The mechanism of resistance to favipiravir in influenza

Daniel H. Goldhill[a,b], Aartjan J. W. te Velthuis[c], Robert A. Fletcher[b], Pinky Langat[b], Maria Zambon[a], Angie Lackenby[a], and Wendy S. Barclay[b,1]

[a]National Infection Service, Public Health England, London NW9 5EQ, United Kingdom; [b]Section of Molecular Virology, Imperial College London, London W2 1PG, United Kingdom; and [c]Division of Virology, Department of Pathology, Addenbrooke's Hospital, University of Cambridge, Cambridge CB2 2QQ, United Kingdom

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Favipiravir is a broad-spectrum antiviral that has shown promise in treatment of influenza virus infections. While emergence of resistance has been observed for many antiinfluenza drugs, to date, clinical trials and laboratory studies of favipiravir have not yielded resistant viruses. Here we show evolution of resistance to favipiravir in the pandemic H1N1 influenza A virus in a laboratory setting. We found that two mutations were required for robust resistance to favipiravir. We demonstrate that a K229R mutation in motif F of the PB1 subunit of the influenza virus RNA-dependent RNA polymerase (RdRP) confers resistance to favipiravir in vitro and in cell culture. This mutation has a cost to viral fitness, but fitness can be restored by a P653L mutation in the PA subunit of the polymerase. K229R also conferred favipiravir resistance to RNA polymerases of other influenza A virus strains, and its location within a highly conserved structural feature of the RdRP suggests that other RNA viruses might also acquire resistance through mutations in motif F. The mutations identified here could be used to screen influenza virus-infected patients treated with favipiravir for the emergence of resistance.

antiviral | resistance | polymerase | influenza | virus

Influenza viruses are single-stranded, negative-sense RNA viruses that have a significant impact on our healthcare systems and cause up to 650,000 human deaths each year (1). Current antiviral options for treatment of influenza are limited by the ease with which influenza viruses can evolve resistance (2). Licensed influenza antiviral drugs include the adamantanes and the neuraminidase inhibitors (NAIs). The adamantanes are M2 ion channel inhibitors, but they are no longer used due to widespread resistance in circulating influenza virus strains (3). NAIs, such as oseltamivir, are currently the only widely available option to treat influenza virus infections (3, 4). However, resistance to oseltamivir has also been observed following prolonged treatment (5). Furthermore, in 2007–8 the seasonal H1N1 influenza virus evolved oseltamivir resistance independently of NAI use (2, 6, 7). Current influenza virus research thus aims to develop new classes of drugs against different viral targets to give options for combination therapies and to minimize the risk of resistance emergence. Novel antiviral strategies are essential to provide a first defense to mitigate novel outbreaks and pandemics before specific vaccines are available (8).

Favipiravir is a nucleoside analog that is a promising antiviral drug targeting the viral RNA-dependent RNA polymerase (RdRP) (9–12). The drug is effective against both seasonal and avian influenza viruses in cell culture and in vivo in experimental animal models, including against strains that are resistant to NAIs (11, 13–16). Favipiravir has already been licensed in Japan for use against emerging influenza viruses resistant to other antivirals and has undergone a phase III clinical trial in the United States (10). During treatment, favipiravir is phosphorylated by cellular enzymes into its active form favipiravir-ribofuranosyl-5′-triphosphate (F-RTP) (12, 17). F-RTP does not strongly affect cellular transcription and therefore has a high selective index (12). As favipiravir targets the influenza RdRP, it could be used in combination with other antivirals such as oseltamivir, and animal studies have indeed shown that the two drugs have a synergistic effect (18, 19). Although favipiravir was initially developed to be effective against influenza virus infections, it has also been shown to be active against a wide range of negative-strand and positive-strand RNA viruses, including many for which no licensed treatment options currently exist (10, 20–22).

Influenza virus RdRP has a high intrinsic mutation rate, facilitating the notorious antigenic drift of influenza viruses in response to the host immune response and enabling them to evolve resistance to antivirals (23). It is therefore desirable that future influenza antivirals should have a high barrier to resistance. The influenza virus RdRP consists of the viral proteins PB1, PB2, and PA. All three subunits contribute to the formation of the polymerase active site, which is strongly conserved and therefore a good target for antiviral drugs as the functional constraints on the amino acids of the active site may preclude the emergence of resistance (24). So far, experimental studies have not found any mutations in influenza viruses that lead to significant resistance against favipiravir (11, 16, 25–29). In addition, no resistant mutants have yet been identified in animal models (19) or in patients treated with favipiravir (30). One study reported a small change in susceptibility to favipiravir in an H3N2 viral mutant selected for resistance to the nucleoside analog ribavirin (31). However, the effect of the resistance mutation was not reproducible in an H1N1 influenza virus strain (32), and this, together with the small change in favipiravir susceptibility, makes the clinical significance of the observed mutation debatable. So far, significant resistance in other RNA viruses to favipiravir has only been reported from experimental evolution studies in chikungunya virus (CV) (33) and enterovirus 71 (EV-71) (34). However, both of these viruses are positive-stranded

Significance

Favipiravir is a broad-spectrum antiviral that has shown promise in treatment of influenza virus infections, in particular due to the apparent lack of emergence of resistance mutations against the drug in cell culture or animal studies. We demonstrate here that a mutation in a conserved region of the viral RNA polymerase confers resistance to favipiravir in vitro and in cell culture. The resistance mutation has a cost to viral fitness, but this can be restored by a compensatory mutation in the polymerase. Our findings support the development of favipiravir-resistance diagnostic and surveillance testing strategies and reinforce the importance of considering combinations of therapies to treat influenza infections.

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Data deposition: The sequence data reported has been deposited in the European Nucleotide Archive, https://www.ebi.ac.uk/ena/data/view/PRJEB27963, under project no. PRJEB27963. The code used to run the sequence analyses can be found at https://github.com/Flu1/Favipiravir.

[1]To whom correspondence should be addressed. Email: w.barclay@imperial.ac.uk.

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RNA viruses, which have lower baseline susceptibility to favipiravir and it is unclear whether robust resistance in negative-strand RNA viruses can be attained.

In the present study, we describe a mutation in the influenza A virus PB1 gene that confers resistance to favipiravir. The fitness cost of this mutation, resulting from a decrease in polymerase activity, is compensated for by a mutation in the PA gene that can restore polymerase activity and viral growth, while maintaining favipiravir resistance. We use in vitro analyses and structural modeling to link favipiravir resistance to a molecular change in the RdRP active site that affects nucleotide incorporation. Our findings suggest that a universal mechanism for favipiravir resistance exists. Together, these observations are important for the use of favipiravir as an antiviral drug against influenza virus infections in the present and future.

Materials and Methods

Methods for cell culture, virus growth, recombinant virus generation, minigenome assays, sequencing, and in vitro replication assays are described in *SI Appendix, Supplemental Materials and Methods*.

Results

Passaging with Favipiravir Led to Evolution of Resistance. Influenza A/England/195/2009 (Eng195) is a prototypical first wave virus from the 2009 H1N1 pandemic. Replication of Eng195 is sensitive to inhibition by favipiravir. Virus yield from Madin-Darby canine kidney (MDCK) cells infected with Eng195 was decreased by three orders of magnitude in the presence of 10 μM favipiravir (Fig. 1*A*). To identify mutations that may confer resistance to favipiravir, we passaged three replicate populations of Eng195 for 10 passages with constant exposure to favipiravir (F1–3) at a dose of 3 or 3.5 μM. This concentration was chosen as it provided a strong selective pressure, resulting in a robust (~99%) reduction in Eng195 virus plaques at 48 h (Fig. 1*A*), while still allowing for a high enough population size to allow for maintenance of the population in presence of favipiravir. Control populations (C1–3) were passaged in parallel in the absence of drug. Each population was titrated every second passage and diluted to maintain approximately a constant and low multiplicity of infection (MOI) over the course of the experiment. The titer of the drug-passaged population showed some oscillation, perhaps due to the generation and subsequent dynamics of defective interfering particles (35), whereas titers of the control-passaged population remained relatively constant (*SI Appendix*, Fig. S1*A*).

After 10 passages, the control- and drug-passaged populations were tested in a virus inhibition assay to determine whether they had altered susceptibility to favipiravir (Fig. 1*B*). While the ancestral virus Eng195, and the three control populations (C1–3) remained susceptible, the populations that had been passaged in the presence of favipiravir showed significant growth in the presence of the drug, most notably populations F2 and F3 whose viral yield after propagation in 3.5 μM favipiravir was significantly higher than Eng195 and not significantly different from the titer attained in the absence of drug (one-way ANOVA; F2: $P < 0.001$ and F3: $P < 0.001$). Next-generation sequencing showed a fixed lysine 229 to arginine (K229R) mutation in PB1 in populations F2 and F3. No fixed mutations were detected in population F1 and we did not analyze this population further. Although, there were no other fixed mutations, there were many polymorphic sites in the F2 and F3 populations that were not present in the control populations. Four plaques were purified from the F2 population, all of which showed resistance to favipiravir in a virus inhibition assay (*SI Appendix*, Fig. S1*B*). Sanger sequencing of the PB1, PB2, and PA genes confirmed the presence of the K229R mutation in PB1 in all four clones and revealed the presence of an additional proline 653 to leucine mutation (P653L) in the PA subunit in all four clones. Further inspection of the next-generation sequencing data of the F2 and F3 populations showed that the P653L mutation was present in both populations, albeit at a low level (6% in population F2 and 5% in F3). The low level of the P653L mutation may have been caused by defective interfering particles that only contained the K229R mutation and not the P653L mutation. Neither the K229R nor the P653L mutation was seen in F1 or any of the control populations.

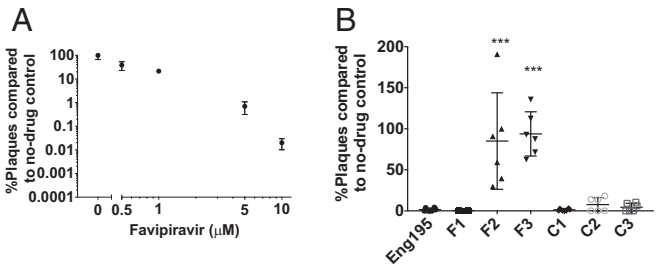

Fig. 1. Experimental evolution leads to resistance to favipiravir following 10 passages. (*A*) Virus inhibition assay of Eng195 by favipiravir. Supernatants were titered following 48 h of growth on MDCK cells. Error bars are ±SD, $n = 3$. (*B*) Virus inhibition assay with 3.5 μM favipiravir for Eng195 and passage 10 populations. Error bars are ±SD, $n = 6$, one-way ANOVA with Dunnett's multiple comparison test, $***P < 0.001$.

K229R Confers Resistance to Favipiravir. To demonstrate whether the PB1 K229R, the PA P653L, or a combination of both mutations was responsible for the favipiravir resistance observed in the F2 and F3 populations, we introduced the K229R and P653L mutations into PB1 and PA expression plasmids and tested the reconstituted polymerase activity of the mutant viral polymerases in presence or absence of favipiravir using a minigenome assay (Fig. 2). In the absence of favipiravir, the PB1 K229R mutant showed a 30-fold reduction in polymerase activity compared with the wild type (Fig. 2*A*). By contrast, the polymerase containing the PA P653L mutation showed a 5-fold higher activity compared with the wild-type polymerase. The luciferase signal in the wild-type polymerase minigenome assay was inhibited in presence of increasing concentrations of favipiravir (3% activity at 100 μM). However, the PB1 K229R mutant showed only a slight decrease in activity in the presence of the drug, implying that the K229R mutation rendered the polymerase resistant to favipiravir (79% activity at 100 μM) (two-way ANOVA, $P < 0.001$) (Fig. 2*B*). The favipiravir sensitivity of the P653L mutant was not significantly different from the wild-type polymerase (two-way ANOVA, $P = 0.08$). Importantly, minigenome assays of a mutant polymerase containing both PA P653L and PB1 K229R mutations showed a 4-fold higher level of polymerase activity compared with wild type, while the favipiravir resistance was similar to PB1 K229R (no loss of activity until >20 μM and 57% activity at 100 μM, two-way ANOVA, $P = 0.53$). This implies that P653L can compensate for the loss of activity in the K229R mutant without affecting favipiravir resistance in cell culture.

To verify the above results, we next generated recombinant Eng195 viruses containing either the PA P653L or PB1 K229R mutation, or both mutations. Multicycle growth curves performed in MDCK cells showed that, in comparison with the wild-type Eng195 virus, the K229R virus was attenuated (one-way ANOVA, $P < 0.001$ at 48 h), while there was no difference in growth for the P653L virus (one-way ANOVA, $P = 0.052$) or the virus containing both mutations (one-way ANOVA, $P = 0.12$) (Fig. 3*A*). At 24 h, the P653L virus titer was significantly greater than Eng195 (one-way ANOVA, $P = 0.003$). In a viral yield experiment in presence of different drug doses, the K229R + P653L double mutant virus was ~30-fold less susceptible to favipiravir than the wild-type virus (IC_{90} 55 μM vs. 1.8 μM) (Fig. 3*B*). Taken together, these results suggest that K229R in PB1 confers resistance to favipiravir to the influenza virus polymerase in cell culture and that P653L in PA compensates for a fitness cost for resistance.

K229R Mutation in PB1 Confers Favipiravir Resistance to RdRP of Other Influenza A Virus Subtypes. To test if the resistance mutations identified above also conferred resistance to other

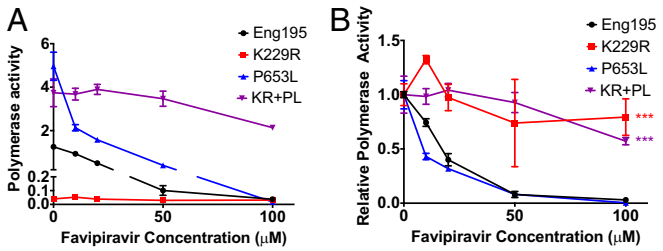

Fig. 2. K229R and P653L combine to give resistance to favipiravir in a minigenome assay. (*A*) Minigenome assay with Eng195, PB1 K229R, PA P653L, and PB1 K229R + PA P653L in presence of increasing concentrations of favipiravir. Polymerase activity is given as Firefly/Renilla. (*B*) Relative polymerase activity to no drug of data presented in *A*. Error bars are ±SD, $n = 3$, two-way ANOVA with Tukey's honestly significant difference (HSD), ***$P < 0.001$.

influenza virus strains, we next engineered the K229R mutation into the PB1 subunit and the P653L mutation into the PA subunit of a historic H3N2 human influenza virus (Fig. 4*A*) and a recently emerged H7N9 avian influenza virus (Fig. 4*B*). Reconstituted polymerases harboring the K229R mutation displayed resistance to favipiravir to a similar degree as Eng195 polymerase in a minigenome assay (Fig. 4 *A* and *B*). Interestingly, the cost to polymerase activity of the K229R mutation in H7N9 and H3N2 was not as severe as for the Eng195 RNA polymerase (Fig. 3*A*). Moreover, by contrast to Eng195, introduction of the P653L mutation in the H3N2 and H7N9 RNA polymerases did not raise polymerase activity. However, similar to Eng195 RNA polymerase, the P653L mutation did not provide resistance to favipiravir in the H3N2 and H7N9 polymerases, but compensated for the small reduction in K229R polymerase activity entirely when combined with K229R in the H7N9 RNA polymerase (Fig. 4*B*) and recovered more than 40% of the activity in the H3N2 RNA polymerase (Fig. 4*A*). Our results suggest that the combination of the PB1 and PA mutations provides a general mechanism by which resistance to favipiravir can be conferred to many, if not all, influenza A viruses.

K229R Prevents Mutations in the Presence of Favipiravir. We and others have shown that favipiravir induces mutations in the viral genome (25, 36). One possible mechanism by which the K229R mutation might confer resistance to favipiravir would be to increase the fidelity of the polymerase, as has been observed for ribavirin resistance in several viral systems (37). To investigate whether the K229R, the P653L, or the double mutant conferred resistance to other nucleoside analogs, such as ribavirin (19), we tested the susceptibility of the double mutant to ribavirin in a minigenome assay. We found no decrease in relative susceptibility to ribavirin compared with the wild-type polymerase (Fig. 5*A*) (two-way ANOVA, $P = 0.95$). This observation suggests that the K229R mutation confers specific resistance to favipiravir and likely does not confer favipiravir resistance by increasing polymerase fidelity. To rule out that the K229R mutation confers resistance to favipiravir by reducing the RdRP error rate, we used next-generation sequencing to quantify viral mutations. To ensure that the provided input template was well defined and that our analysis was not complicated by selective pressures or loss of RNA expression resulting from mutations induced in virally encoded polymerase genes, we sequenced the population of RNAs replicated by the viral polymerase in a minigenome assay. To ensure that we were able to identify polymerase errors correctly, ignore sequencing errors, and count every mutation only once, we used primer ID, a method in which a pool of barcoded primers is used during reverse transcription to label each viral RNA with a unique molecular identifier (UMI) (38). Although this method can be used to construct a consensus sequence for each unique cDNA molecule to remove PCR or sequencing errors, the method is still sensitive to errors introduced by the reverse transcriptase. To minimize this issue, we measured the mutation rate of the polymerase mutants relative to the wild type in the absence of drug.

In the absence of favipiravir, we observed no large differences between the relative mutation frequency in RNA products produced by the different polymerases (Fig. 5*B*). However, as the favipiravir concentration was increased, we observed a higher mutation frequency in wild-type polymerase products relative to the mutation frequency in the absence of drug. As we and others have previously reported, this was due to transition mutations (*SI Appendix*, Fig. S2) (25, 28, 36). The mutation frequency observed for the P653L mutant was higher compared with the wild-type polymerase at each drug concentration (two-way ANOVA, $P < 0.001$). By contrast, the polymerase containing the PB1 K229R mutation produced RNA that contained significantly fewer errors in the presence of favipiravir compared with the wild type (two-way ANOVA, $P < 0.001$), even at high drug concentrations. The double mutant containing both PB1 K229R and PA P653L polymerase mutations displayed a similar relative mutation frequency as the PB1 K229R mutant (two-way ANOVA, $P = 0.83$).

The K229R Mutant Shows Reduced Favipiravir Incorporation During Transcription in Vitro. To study the effect of the PB1 K229R and PA P653L mutations in more detail, we expressed recombinant polymerases harboring either of the single mutations or both mutations together and purified the recombinant polymerases using a Protein A tag on the C terminus of the PB2 subunit. All were able to form heterotrimeric complexes with equal ratios of PB1, PB2, and PA, as shown by SDS/PAGE followed by silver staining or Western analysis (*SI Appendix*, Fig. S3).

We next measured the activity of the wild-type and mutant polymerases in a transcription assay, in which a ^{32}P-radiolabeled capped primer is extended after base pairing with a short template representing the influenza virus promoter (Fig. 6*A*). In this assay, a capped primer terminating in A-G, the primer sequence that is preferred during influenza virus infections in cell culture, preferentially initiates from the 3′ UC (G product), but can also initiate from the 3′ U (C product) (39) (Fig. 6*A*). Analysis of the total polymerase activity summing both products in the presence of all four nucleotides showed no significant difference between the mutant and wild-type polymerase (Fig. 6*B*).

We next modified the in vitro transcription assay to assess the incorporation of favipiravir into nascent RNA, by performing the assay in the absence of ATP and GTP with which F-RTP would normally compete. When CTP was the only nucleotide provided, we observed extension of the primer to a +1 product by incorporation of CTP opposite 3G (Fig. 6*C*). Further extension of the RNA product was observed in the presence of CTP + GTP for both polymerases. However, in the presence of CTP + F-RTP, only the wild-type polymerase generated a +2 product (Fig. 6*D*). Since both possible +2 products, whether a G product (Cap-AGCF) or, the much less likely, C product (CapAGFC) require F-RTP

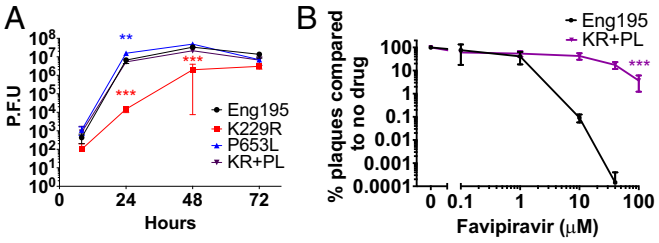

Fig. 3. PB1 K229R has a cost to viral growth that is rescued by PA P653L. (*A*) Viral growth curves on MDCKs at MOI = 0.002 for Eng195, PB1 K229R, PA P653L, and PB1 K229R + PA P653L. (*B*) Virus inhibition assay for Eng195 and PB1 K229R + PA P653L in presence of increasing concentrations of favipiravir. Error bars are ±SD, $n = 3$, one-way ANOVA with Dunnett's multiple comparison test, **$P < 0.01$, ***$P < 0.001$.

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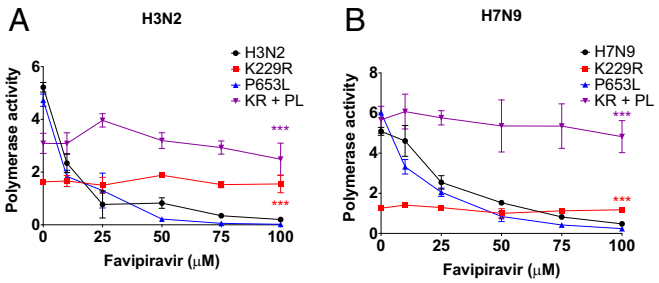

Fig. 4. PB1 K229R confers resistance to favipiravir in other influenza A polymerases. (*A*) Minigenome assay with H3N2 A/Victoria/3/1975, PB1 K229R, PA P653L, and PB1 K229R + PA P653L in presence of increasing concentrations of favipiravir. (*B*) Minigenome assay with H7N9 A/Anhui/1/2013, PB1 K229R, PA P653L, and PB1 K229R + PA P653L in presence of increasing concentrations of favipiravir. Error bars are ±SD, $n = 3$, two-way ANOVA with Tukey's HSD, $***P < 0.001$.

incorporation, this suggests that the K229R mutation prevented incorporation of F-RTP in vitro.

The K229R Mutant Shows Reduced Favipiravir Incorporation During Replication in Vitro. To confirm that the K229R mutant provides resistance to F-RTP in vitro, we performed a replication assay. In contrast to transcription initiation, influenza virus replication does not use a capped RNA primer and does not create different extension products. To assess F-RTP incorporation during replication, we performed an ApG primed reaction that used [α -^{32}P]GTP incorporation as readout (Fig. 6*E*). In the absence of F-RTP, both polymerases efficiently produced a full-length 14-nt product (+12) (Fig. 6*F*). F-RTP effectively competed with labeled GTP in reactions containing the wild-type polymerase, resulting in a reduction of the measured signal. In contrast, in reactions with the K229R polymerase efficient GTP incorporation was observed even when F-RTP was present (Fig. 6 *F* and *G*), confirming that the K229R mutation reduces F-RTP incorporation in vitro. When the same experiment was repeated with the polymerase with single P653L mutation or the double mutant, the polymerase with the K229R mutation in PB1 incorporated GTP in presence of F-RTP, but P653L alone did not (Fig. 6*G*).

PB1 Amino Acid 229 Resides in Motif F. To gain structural insight into the mechanism of favipiravir resistance in influenza A viruses, we mapped the PB1 K229 and PA P653 residues onto the bat H17N10 influenza A polymerase structure using sequence alignment (40) (Fig. 7*A*). We found that PB1 K229 is conserved among influenza A virus strains and forms part of motif F, which contributes to NTP binding (41). By contrast, PA P653 varies among influenza virus polymerases and is equivalent to A648 in the bat influenza virus.

Superposing initiation and elongation complexes from other RdRPs onto the bat influenza A virus polymerase active site showed that K229 is likely to interact with the incoming base (Fig. 7*B*). Modeling of the K229R mutation into the bat influenza polymerase structure shows that it reduces the NTP binding space of the active site (Fig. 7*C*), in line with a previous suggestion that this mutation may impede NTP access to the active site (42).

PA P653L Preferentially Initiates Transcription from 3′ U. Next, we considered what effects the P653L mutation in PA might have on the polymerase. The structural alignment shows that P653 resides at the end of a helix that establishes contacts between motif F to the thumb domain and may therefore affect the position and orientation of residues in motif F and their interaction with incoming NTPs (Fig. 7*B*). In a transcription assay with labeled capped primer terminating in AG and 3′ 4U template we noted significantly less G-product relative to C-product synthesis by the P653L-containing mutants (*SI Appendix*, Fig. S4). This suggests that the P653L mutation had changed either how the template is positioned in the active site, how the primer is bound by the polymerase, how important Watson–Crick base pairing is for base pairing between the template and the incoming base, or a combination of these possibilities.

Discussion

Previous studies suggested that influenza virus resistance against favipiravir was unlikely to evolve and multiple passaging studies failed to find resistant viruses (25, 26). Pauly and Lauring suggest that there is only a narrow window in which resistance to nucleoside analogs can evolve and that too high a concentration of such a drug will lead to lethal mutagenesis and population extinction (43). We propose that our study succeeded in evolving resistance by maintaining a sufficient population size of virus, while maintaining selective pressure through continuous exposure to favipiravir. Importantly, we found that a combination of two mutations in the influenza A virus RNA polymerase were necessary for robust resistance. The key mutation was K229R in the PB1 subunit, which prevented the incorporation of favipiravir into nascent viral RNA by polymerases from H1N1, H3N2, and H7N9 influenza A viruses albeit at a cost to the RNA polymerase activity in cell culture assays. This cost was compensated for by a P653L mutation in the PA subunit. In the context of infectious virus, PB1 K229R reduced the mutagenic effect of favipiravir at a cost to growth and this effect could be alleviated by PA P653L. The combination of the two mutations led to a virus that was 30-fold less susceptible to favipiravir relative to the wild-type virus and not impaired in replication kinetics. The fitness cost imparted by the K229R mutation in PB1 and the requirement for a second compensatory mutation to restore viral fitness may explain why resistance has not been observed in other studies.

Although many nucleoside analogs have pleiotropic effects, for influenza, as well as other viruses, the majority of mutations that have been described to give resistance to ribavirin or other nucleoside analogs have increased the fidelity of the virus (31, 37, 44). We observed that the K229R mutation did not confer any resistance to ribavirin or increase polymerase fidelity in general. This further suggests that favipiravir has a different mechanism of action from ribavirin, which may help explain the synergistic effect of favipiravir and ribavirin when used in combination (21, 45, 46).

Our structural modeling showed that K229 resides in motif F of the fingers subdomain, while PA P653 resides in the so-called thumb subdomain of the RNA polymerase. K229 is strictly conserved in the PB1 subunit of RNA polymerases of different influenza subtypes and within motif F of other single-stranded RNA viruses (47). Our modeling shows that K229 likely interacts with the base of the NTP that is added to the growing RNA

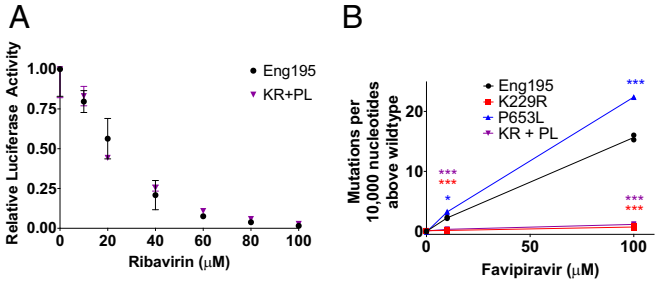

Fig. 5. PB1 K229R does not confer resistance to ribavirin but prevents mutations caused by favipiravir. (*A*) Minigenome assay showing relative polymerase activity for Eng195 and K229R + P653L in presence of increasing concentrations of ribavirin. Error bars are ±SD, $n = 3$, two-way ANOVA. (*B*) Individual mutations per 10,000 sequenced nucleotides above the control (0 μM, Eng195) measured by primer ID at 0, 10, and 100 μM favipiravir from RNA expressed in a minigenome assay with Eng195, PB1 K229R, PA P653L, or PB1 K229R + PA P653L polymerase. $n = 2$, two-way ANOVA with Dunnett's multiple comparison test. $*P < 0.05$, $***P < 0.001$.

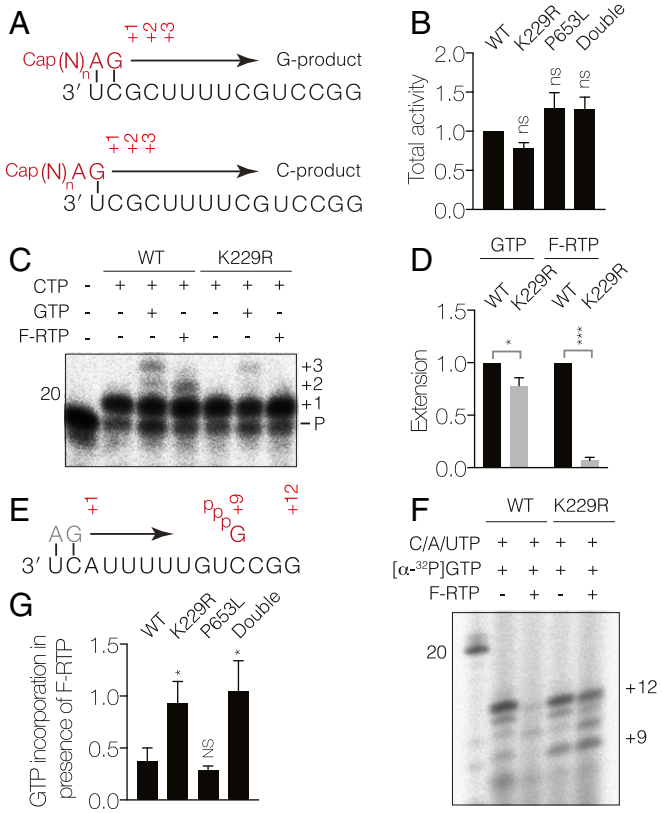

Fig. 6. Resistance to favipiravir triphosphate incorporation by the influenza A virus RNA polymerase in vitro. (*A*) Schematic showing how a radiolabeled capped primer (red) binds the 3′ terminus of the 3′ "4C template." Initiation can start at 3′ G3 of the template giving the G product or at 3′ C2 giving the C product. (*B*) Total polymerase activity of the wild-type influenza A virus polymerase and the three mutants on the 3′ 4C template quantified by combining C and G products. (*C* and *D*) Gel analysis and quantitation of the activity of the wild type or PB1 K229R mutant influenza A virus RNA polymerase on the 3′ 4C template provided with CTP and either GTP or 500 μM F-RTP. The radiolabeled capped primer signal is indicated with P. (*E*) Schematic showing how an ApG dinucleotide primer (gray) binds the 3′ terminus of "promoter up" template. Initiation starts at 3′ A3 of the template by the incorporation of UTP (+1 product). The first [α -^{32}P]GTP is incorporated opposite C11 (+9 product). (*F*) Gel analysis of the replication activity of the wild type or PB1 K229R mutant polymerase on the promoter up template in the absence or presence of 500 μM F-RTP. (*G*) Quantitation of the replication assay performed with wild type, K229R, P653L, or double mutant polymerase. Error bars are ±SD, $n = 3$, one-way ANOVA with Dunnett's multiple comparison test in *B* and *G*, and two-sided *t* test in *D*, $*P < 0.5$, $***P < 0.001$, ns, not significant.

molecule. Our in vitro studies using F-RTP (Fig. 6) imply that the K229R mutation prevents F-RTP incorporation into nascent RNA during both viral transcription and replication. The increased side chain size of arginine compared with lysine reduces the size of the NTP binding pocket at position 229 of the active site. We hypothesize that the diminished space precludes correct orientation of the analog and interferes with its incorporation (Fig. 7*C*). This occlusion of the NTP binding pocket likely reduces the efficiency of normal NTP incorporation in cell culture as well and may account for reduction in both polymerase activity and viral growth of the single K229R mutant (Figs. 2*A* and 3*A*).

P653 is part of the thumb subdomain and interacts with motif F (Fig. 7*B*), effectively closing the active site. It is tempting to speculate that the proline-to-leucine mutation makes the connection between the fingers and thumb subdomains more flexible, which could alleviate the decrease in nucleotide binding space that results from the K229R mutation. The increase in mutation frequency that we observe for P653L in the presence of favipiravir (Fig. 5*B*) suggests that this mutation alone reduces polymerase fidelity, which is supported in part by the change in transcription initiation mode in vitro (*SI Appendix*, Fig. S4). As the P653L mutant is not critical for favipiravir resistance per se, we will address the contribution of P653 to polymerase activity in more detail elsewhere. It is interesting to note that mutations interacting epistatically in PA and PB1 have previously been shown to provide resistance to other nucleoside analogs without any change in the fidelity of the RdRP (32). However, the mechanism of resistance described by Pauly et al. (32) is likely different from our findings as the location of the mutations is distant from K229R and the site of nucleotide incorporation.

Despite favipiravir having been shown to inhibit a wide range of RNA viruses, there are few published reports of resistance. Interestingly, Delang et al. (33) showed that the key mutation that led to favipiravir resistance of chikungunya virus was also a K-to-R mutation in motif F of the viral polymerase and this mutation is equivalent to the PB1 K229R mutation we report here. We found a 30-fold increase in resistance due to the K-to-R mutation at this position in the influenza A virus polymerase, compared with an ∼2× increase in resistance in the chikungunya mutants. However, bearing in mind that favipiravir is ∼100× less effective against chikungunya than against influenza virus, this discrepancy is not unexpected. Analysis of a single K-to-R mutation in motif F of the Coxsackie B3 virus polymerase showed that this mutation was incompatible with viral growth (42). This implies that the fitness cost of this mutation may be too large in some RNA viruses and that appropriate compensatory mutations would be required to tolerate resistance. Given the conservation of motif F in RdRPs, we suggest that K-to-R mutation at the same position, may lead to favipiravir resistance in polymerases of a wide range of viruses.

Although, we have demonstrated a mechanism of resistance to the mutagenic effect of favipiravir, it is possible that favipiravir

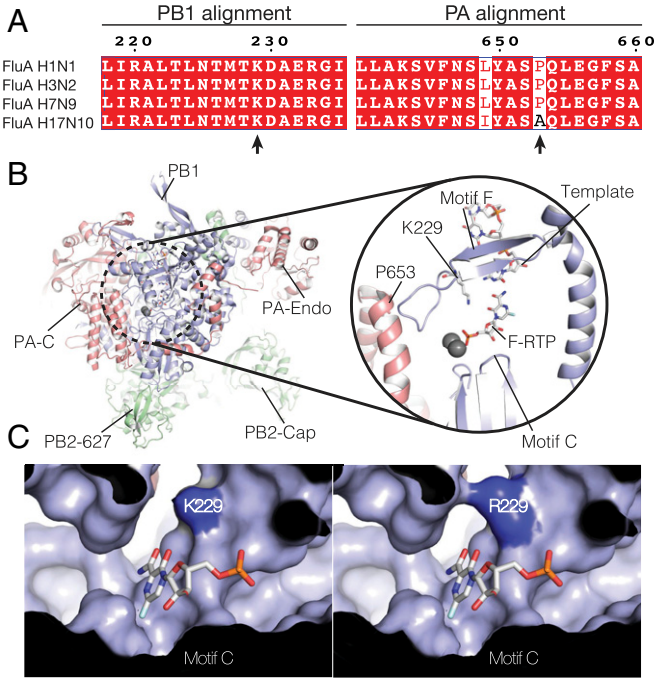

Fig. 7. Alignment and structural modeling of the PB1 K229R mutation. (*A*) Sequence alignments of PB1 and PA subunits of the influenza virus RdRP. Mutations present in favipiravir-resistant virus indicated by black arrows. (*B*) Cartoon model of F-RTP binding the influenza A virus RdRp. Motifs C and F are indicated as well as the helix of the PA C terminus (PA-C) that contains residue P653. (*C*) Surface models of F-RTP binding by the wild-type influenza A virus and by the K229R mutant. The location of motif C is indicated.

has other nonmutagenic mechanisms of action. Several papers have described that favipiravir could act as a chain terminator under certain circumstances (16, 36, 48) and Dulin et al. (49) recently showed how T-1106, a compound closely related to favipiravir, caused long pauses and backtracking in the RdRP of poliovirus. Additional research would be needed to determine whether the nonmutagenic mechanisms of favipiravir play a significant role in inhibiting influenza and whether the K229R mutation could also provide resistance to these alternative mechanisms.

Our research has implications for the treatment of influenza virus infection. The double mutant polymerase containing both P653L and K229R has wild-type polymerase activity while still preventing the incorporation of favipiravir, and it will be important to assess the transmissibility and pathogenicity of this virus in future studies. Although it remains unknown whether the two mutations found here would confer resistance in a clinical setting, use of current drugs for influenza is hampered by the evolution of resistance. A(H7N9) is an avian influenza virus that has emerged in China and has been assessed as having high pandemic potential (50). Human A(H7N9) cases have been treated with oseltamivir, a NAI, but resistance has been observed and these resistant viruses are transmissible between ferrets (50). Favipiravir is currently licensed in the event of a pandemic of NAI-resistant H7N9. PB1 K229 and PA P653 are conserved in H7N9 as well as other circulating influenza A viruses and we show here that mutations at these positions lead to resistance in the H7N9 polymerase. It is therefore possible that favipiravir resistance may evolve through these mutations in H7N9 and other influenza A virus subtypes. We suggest that if favipiravir is used to treat patients, these sites should be monitored for the potential evolution of resistance, and that using combinations of antiviral drugs that minimize the chance of resistance emerging is recommended.

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