indicated NRTKIs were added to the medium at either $[0.5x$ or $1x]_{max}$. At 30 hpt

313   (24 h of treatment), luciferase activity was measured, normalized and represented as

314   above. In this context, AC (NL09 only), IB, NI and SA treatments significantly reduced

315   polymerase activity (**Fig. 8B**). In contrast to what we observed in infected cells, the

316   magnitude of reduction in polymerase activity was comparable in NL09- and NL11-

317   minigenome transfected cells. Interestingly, polymerase activity was significantly higher

318   in untreated H1N1 (NL09) than H3N2 (NL03) minigenome-transfected cells.

319

320   **NRTKIs do not affect innate immune responses during IAV infections**

321 Given that our NRTKIs reduced viral titers by affecting either viral entry or replication,

322 we speculate whether these effects were coupled to altered innate immune signaling.

323 Activation of STAT3 by IFN type-I, -II, and -III as well as by interleukins results in

324 phosphorylation of STAT3 at Y705. We assessed the level of pSTAT3 (pY705) in A549

325 cells infected with either NL09 or NL11 (MOI=1) in the presence or absence of NRTKIs

326 at $[1x]_{max}$. At 18 and 48 hpi, total proteins were isolated from whole cell lysates, separated

327 by SDS-PAGE and analyzed by immunoblotting. We observed a decrease in the relative

328 pSTAT3/total STAT3 ratio in untreated NL09-infected cells at 18 and 48 hpi (**Fig. 9A**)

329 compared to mock-infected cells (18 h = 159%, 48 h= 225%). This reduction was less

330 striking in NL11-infected cells (**Fig. 9B**). Only DF treatment resulted in a significant

331 reduction of pSTAT3 relative to untreated infected cells (NL09 = 5% to 8%, NL11 = 5%

332 to 14% of untreated). None of the other NRTKIs showed a significant effect.

333 Phosphorylation of NFkB p65 (pNFkB) at S536 results in activation of the NFkB

334 pathway. We did not detect an increase in the relative pNFkB/total NFkB ration in NL09-

335 infected cells compared to mock-infected cells at either 18 or 48 hpi following treatment

336 with any of the NRTKIs (**Fig. 10A**). In contrast, we observed a slight increase in the

337 pNFkB/total NFkB ratio in untreated NL11-infected cells at 18 and 48 hpi (**Fig. 10B**)

338 compared to mock-infected cells (18 h = 75%, 48 h= 89%). Although we did observe a

339 reduction in NFkB activation following DF treatment of NL11-infected cells (18 h = 64%,

340 48 h = 68% of untreated), this reduction was not statistically significant. We next

341 confirmed that NFkB signaling is not impaired in our system. Mock-infected cells were

342 treated with high-molecular weight poly(IC), a synthetic dsRNA, at either 50 or 200 ng/ml.

343 Following infection of cells with NL09 or NL11 (MOI=1), cells were treated with 200 ng/ml

344   poly(IC). At 18 and 48 hpi, total proteins were isolated from whole cell lysate, separated

345   by SDS-PAGE and analyzed by immunoblotting. At 18 hpi, the relative pNFkB/total-NFkB

346   ratio significantly increased in response to poly(IC) stimulation with both low and high

347   concentrations (~7-fold relative to untreated 18 h mock) (**Fig. 10C**). Similarly, poly(IC)

348   treatment of NL09 or NL11 infected cells had a robust increase in NFkB activation (NL09

349   = ~10-fold, NL11 = ~17-fold relative to untreated 18 h mock) (**Fig. 10C**). By 48 hpi, the

350   observed NFkB activation was back to untreated-levels in mock, NL09 and NL11 infected

351   cells. These data further support our results that IAV-induced NFkB activation is limited

352   later during infection; likely suppressed by the viral NS1 (**Fig. 10C**).

353

354   **DISCUSSION**

355   Despite their clear susceptibility to rapidly arising resistance mutations, virus-targeted

356   antivirals are still the only available class of antivirals against respiratory viruses such as

357   influenza. In this study, we screened FDA approved SMKIs currently in clinical use

358   against cancers and autoimmune diseases for their antiviral potential against IAV

359   infections. Six of the eight NRTKIs we tested showed a potent inhibition of pandemic

360   A(H1N1)pdm09 and seasonal A(H3N2) IAV strains with little to no impact on cell viability

361   *in vitro*. We further validated these NRTKI candidates using a faithful *ex vivo* model of

362   human PCLS. We identified the step(s) of the viral replication cycle affected by each

363   compound. In doing so, we provide valuable information on the interplay of signaling

364   pathways regulating these steps and the likely kinases involved.

365   Kinase dysfunction often leads to malignancies and tumor immune evasion.

366   Therefore, kinases have been heavily targeted for cancer treatments using selective

367   inhibitors [26]. At the molecular level, most kinases regulate signaling pathways via catalytic

368   and/or protein-scaffolding activities. Phosphorylation often triggers changes in protein

369   conformation, enzymatic activity or subcellular localization; all of which allow fine-tuning

370   of protein-protein interactions to mediate stimuli-specific responses [53]. Indeed, host

371   kinases play a critical role in IAV entry, replication, and release as well as viral

372   evasion/suppression of hosts immune responses. These processes often require

373   phosphorylation of viral proteins by mostly unidentified kinases [54-65].

374   We carried out our initial screening using A549 cells (ATII lung adenocarcinoma);

375   however, due to potential biases associated with aberrant expression and kinase activity

376   of cancerous cell-lines, we validated candidate NRTKIs using human PCLS (hPCLS).

377   Unlike 2D monolayers or 3D well-differentiated air-liquid interface (ALI) cultures, PCLS

378   preserve the native lung tissue architecture, cellular composition including endothelial,

379   ATI and ATII epithelial cells, fibroblasts and maintain the native extracellular matrix [33-35].

380   Moreover, the tissue tropism and infectivity of certain viruses may not be accurately

381   represented *in vitro* due to the absence of relevant cell-cell interactions that can influence

382   infectibility and host responses [66]. Accordingly, we observed a similar discrepancy in

383   which the strain-dependent differences observed in NRTKI-treated A549 cells were not

384   observed in hPCLS, suggesting that the variations between IAV strains in A549 might be

385   an *in vitro* artifact. Moreover, we observed a wide tissue tropism in hPCLS which suggests

386   that while ATII cells may support more efficient infection, other cell-types of the lung are

387   readily infectible as well. Nevertheless, we observed robust viral titer reductions in both

388   systems following NRTKI treatment that were not readily explained by a reduction in

389   infectivity or cell viability. Considering that the smallest reductions in viral titers were

390 observed following Defactinib (DF) < Acalabrutinib (AC) < Saracatinib (SA) < Bosutinib

391 (BO) treatment, hPCLS infectivity was reduced by ~50% by each of those NRTKIs.

392 Similarly, Ibrutinib (IB) and Nilotinib (NI) had the largest effect on viral titers but increased

393 infectivity (NI:~22%, IB=~7%). Together our data suggest that reduction in infectivity of

394 either A549 cells or hPCLS does not fully account for the potent reduction in viral titers

395 and supports a *bona fide* effect of NRTKIs on either viral entry or RNA replication.

396   Although there is a growing body of *in vitro* and *in vivo* evidence to support the use of

397 kinase inhibitors, not a single SMKI has been approved or licensed for the treatment of

398 influenza virus infection so far [4,13,14,17,60]. SMKI selectivity remains a contentious topic and

399 has been a hurdle to the pursuit of kinase inhibitors as antivirals. Assumptions made

400 regarding "off-target effects" are attributed to changes in phosphorylation or activation of

401 proteins/pathways besides the intended target. While compounds still in the pre-clinical

402 development phase require target validation, the selectivity of compounds in clinical use

403 has been heavily investigated [26,67-69]. The ever-expanding kinase-substrate interaction

404 map highlights the extensive crosstalk between signaling pathways. Therefore, inhibition

405 of an "off-target" kinase or pathway, cannot be oversimplified and attributed to promiscuity

406 of the SMKI in question; rather it is more likely evidence of an interaction between the

407 intended target and the affected "off-target" signaling node. Two seminal studies

408 collectively examined selectivity of over 170 SMKIs against more than 440 kinases,

409 covering ~80% of the human kinome. In the first study, Davis et al. compared inhibitor-

410 kinase binding affinities, whereas Anastassiadis et al. used functional kinase inhibition

411 assays in the second study. They determined that while classes of SMKIs may inhibit

412 multiple kinases within a single subfamily, inhibitors are selective against kinases outside

18

413   that subfamily [68,69]. However, a limitation of those studies is that they were carried out

414   using truncated or fused recombinant proteins that may adopt altered conformations in

415   the absence of regulatory domains (i.e., regulatory subunit of PI3K) or binding partners

416   that may influence availability of substrate or ATP binding sites [26]. Moreover, selectivity

417   of clinically approved SMKIs was validated by super resolution microscopy (dSTORM) to

418   show the superior specificity and selectivity of fluorescently labeled SMKIs like Gefitinib

419   (EGFR inhibitor), over either a fluorescently labeled EGF ligand or an EGFR monoclonal

420   antibody [70].

421   In addition to their therapeutic uses, kinase inhibitors are used as molecular beacons

422   to probe host signaling pathways and delineate how they are regulated by kinases. It is

423   not surprising that NRTKIs which target known effectors of IAV entry have a significant

424   effect on this step of the replication cycle. Indeed, we previously showed that targeting

425   FAK using the pre-clinical inhibitor Y15, led to inhibition of PI3K-mediated endosomal

426   trafficking of virions [28]. Using the FDA-approved FAK inhibitor DF, we saw comparable

427   effects on actin reorganization and viral entry as we previously observed using Y15 [28].

428   This is consistent with the fact that the most prominent effect of DF on viral replication in

429   both A549 and hPCLS was at earlier time-points when reduction in viral entry may have

430   a larger impact than at later time-points. Indeed, we observed a reduction in infectivity in

431   response to DF treatment which is consistent with the observed reduction of viral entry.

432   Bruton's tyrosine kinase (BTK) activity regulates survival, proliferation and

433   inflammatory responses in B-cells, as well as epithelial cells [71]. Cell-specific BTK isoforms

434   have recently been implicated in PI3K and PLCγ signaling that are either pro- or anti-

435   apoptotic [72]. IB and AC are two high-affinity irreversible inhibitors of BTK; IB also inhibits

436   EGFR activity [73,74]. IB treatment reduces excessive neutrophil infiltration, acute lung injury

437   (ALI) and subsequent ARDS; ultimately resulting in increased survival of mice severely

438   infected with IAV [27]. Given that IB inhibits both EGFR and BTK whereas AC selectively

439   inhibits BTK, the IB-specific reduction in viral entry we observed, suggests this effect is

440   mediated largely through inhibition of EGFR signaling. This is consistent with IAV-induced

441   EGFR signaling which facilitates viral entry and activation of downstream pathways (Src,

442   PI3K and ERK) that promote efficient replication [75].

443   BO, along with NI and SA, are second-generation Src inhibitors. BO also inhibits Abl

444   kinase and to a lesser extent BTK. Src orchestrates signaling across multiple pathways

445   that regulate proliferation, survival, cell-cell communication, innate immune responses

446   and apoptosis [25]. Growth factor RTKs like PDGFR and EGFR induce Src-mediated

447   activation of PI3K/AKT, Ras-Raf-MEK-ERK, FAK, and STAT3 [76]. Accordingly, Src plays

448   a mostly proviral role during IAV infections that is modulated by the viral NS1 protein [13,77].

449   In contrast, Abl's role in human IAV infections is not clear; however, Abl inhibition by some

450   avian IAVs results in significant pathology *in vitro* and *in vivo* [78,79]. We observed a

451   significant reduction in viral titers following BO treatment of hPCLS and A549 cells which

452   also resulted in a stark reduction in viral entry, largely due to disruption of the actin

453   network. Despite the increase in cell viability during infection, we observed a complete

454   absence of detectible actin filaments suggesting either enhanced depolymerization of F-

455   actin or altered actin dynamics. Consequently, BO inhibition of Src activity can lead to

456   actin depolymerization due to retention of alpha and β-catenin at the cell membrane [80,81].

457   To dissect the effect of our NRTKIs on polymerase activity, we employed a

458   polymerase activity reporter system [82]. Interestingly, in the context of viral infections, we

20

459   detected higher polymerase reporter activity in NL11 (H3N2)-infected cells. In contrast,

460   significantly higher polymerase activity was detected using NL09 (H1N1) minigenome.

461   Faster kinetics may be more susceptible to NRTKIs as a reduction in replication rate

462   results in exponential differences with time. This suggest that polymerase activity of NL09

463   is higher than NL11 and that the faster kinetics in virus replication observed in NL11

464   infected cells may be due to more efficient virus entry, release, or immune evasion than

465   NL09.

466   In addition to its role in viral entry, we previously demonstrated that FAK regulates the

467   polymerase activity *in vitro* of multiple IAV strains using Y15 as well as dominant-negative

468   kinase mutants [29]. However, in contrast to our previous studies, we only observed a

469   modest and non-significant effect on polymerase activity following DF treatment. O'Brien

470   et al. showed that Y15 was a significantly more potent and selective inhibitor of FAK

471   activity than DF which also targets the FAK related kinase Pyk2 [83]. The disparity between

472   a given SMKI's binding affinity (K_d) and its functional inhibitory concentrations can also

473   be observed in the case of a single inhibitor targeting multiple kinases. For instance, the

474   K_d of the multi-kinase inhibitor Sunitinib for TrkC is 5.1 µM, but a 10-fold lower

475   concentration (0.5 µM) is sufficient to inhibit >97% of its activity. In contrast, Sunitinib's

476   K_d for PAK3 is 16 nM, but not even a 30-fold higher concentration (0.48 µM) has an effect

477   on its activity [67]. Therefore, the difference in potency of FAK inhibition by DF vs Y15 may

478   account for the limited effect of DF treatment on IAV polymerase activity we observed.

479   Inhibition of Src by SA, BTK and EGFR by IB, and BTK by AC had less of a significant

480   effect on IAV polymerase activity indicating that the contribution of these kinases to host

481   innate immune responses does not directly affect RNA replication. In contrast, inhibition

482   of Abl and PDGFRα by NI treatment had the most significant reduction in IAV polymerase

483   activity that was also strain independent. These data point to a role of PDGFRα in

484   facilitating efficient IAV polymerase activity. This is consistent with previous findings that

485   show inhibition of PDGFRα by the RTK inhibitor A9, blocks RNA synthesis of all viral RNA

486   species (vRNA, cRNA and mRNA) independently of NFkB signaling [56].

487   The NFkB pathway typically mediates inflammatory/antiviral responses to viral

488   infections and accordingly, it plays a critical role in IAV replication and pathogenesis.

489   Several reports indicate that IAVs modulate antiviral NFkB activity to facilitate viral

490   replication. Inhibition of NFkB results in reduced viral titers partly due to a disruption of

491   vRNP nuclear export [84-86]. Consistent with previous data, we did not observe a robust

492   induction in NFkB phosphorylation, most likely due to the immuno-suppressive role of the

493   viral NS1 protein [87]. However, we observed a clear induction of NFkB activation by

494   poly(IC) treatment alone or in combination with IAV infection at 18 h but not 48 h.

495   In addition to its role in actin reorganization, FAK modulates the cellular immune

496   response by regulating various T-cell-, B-cell-, and macrophage-functions as well as RIG-

497   I-Like antiviral signaling [88-91]. We previously demonstrated FAK-dependent regulation of

498   NFkB signaling and polymerase activity *in vitro* and NFkB-dependent pro-inflammatory

499   responses *in vivo* [30]. In that study, FAK inhibition resulted in increased survival, reduced

500   viral load and reduction in a severe infection model. Surprisingly, DF treatment had no

501   significant effect on NFkB phosphorylation; this again, is likely due to the difference in

502   FAK inhibition potency between Y15 and DF. Similarly, none of the other NRTKIs

503   influenced NFkB activation suggesting that the mechanism by which these NRTKIs inhibit

504   virus replication is independent of the NFkB-pathway. Considering the transient and

505   biphasic nature of NFkB activation, we cannot rule out that strain-dependent differences

506   in kinetics did not affect the magnitude or duration of NFkB activation we observed [92,93].

507   An emerging regulator of IFN and inflammatory responses is STAT3. A wide range of

508   cytokine, growth factor, and RTKs activate STAT3 via JAK1/2/3 and Tyk2-dependent

509   phosphorylation at Y705 (STAT3pY705) [94]. The role of STAT3 is not fully understood with

510   opposing functions dependent on pathway partner; IL-6 mediated STAT3 activation is

511   proinflammatory while IL-10 mediated STAT3 activation is anti-inflammatory [94,95].

512   Although STAT3 is dispensable for IFN signaling, it is activated by IFN-I and serves as a

513   negative regulator to fine-tune the IFN response (reviewed in [95]). Because STAT3

514   activation upregulates anti-apoptotic factors, H5N1 mediated STAT3pY705 allows

515   prolonged viral production through delay of apoptosis; H1N1 is less efficient at

516   STAT3pY705 and triggers apoptosis earlier [96,97]. Although the mechanism of differential

517   suppression of STAT3 activation by IAV is not clear, it has been suggested to be mediated

518   by NS1 [87]. Interestingly, EGFR activation can result in Src/FAK/BTK mediated activation

519   of STAT3, thereby modulating the IFN and proinflammatory responses. As expected of

520   H1N1 and H3N2 infections [96,97], we observed a suppression of STAT3pY705 in untreated

521   cells that was comparable to that observed following treatment with most NRTKIs.

522   Surprisingly, we observed significant suppression of STATpY705 following DF treatment

523   (85-90% of untreated infected cells). Considering that DF inhibits both FAK and Pyk2, it

524   is tempting to speculate that STAT3pY705 requires FAK/Pyk2 activity during IAV

525   infection. Indeed, Pyk2 kinase activity was required to induce EGFR/Src-mediated

526   STAT3pY705 [98]. Interestingly, STAT3pS727 which is required for full transcriptional

23

527   activity of STAT3, points to an indirect Pyk2 mechanism possibly through JNK, p38 or

528   ERK activation [98].

529   In summary, we have demonstrated that NRTKs are host cell factors required for

530   efficient IAV replication and represent promising drug targets for the development of the

531   next generation of antivirals. Because these inhibitors target host factors, their therapeutic

532   window is likely to be different than that of virus-targeted antivirals. To our surprise, our

533   tested NRTKIs directly affected steps of the virus replication cycle with limited effects on

534   tested host responses. Importantly, our results were validated using an *ex vivo* lung tissue

535   model from several donors. Given that most of our PCLS were obtained from lung cancer

536   tumor resections, our donors tend to be older, are often smokers and suffer from either

537   COPD or other respiratory pathologies. Although at first glance this may seem like a

538   limitation of our model, we believe that these donors represent the "at risk" populations

539   that would most benefit from IAV antivirals. Therefore, our data obtained from donor

540   PCLS using these already FDA-approved NRTKIs as IAV antivirals is highly applicable to

541   clinical settings. In contrast to virus-directed IAV antivirals which are susceptible to

542   resistance mutations, our data indicate a high genetic barrier for resistance to our tested

543   NRTKIs. This is based on the stability of IAV inhibition after 5 passages under selective

544   pressure by each of our six validated inhibitors. Although we cannot rule out NRTKIs-

545   selected mutations, no resistance/adaptive variants were detected. Additionally, their

546   established safety and bioavailability data further warrants the evaluation of these

547   compounds as potential influenza treatments. Given that IAV infections are typically

548   restricted to the respiratory tract, localized delivery of the kinase inhibitors can further limit

549   potential cytotoxic effects. Finally, the local microenvironment must be considered to elicit

550   a balanced immune response. Likewise, the effect of promising kinase inhibitors on

551   resident and infiltrating immune cells must be investigated to avoid opposite or

552   unintended consequences. Considering that many viruses, including respiratory viruses,

553   utilize the same (or related) host kinases to facilitate their replication and transmission,

554   our studies have broader implications for the potential use of these SMKIs to treat

555   infections by other viruses in addition to IAV infections.

MATERIALS AND METHODS

Cells and Viruses. Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, and 1% nonessential amino acids (NEAAs). A549 cells were cultured in F-12 K-Nut Nutrient Mix medium (Gibco) supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM Glutamax. All cells were incubated at 37°C and 5% CO_2.

The pandemic H1N1 strain A/Netherlands/602/09 (NL09) and seasonal strain H3N2 A/Netherlands/241/11 (NL11) influenza viruses were obtained from the Repository of the National Influenza Center at the Erasmus Medical Center in Rotterdam, the Netherlands, and were grown on MDCKs for 48h at 37 °C. Virus stocks and culture supernatants were stored at -80°C until further use. Virus yields were titrated on MDCK cells by 50% tissue culture infectious dose ($TCID_{50}$)/ml method as described by Reed and Muensch [99].

Human precision-cut lung slices (PCLS). Human PCLS for *ex vivo* studies were generated from lung tissues obtained from patients undergoing surgical operations at Hannover Medical School. Tissues used for PCLS generation that were obtained from lung tumor resections were confirmed as tumor-free by an experienced pathologist. The freshly obtained lung tissues were processed into circular slices that were 300 microns thick and 8 mm in diameter as previously described [34]. All donors provided informed consent as approved by the Hannover Medical School Ethics Committee (Ethics vote #8867_BO_K_2020). PCLS were maintained in DMEM/F12 medium (ThermoFisher)

578   supplemented with 2 mM of HEPES, 1 × GlutaMAX (Gibco), 100 U/ml penicillin and 100

579   μg/ml streptomycin in a humidified 37°C and 5% CO_2 incubator.

580

581   ***Inhibitors.*** Small molecule kinase inhibitors (SMKI) were all purchased from Selleckchem

582   (TX, USA). Inhibitors were diluted in DMSO to a stock concentration of 10mM and stored

583   at -20°C upon usage.

584

585   ***In vitro and ex vivo cytotoxicity assays.*** *In vitro* cytotoxicity of SMKIs on mock and/or

586   virus infected A549 cells was determined using CellTiter-Glo 2.0 (CTG) Cell Viability

587   Assay (Promega) according to manufacturer protocols.

588   Cytotoxicity of SMKIs on mock and/or virus-infected PCLS was determined using the

589   LDH-Glo Cytotoxicity Assay (Promega) according to manufacturer's protocols.

590   Supernatants of SMKI-treated and untreated hPCLS were collected and completely

591   replaced with fresh pre-warmed infection medium containing SMKIs at the indicated

592   concentrations. LDH levels were relative to the positive control (treated with 1% triton-X

593   100 for 30 min at 37°C).

594

595   ***Virus infections.*** A549 cells were plated on the day prior to infection so they were 80-

596   90% confluent on the day of infection. For infections, viruses were diluted in infection

597   medium (F12K containing 0.1% [vol/vol] bovine serum albumin [BSA] and 50 ng/μl TPCK-

598   treated trypsin). The cells were inoculated with the virus at the indicated multiplicity of

599   infection (MOI) for 1h at 37°C. The cells were washed twice with phosphate-buffered

600   saline containing Mg^{2+}/Ca^{2+} (PBS+/+) to remove unbound virus and incubated in infection

601   medium at 37°C in the presence or absence of SMKIs at the indicated concentrations.

602   Supernatants were collected at 0, 24, 48, 72 hours post-infection (hpi), and viral titers

603   were determined by $TCID_{50}$ assay in MDCK cells [99]. GraphPad's Heatmap (Prism)

604   function was used to visualize the fold-reduction in viral titers.

605

606   ***Immunofluorescent staining and imaging.*** To visualize virus infection, infected cells

607   were fixed with 4% paraformaldehyde (4% PFA) for 30 minutes at room temperature (RT),

608   permeabilized with 0.1% Triton X-100 for 15 minutes at RT, washed with PBS and blocked

609   with heat inactivated 5% horse serum in PBS (PBS-HS) at RT for 1h. Cells were then

610   incubated with mouse monoclonal antibodies to IAV nucleoprotein (clone HB65, ATCC)

611   diluted in PBS-HS at 0.2 µg/ml overnight at 4°C under constant agitation. Cells were

612   washed and incubated with AlexaFluor-594 conjugated goat anti-mouse IgG antibody (0.2

613   µg/ml; ThermoFisher) and NucBlue Live ReadyProbes Reagent (ThermoFisher) for 1h at

614   RT under constant agitation. Cells were washed 3 times with PBS, images were captured

615   using a Leica DMi8 fluorescence microscope and quantitative analysis was performed

616   using ImageJ Threshold, Watershed, and Particle Analyser tools.

617   Using a counting macro that we adapted from [100], we quantified the number of nuclei

618   and the number of separate infected cells by analyzing the RAW image data for each

619   channel (n=4). The nucleus count was used to define the total cell number per 0.6 mm^2.

620   The NP staining was used to define the number of infected cells per 0.6 mm^2. The ratio

621   of infected to total cells was used to calculate Relative Infectivity. The total number of

622   cells based on nuclei detected relative to mock-infected cells treated with the respective

623   NRTKI was used to determine Relative Viability. GraphPad's Heatmap (Prism) function

624   was used for visualization.

625

626   ***Immuno-histochemistry staining.*** Mock- and virus-Infected PCLS were inactivated by

627   fixation in 4% PFA/PBS and paraffin-embedded into blocks. Tissue sections (2 µm thick)

628   were cut from the paraffin-embedded blocks and subjected to Hematoxylin & Eosin (HE)

629   staining using standard protocols. Immunostaining for IAV antigen was done using a

630   HRP-conjugated anti-IAV NP antibody. Histological analysis was performed by an

631   experienced pathologist blinded to clinical data and experimental setup using a routine

632   diagnostic light microscope (BX43, Olympus). Representative images were acquired with

633   an Olympus CS50 camera using Olympus CellSens software (Olympus). Semi-

634   quantitative analysis of IAV NP signal was performed for all tested NRTKIs using FIJI

635   image-analysis software.

636

637   ***Polymerase activity assay.*** Semi-confluent (~70–80%) A549 cells (8×10^4 cells in 24-

638   well plates) were transfected using Lipofectamine LTX with The pPOLI-358-FFLuc

639   reporter plasmid, which encodes a firefly luciferase gene under control of the viral

640   nucleoprotein (NP) promoter (kindly provided by Megan Shaw) [50-52]; the Lonza

641   pmaxGFP™ expression vector, was used as a transfection control.

642   For minigenome polymerase activity, a mix of plasmids encoding the PB2, PB1, PA, and

643   NP genes of NL09 or A/NL/213/03 (H3N2) IAVs in quantities of 0.35, 0.35, 0.35, and 0.5

644   µg, respectively, were co-transfected with the reporter and control plasmids. At 6h post-

645   transfection (hpt), the indicated SMKIs were added at 1x and 0.5x concentrations (see

646 Table 1) and at 30 hpt (24h of treatment), luciferase reporter activity was detected using

647 the One-Glo luciferase assay system (Promega). GFP mean fluorescence intensity (MFI)

648 and luciferase luminescence were measured using a Tecan multi-mode plate reader.

649 To measure polymerase activity during IAV infection, cells were infected at an MOI of

650 1 with NL09 or NL11 at 24h post-transfection (hpt) of the pPOLI-358-FFLuc reporter and

651 the GFP plasmids in the presence or absence of SMKIs at the indicated concentrations

652 as described above. At 48 hpt (24 hpi), luciferase reporter activity was detected using the

653 One-Glo luciferase assay system (Promega). GFP mean fluorescence intensity (MFI) and

654 luciferase luminescence were measured using a Tecan multi-mode plate reader.

655

656 ***Viral entry assay and confocal microscopy.*** A549 cells were seeded on 12.5-mm

657 coverslips in 24-well plates. On the day of infection, cells were washed 3 times with

658 PBS+/+ and incubated in infection medium in the presence or absence of kinase inhibitors

659 for 2h. The cells were chilled on ice for 15 min and inoculated with virus (MOI=10) in the

660 presence or absence of the indicated SMKI concentrations at 4°C and on ice for 30 min.

661 To limit receptor activation due to continuous viral-receptor engagement/internalization

662 following the 4°C adsorption and to gently warm up the cells, unbound/noninternalized

663 virus was removed by washing the cells twice with RT PBS+/+. The cells were then

664 incubated with prewarmed infection medium containing the respective SMKIs at 37°C for

665 30 min. Cells were then fixed in 4% PFA for 30 min, permeabilized with 0.1% Triton X-

666 100 at RT for 15 min, washed in PBS, and incubated overnight at 4°C in blocking buffer

667 (PBS-HS). The cells were then incubated with anti-IAV NP antibody (clone HB65, ATCC)

668 diluted in blocking buffer for 1h at RT, washed 3 times with PBS, and incubated for 1h at

669   RT with AlexaFluor488-conjugated donkey anti-mouse IgG secondary antibody (0.2

670   μg/ml; ThermoFisher) diluted PBS-HS. Cell nuclei and F-Actin were stained with NucBlue

671   Live ReadyProbes (ThermoFisher) and ActinRed-555 ReadyProbes Reagent

672   (ThermoFisher), respectively. Coverslips were mounted with Prolong mounting medium

673   (Invitrogen), and cell images were acquired with a Leica TSC SP5 laser-scanning

674   confocal system mounted on an upright Leica DM6000 CFS using a 63x oil immersion

675   objective. The images were merged and analyzed with Leica LAS software using identical

676   imaging settings across all experiments.

677

678   ***NRTKI resistance analysis.*** To assess the resistance barrier for our NRTKIs, we

679   passaged our viruses five times in the presence or absence of submaximal inhibitor

680   concentrations (0.5x; see table 1). The parental viruses were also passaged under the

681   same culture conditions in parallel in the absence of NRTKIs. Semi-confluent MDCK cells

682   (~10^6 cells/well in 6-well plates) were infected with the pandemic H1N1 strain

683   A/Netherlands/602/09 (NL09) and seasonal strain H3N2 A/Netherlands/241/11 (NL11) at

684   MOI 0.001. At each passage, the cultures were maintained in 3 ml MDCK infection media

685   at 37°C for 72h, in the presence or absence of the $[0.5x]_{max}$ (see Tab. 1) of respective

686   candidate NRTKIs. Supernatants were harvested, clarified by centrifugation at 500 x g for

687   5 min at 4°C, and stored at −80°C until titration by $TCID_{50}$ assay on MDCK cells. For the

688   subsequent passage, cells were infected by using virus from the previous passage at

689   MOI=0.001.

690

691   ***SDS-PAGE and immunoblotting.*** Proteins were isolated from whole cell lysates using

692   the M-PER Mammalian Protein Extraction Reagent (ThermoScientific). Proteins were

693   quantified by Badford Assay, separated by 8% SDS-PAGE, transferred onto a PVDF

694   membrane and blocked overnight in blocking solution (TBS pH7.6, 0.05% Tween-20, and

695   5% w/v of nonfat dry milk). Primary antibodies were diluted in blocking solution overnight

696   at 4°C: phos-NFkB p65 (Ser536) (93H1) Rabbit mAb (1:1000) (Cell Signaling), phos-Stat3

697   (Tyr705) (D3A7) rabbit mAb (1:1000) (Cell Signaling), Influenza A virus NP Antibody

698   (PA5-32242) rabbit pAb (1:20,000) (ThermoScientific). Beta-Actin (BA3R) mAb (1:5000)

699   was used as a loading control. HRP-conjugated secondary anti-rabbit or anti-mouse

700   antibodies (1:20,000) were diluted in blocking solution for 1 hour at room temperature.

701   Proteins were detected by chemiluminescence using the SuperSignal West Pico Plus and

702   SuperSignal™ West Femto Maximum Sensitivity Substrate. Band density was measured

703   using a Li-Cor C-DiGit scanner and analyzed using Image Studio™ (Li-Cor). When

704   necessary, the imaged membrane was subsequently stripped using a mild water-based

705   stripping solution (1.5% glycine; 0.1% SDS; 1% Tween-20; pH 2.2) and restained for total

706   proteins using NFkB p65 (L8F6) mouse mAb (1:1000) (Cell Signaling) or Stat3 (124H6)

707   mouse mAb (1:1000) (Cell Signaling).

708

709   **Statistical analyses.** Statistical analyses with GraphPad Prism 9 included multiple *t* test,

710   Brown-Forsythe and Welsh's ANOVA tests and Dunnett's T3 test for multiple

711   comparisons. Values are represented as means standard deviations (SD) or standard

712   error of the mean (SEM), with a *P* value of 0.05 considered statistically significant (ns =

713    $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$). The performed

714   tests and given significances are provided in the figure legends.

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AUTHOR CONTRIBUTIONS

HE conceived the project. HE, RM and GR designed the experiments and supervised the project. RM, SS, MB performed the experiments. HE, RM, conducted data analysis. CW, MK and DJ generated and provided human PCLS. CW and MK provided histopathological and immunohistochemical evaluation of PCLS. RM, GR and HE wrote the manuscript with input from all the authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1027 **FIGURE LEGENDS**

1028 **Figure 1**. Main target specificity of candidate NRTKIs used in this study.

1029

1030 **Figure 2:** Effect of SMKI treatment on NL09 and NL11 replication at MOI 1. A549 cells

1031 were infected with pandemic H1N1 strain NL09 and seasonal H3N2 strain NL11 at MOI1

1032 and incubated for 72h in the presence of SMKI concentrations of 0.25x, 0.5x and 1x with

1033 1x being the highest non-toxic concentration under infection conditions (Tab. 1). At 24,

1034 48, and 72 hpi, supernatants were collected and viral titers quantified by $TCID_{50}$/ml assay

1035 (n = 4). Means ±SD are shown. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns,

1036 not significant (P>0.05).

1037

1038 **Figure 3.** NRTKIs effects on cell viability and infectivity during infection. A549 cells were

1039 infected with pandemic H1N1 strain (NL09) and seasonal H3N2 strain (NL11) (MOI=1)

1040 and incubated in the presence of SMKIs (0.5x of the highest non-toxic concentration) for

1041 48h. A) Fluorescence microscopy pictures were captured using a Leica DMi8

1042 fluorescence microscope (representative field shown from an n=4/condition). Virus-

1043 infected cells were detected by anti-IAV NP antibody (red), and nuclei were detected

1044 using NucBlue Live ReadyProbes (blue). B) Heatmap visualization of NRTKIs affect cell

1045 viability (blue/green) and infectivity (red) relative to untreated infection in A549 cells.

1046 Images were quantified using ImageJ software suite. Data is based all fields represented

1047 in (A) (n=4/condition).

1048

1049   **Figure 4.** MOI-independent effect of NRTKIs on IAV infection. A549 cells were infected

1050   with pandemic H1N1 strain (NL09) and seasonal H3N2 strain (NL11) at either (MOI=0.1

1051   or 3) and incubated for 72h in the presence of NRTKIs at 0.25x (gray) and 0.5x (white) of

1052   highest-toxic concentrations. At 24, 48, and 72 hpi, supernatants were collected and viral

1053   titers quantified by $TCID_{50}$/ml assay (n=4). Means ±SD are shown. *, P<0.05; **, P<0.01;

1054   ***, P<0.001; ****, P<0.0001; ns, not significant (P>0.05).

1055

1056   **Figure 5.** Effect of NRTKI treatment on *ex vivo* IAV infection. A) hPCLS were infected

1057   with pandemic H1N1 strain (NL09) and seasonal H3N2 strain (NL11) at increasing doses

1058   (10^4, 10^5 and 10^6 $TCID_{50}$/200 ul). At 2, 16, 24, 48, 72, 96, and 144 hpi culture supernatants

1059   were sampled and viral titers quantified by $TCID_{50}$/ml assay. The supernatants were

1060   replenished after collection at every time point (8 donors; n=24/virus); means ±SEM are

1061   shown. B) Heatmap visualization of NRTKI cytotoxicity based on LDH release of hPCLS

1062   normalized to DMSO control, and relative to 1% Triton-X 100 treated cells; NRTKIs were

1063   treated with 1x and 10x of highest cytotoxic concentration on A549 cells (Fig. 1) for up to

1064   144h. At every time point, LDH release was determined using LDH-Glo Cell Viability

1065   Assay (8 donors / n=24). C) PCLS were infected with NL09 or NL11 (10^5 $TCID_{50}$ / 200 ul)

1066   and incubated for 120h in the presence of NRTKIs (Defactinib 50uM; Acalabrutinib 5uM;

1067   Ibrutinib 5uM; Bosutinib 5uM; Nilotinib 10uM; Saracatinib 0.125uM). Growth curves from

1068   supernatants collected at 2, 12, 24, 48, 72, and 120 hpi were quantified by $TCID_{50}$/ml

1069   assay (3 donors; n=6/condition); means ±SEM are shown. D) NL11 infected hPCLS were

1070   fixed 120 hpi and the PFA-fixed paraffin-embedded (PFPE) PCLS were were cut into 2

1071   µm thick section. Shown is H&E staining and viral anti-NP immunohistochemical staining

1072   (brown). Original magnification 10x. E) Semi-quantitative analysis of virus infected (anti-

1073   NP staining) was performed for all tested NRTKIs using FIJI image-analysis software

1074   platform and normalized to whole section areas.

1075

1076   **Figure 6.** Stability of NRTKI treatment effect. (A) Effect of SMKI treatment on NL09 and

1077   NL11 replication at MOI=0.001 over serial passages was determined by growth curves

1078   using MDCK cells infected with pandemic H1N1 strain (NL09) or seasonal H3N2 strain

1079   (NL11) at MOI=0.001 and incubated for 72h in the presence of the highest non-toxic

1080   NRTKI concentrations (n=2). At 72 hpi, samples were collected, and viral titers were

1081   assessed by $TCID_{50}$/ml assay on MDCK cells (n = 4). MDCK cells were infected as before

1082   at MOI=0.001 according to the assessed viral titers. Means ±SD are shown. (B) NL09

1083   and NL11 virus stocks were pre-incubated with control (DMSO) or the 1x concentration

1084   of the respective NRTKI for 4h at 37°C. A549 cells were then infected using a 1:1000

1085   dilution of the NRTKI pre-incubated virus stocks and incubated for 72h. At 72 hpi, samples

1086   were collected, and viral titers were assessed by $TCID_{50}$/ml assay (n=3). Means ±SD are

1087   shown. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, not significant (P>0.05).

1088

1089   **Figure 7.** The effect of NRTKI on viral entry. A549 cells were pretreated with NRTKIs for

1090   2h and then infected with the NL09 and NL11 strains (MOI=10) for 0.5h in the presence

1091   or absence of the inhibitors. The cells were fixed and permeabilized. Virions were

1092   detected by anti-NP (green) antibody, F-Actin was detected by ActinRed-555 (red), and

1093   nuclei were detected using NucBlue Live ReadyProbes (blue). and virion localization was

1094   assessed by confocal microscopy using a 63x oil immersion objective (n = 2).

51

1095

1096 **Figure 8.** NRTKIs effects on IAV RNA replication. (A) A549 cells were transfected with

1097 pPOLI-358-FFluc and pmaxGFP plasmids. At 24 hpt, cells were either infected with NL09

1098 or NL11 at MOI=1 in the presence or absence of indicated NRTKIs. At 48 hpt (24 hpi),

1099 luciferase activity was measured and normalized to GFP expression (MFI). (B) A549 cells

1100 were transfected with pPOLI-358-FFluc and pmaxGFP plasmids and co-transfected with

1101 either NL09 or NL03-minigenome plasmids. At 6 hpt, the indicated NRTKIs were added

1102 to the medium. At 30 hpt (24 h of treatment), luciferase activity was measured and

1103 normalized to GFP MFI. Bars indicate values relative to infected untreated cells

1104 normalized to GFP. For each system luminescence of untreated infected A549 cells

1105 relative to GFP displays the replication kinetics of the respective polymerase complex. All

1106 measurements were taken in triplicates from triplicate samples (n=3). Error bars indicate

1107 ± standard deviation (SD). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, not

1108 significant (P>0.05). P-values determined by Brown-Forsythe and Welsh ANOVA

1109 compared to untreated.

1110

1111 **Figure 9.** Effect of NRTKIs treatment on STAT3 activation. A549 cells were infected with

1112 NL09 (A) or NL11 (B) at MOI=1. Total proteins were isolated from whole cell lysate at 18

1113 and 48 hpi and immunoblot assay was performed for phospho- and total STAT3, IAV-NP

1114 and bActin. Chemiluminescence was detected and quantified using the Li-Cor C-DiGit

1115 and Image Studio 5.1 CLX software. All measurements were taken from two western blots

1116 from two independent experiments (n=2). All values are relative to untreated virus-

1117 infected cells. P=phosphor, T=total. Error bars indicate ± standard deviation (SD). *,

1118   P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns, not significant (P>0.05). P-values

1119   determined by students t-test compared to untreated virus-infected cells.

1120

1121   **Figure 10.** Effect of NRTKIs on NFkB activation. A549 cells were infected with NL09 (A)

1122   or NL11 (B) at MOI=1. Protein was isolated from whole cell lysate at 18 and 48 hpi and

1123   immunoblot assay was performed for pNFkB p65, panNFkB p65, IAV NP and bActin.

1124   Detected signal was quantified using the Li-Cor C-DiGit and Image Studio 5.1 CLX

1125   software. (C) NL09, NL11 or mock infected cells were treated with poly(IC) at

1126   concentrations of 50 ng/ml and 200 ng/ml. Total proteins were isolated from whole cell

1127   lysate at 18 and 48 hpi and immunoblot assay was performed for phospho- and total

1128   NFkBp65, IAV NP and bActin. All measurements were taken from two western blots from

1129   two independent experiments (n=2). All values are normalized to bActin and relative to

1130   untreated of the respective time-point. Error bars indicate ± standard deviation (SD).

1131   P=phosphorylated and T=total. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001; ns,

1132   not significant (P>0.05). P-values determined by students t-test compared to untreated

1133   virus infected cells.

1134

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Table 1. Main targets of FDA-approved NRTKIs used

NRTKIs	Main target	1x dose (µM)	FDA approved conc. (µM)*	Reference(s)
Defactinib (VS-6063)	FAK, Pyk2	5	1.26**	[47]
Acalabrutinib (ACP-196)	Btk	0.5	0.69	[43, 44]
Ibrutininib (PCI-32765)	Btk, EGFR	0.5	2.05	[45, 46]
Bosutinib (SKI-606)	Abl, Src, Btk	5	1.82	[40, 41]
Nilotinib (AMN-107)	Abl, PDGFRα	1	2.44	[38, 39]
Saracatinib (AZD0530)	Src	0.125	0,37	[42]
Ruxolitinib (INCB018424)	JAK1/2	5	0.21	[37]
Tofacitinib (CP-690550)	JAK1/2/3	10	0.1	[36]

*   FDA approved concentrations calculated from orally administered dosage per 24h based on average adult person's weight according to WHO (62.0kg).

**   Defactinib FDA-approved concentration based on dosage information of orphan drug clinical studies.

Figure 1

Figure 2

A)

B)
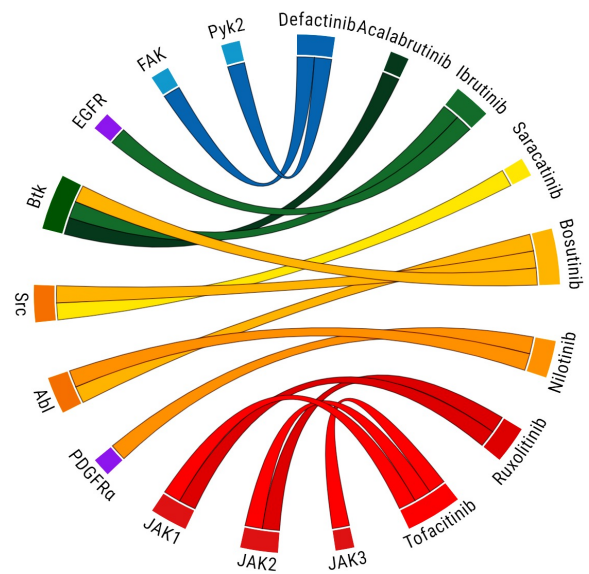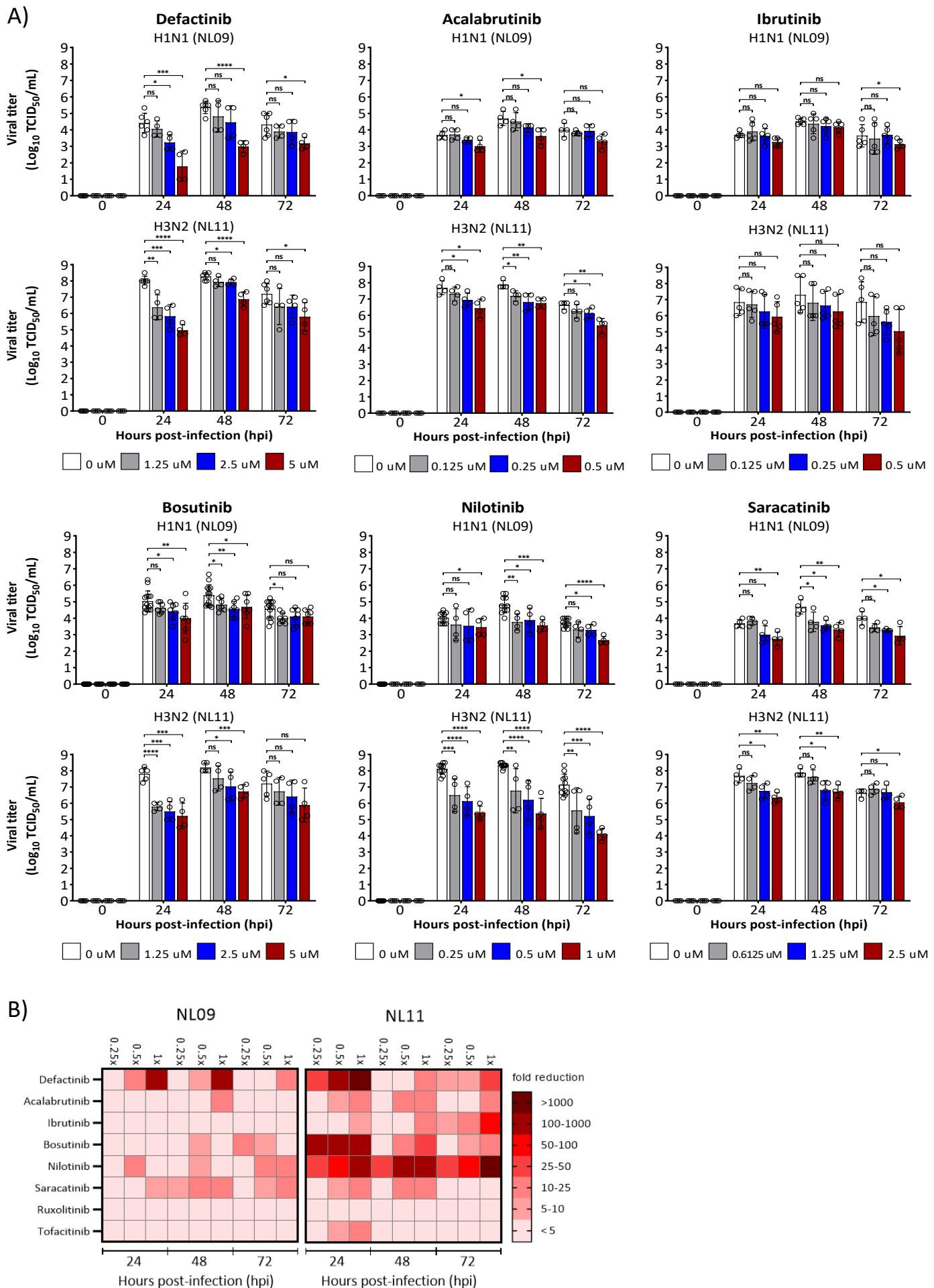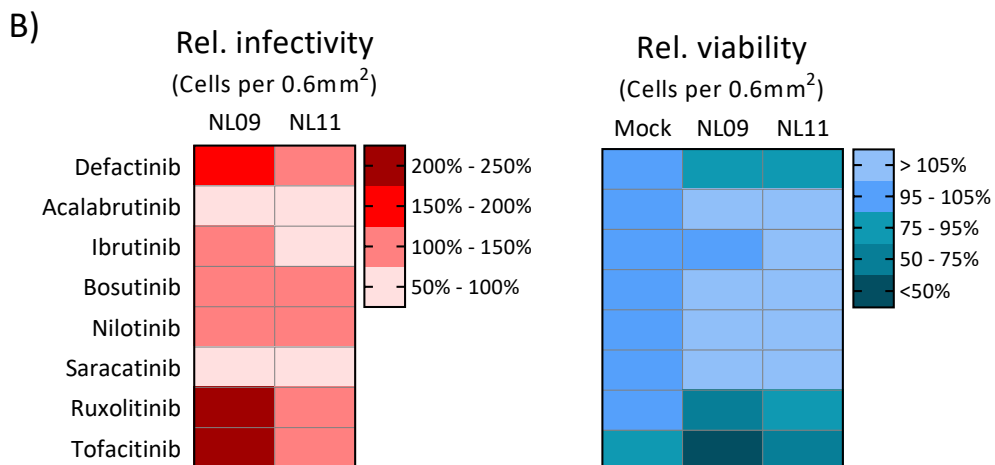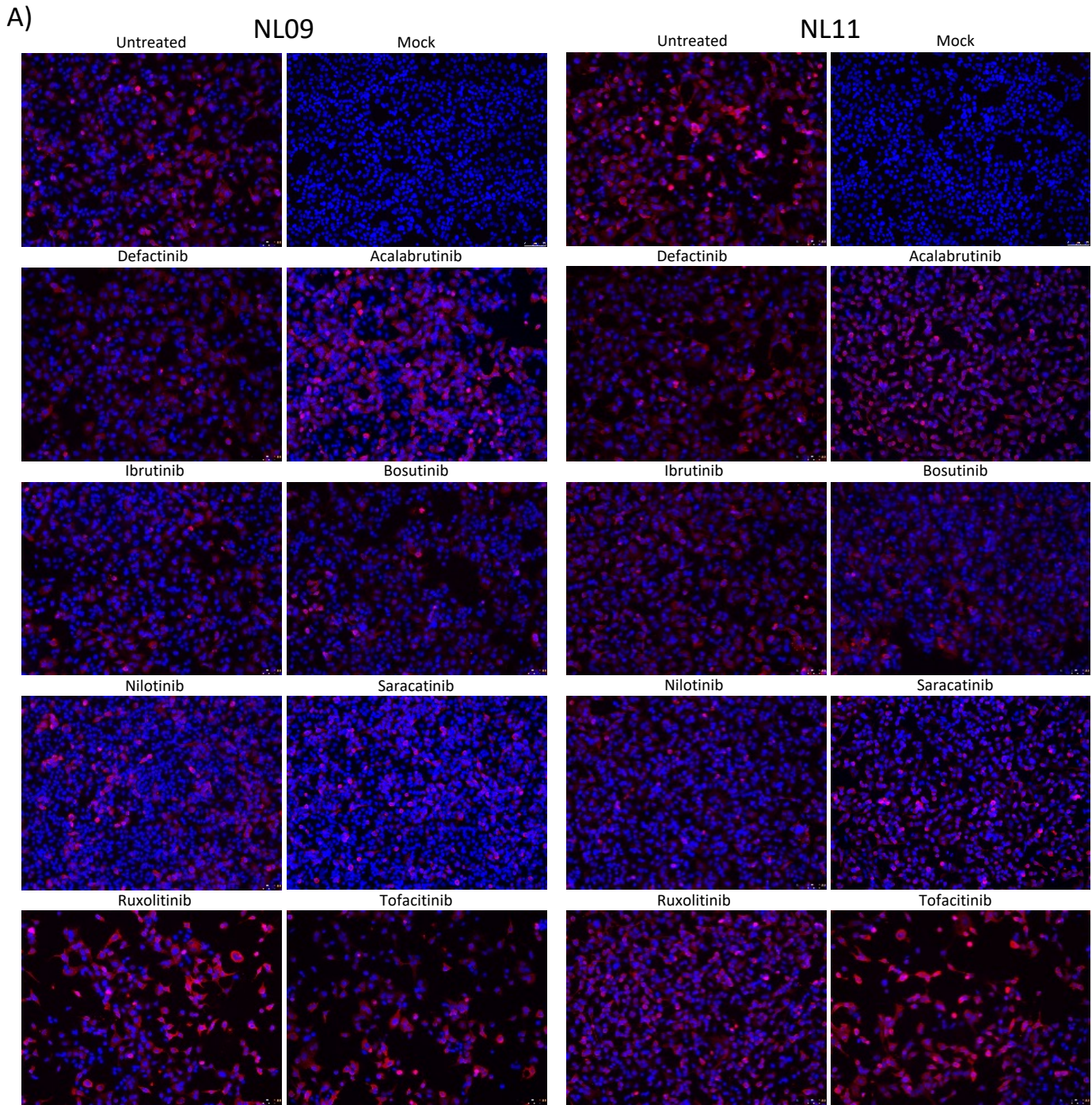
Figure 3

Figure 4

Figure 5

A)

B)
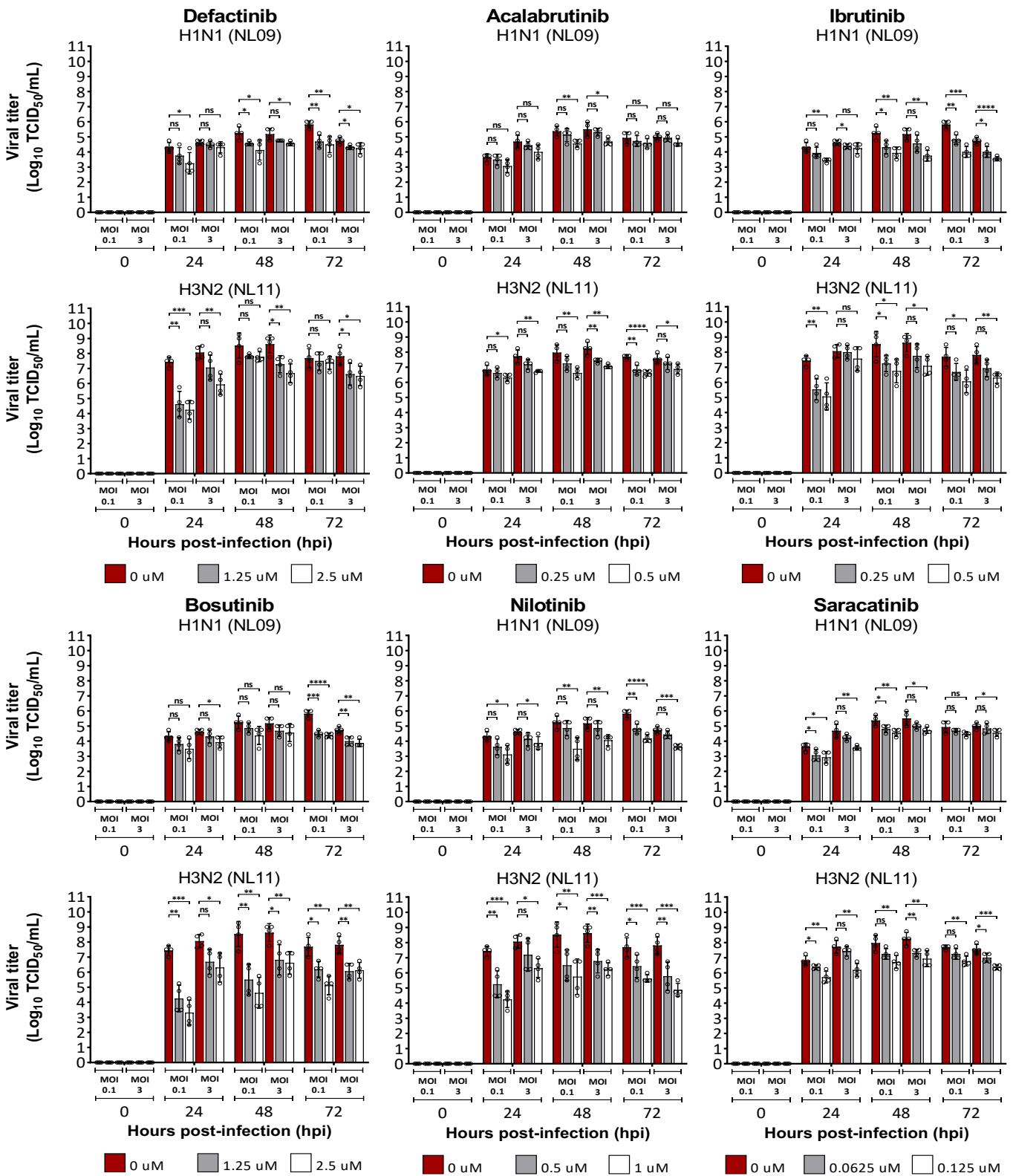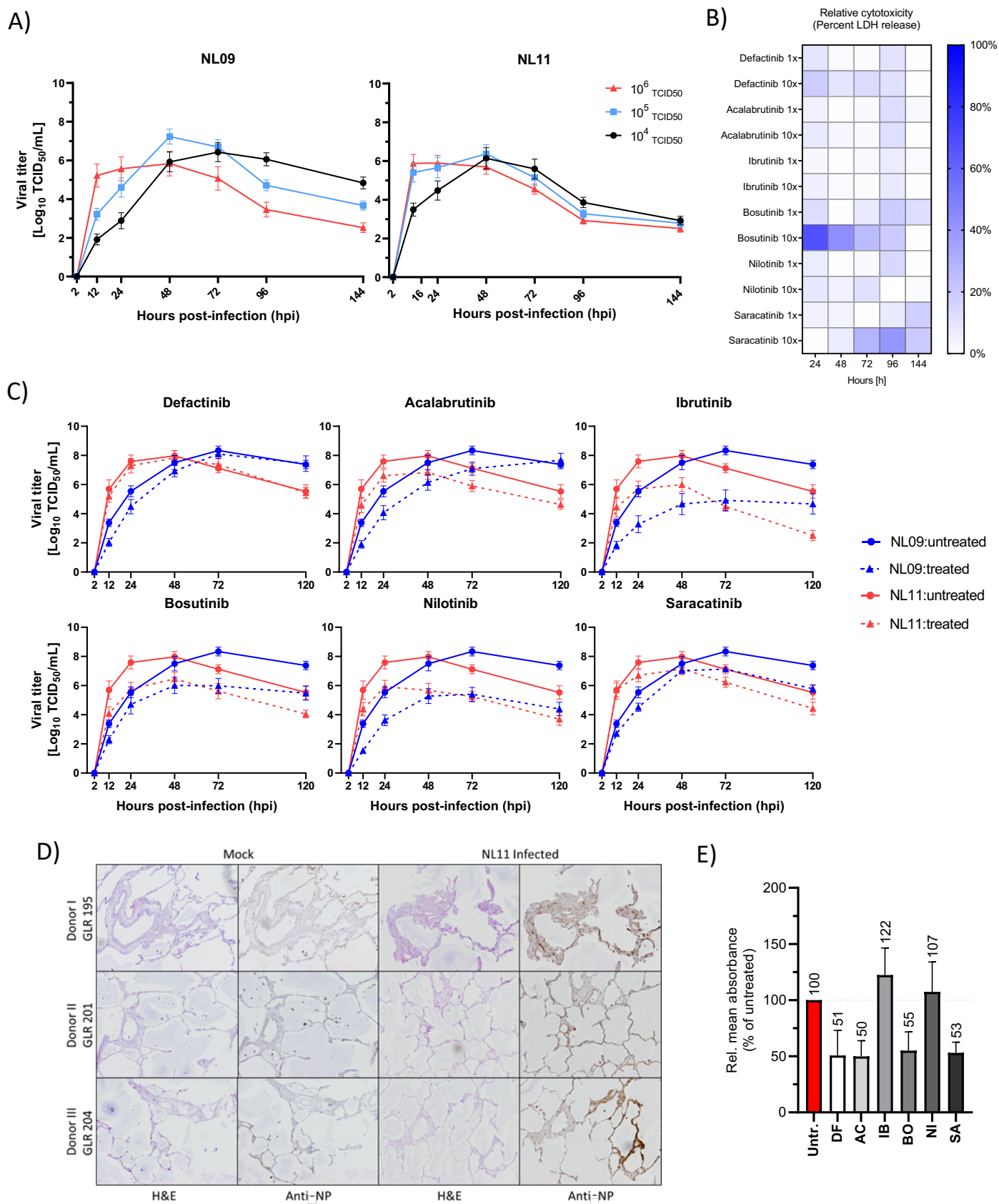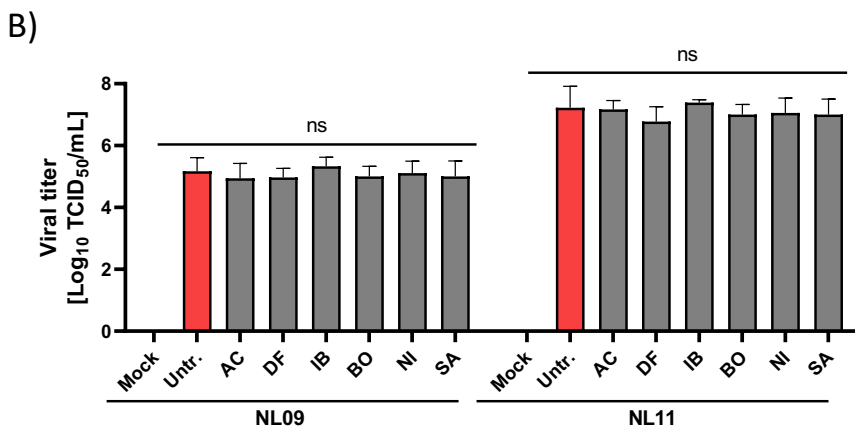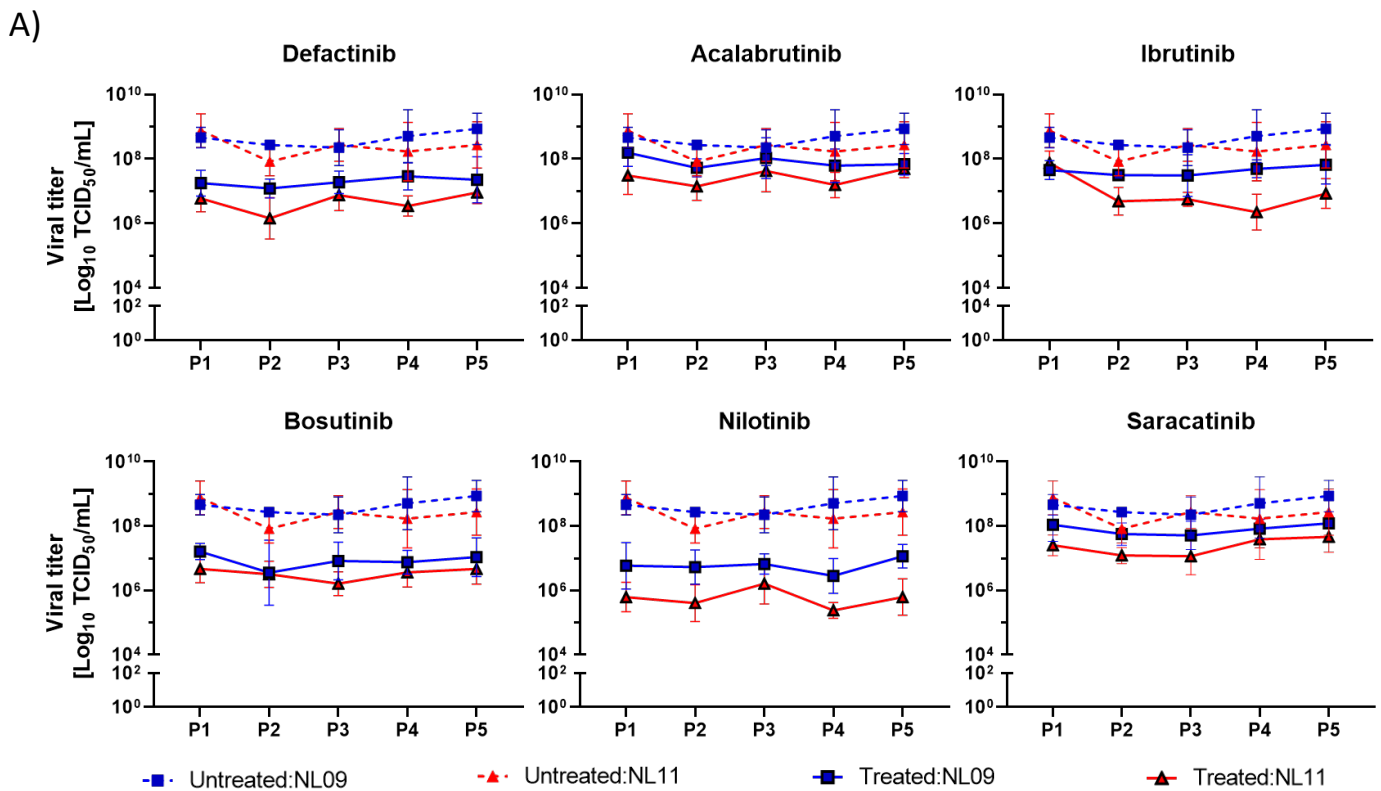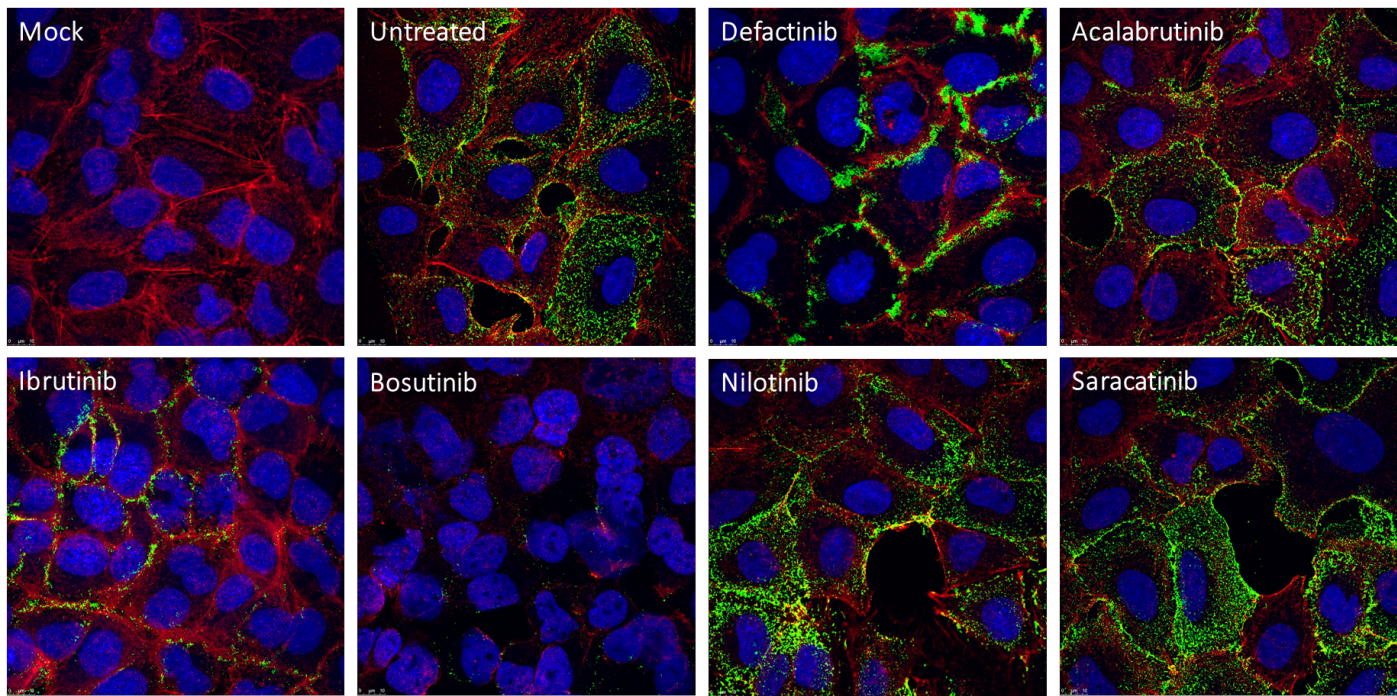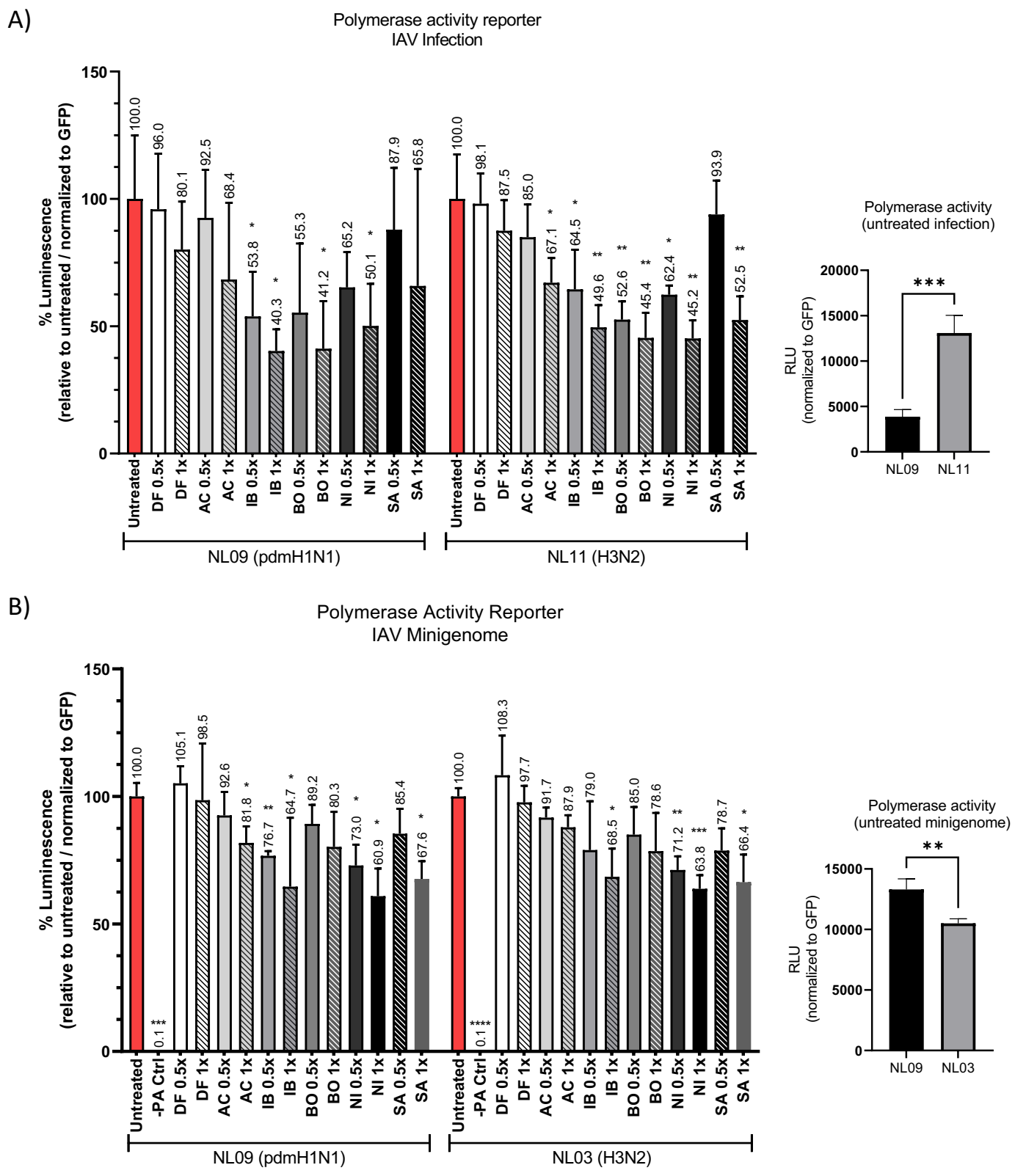
Figure 6

Figure 7

Figure 8

Figure 9

Figure 10