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Received: 22 October 2021

Accepted: 26 January 2022

Accelerated Article Preview

Published online: 07 February 2022

Cite this article as: Schultz, D. C. et al.

Pyrimidine inhibitors synergize with nucleoside analogues to block SARS-CoV-2. *Nature* <https://doi.org/10.1038/s41586-022-04482-x> (2022).

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Pyrimidine inhibitors synergize with nucleoside analogues to block SARS-CoV-2

<https://doi.org/10.1038/s41586-022-04482-x>

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The SARS-CoV-2 virus has infected more than 261 million people and led to more than 5 million deaths in the last year and a half¹ (WHO.org). SARS-CoV-2-infected individuals typically develop mild to severe flu-like symptoms, while infection of a subset of individuals leads to severe to fatal clinical outcomes². While vaccines have been rapidly developed to combat SARS-CoV-2, there has been a dearth of antiviral therapeutics. There is an urgent need for therapeutics which has been amplified by the emerging threats of variants that may evade vaccines. Large-scale efforts are underway to identify antiviral drugs and we screened ~18,000 drugs for antiviral activity using live virus infection in human respiratory cells and validate 122 drugs with antiviral activity and selectivity against SARS-CoV-2. Amongst these candidates are 16 nucleoside analogues, the largest category of clinically used antivirals. This included the antivirals remdesivir and molnupiravir, which have been approved for use in COVID-19. RNA viruses rely on a high supply of nucleoside triphosphates from the host to efficiently replicate, and we identified a panel of host nucleoside biosynthesis inhibitors as antiviral. Moreover, we found that combining pyrimidine biosynthesis inhibitors with antiviral nucleoside analogues synergistically inhibits SARS-CoV-2 infection in vitro and in vivo against emerging strains of SARS-CoV-2 suggesting a clinical path forward.

SARS-CoV-2 is a coronavirus, which is a family of single stranded positive sense RNA viruses, at least seven of which infect humans. RNA viruses including coronaviruses replicate using a virally encoded RNA-dependent RNA polymerase (RdRp), and nucleoside analogs which are incorporated by the RdRp into the growing viral RNA chain, are a large class of approved direct acting antivirals³. Depending on the analog, incorporation can lead to chain termination or mutagenesis ultimately inhibiting viral replication⁴. Importantly, RdRps have conserved structures, and thus nucleoside analogs can show broad activity across related and unrelated viruses^{5,6}. Therefore, repurposing efforts have identified nucleoside analogs that are active against newly emerging viruses and such efforts discovered that the nucleoside analog remdesivir (VekluryTM) inhibits SARS-CoV-2 replication becoming the first approved antiviral therapeutic against this novel coronavirus^{7,8}.

Since all viruses, including SARS-CoV-2, are dependent on diverse cellular factors and metabolic products for their replication, the identification of host directed antivirals also shows promise. In particular, host nucleoside biogenesis is required for viral replication as RNA viruses require high levels of nucleoside triphosphates (NTPs) for their growth. Widespread efforts are underway to identify essential host

pathways that are druggable, and to repurpose therapeutics against these host targets. Respiratory epithelial cells are the major cellular target for SARS-CoV-2 *in vivo*. We and others have found that the cellular entry pathway as well as other host-dependent steps in the viral lifecycle show cell-type specific differences and we were most interested in those antivirals that would be active in the respiratory tract⁹. Therefore, we utilized the human respiratory cell line Calu-3 for these studies. We set out to screen small molecule libraries that contained approved drugs, drugs in clinical trials and drugs with known targets to uncover both direct-acting and host directed antivirals using the ancestral SARS-CoV-2 virus (WA1) and a cell-based, high-content assay in respiratory cells. We optimized a microscopy-based assay to achieve robust screening parameters ($Z' > 0.5$) using vehicle (DMSO) and remdesivir (10 μ M) as controls on each plate. We infect Calu-3 cells pre-treated with compounds and quantify infection of SARS-CoV-2 48h post infection (hpi) using an antibody to dsRNA, a viral replication intermediate⁹. In addition, we quantified the number of cells in each well to remove drugs that are cytotoxic.

We screened ~18,000 drugs from three repurposing libraries: an in-house PENN library of ~3,500 drugs, ~3,400 drugs from the NCATS

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repurposing collection chosen to avoid overlap with the PENN library, and the ReFrame collection of ~11,300 drugs most of which have been tested in humans¹⁰. Altogether, we have screened a large fraction of the drugs that can potentially be rapidly repurposed in respiratory cells with live virus complementing previous screening efforts^{9,11,12}. We screened the PENN library in duplicate at 0.8 μ M and identified 77 drugs that inhibit infection >60% and that had little toxicity (>80% cell number) in each screen; the NCATS library at 0.8 μ M and identified 45 drugs that inhibit infection >60% and had little toxicity (>80% cell number); and the ReFrame library in duplicate at 3.6 μ M and identified 135 compounds that inhibit infection >60% and had low toxicity (>60% cell number) in both replicates (Extended Data Fig. 1a–c). We validated the candidates by repurchasing powders (PENN, NCATS) or testing pre-spotted validation plates (ReFrame) followed by in house created dose-response studies. This allowed us to determine potency (EC50s) and toxicity (CC50s) of each compound and focus on the 122 non-redundant compounds that had a selective index (SI) greater than 3 in Calu-3 cells (Fig. 1a, Supplementary Table 1 (and 102 drugs had a SI >10)). These 122 compounds fall into a number of general categories with nucleoside analogs accounting for 13% of the validated candidates.

Nucleoside analogs are a common class of drugs that are synthetic analogs that mimic their physiological counterparts and can be incorporated by cellular polymerases into DNA or RNA to inhibit cell division^{4,13}. In addition, these can act as antimetabolites that deplete the supply of deoxynucleotides needed for DNA replication or nucleotides for RNA synthesis through inhibition of nucleoside biosynthesis enzymes⁴. Nucleoside analogs and antimetabolites are generally used to treat cancer and for immunosuppression. A subgroup of nucleoside analogs are direct-acting antivirals as they are incorporated specifically by viral polymerases leading to defects in viral nucleic acid replication. Additional nucleoside analogs have been shown to have antiviral activity, likely through their antimetabolite activity where they are not incorporated but rather compete with cellular enzymes for their natural ligands. There are two direct acting antiviral nucleosides shown to block SARS-CoV-2 infection in vitro and in animal models and have been approved for use in humans^{7,14}. Remdesivir is an adenosine analog incorporated by the viral RdRp leading to delayed chain termination^{15,16}. Molnupiravir is a cytosine analog incorporated by the viral RdRp leading to increased viral mutagenesis and antiviral activity^{14,17,18}. Importantly, we identified remdesivir, the active metabolite of remdesivir (Gs-441524), and molnupiravir as antiviral in our unbiased screens (Fig. 1a–b). We identified 13 additional nucleoside analogs with antiviral properties against SARS-CoV-2 (Fig. 1b, Extended Data Fig. 2). To determine the breadth of antiviral activity of the nucleosides, we tested a panel of cell lines that are permissive to infection with SARS-CoV-2 including human respiratory A549-expressing human ACE2, human intestinal epithelial Caco-2 cells, human hepatocyte Huh7.5 and African green monkey Vero cells (Fig. 1c, Extended Data Fig. 1d). The known direct acting antivirals remdesivir and molnupiravir show activity across diverse cell types, with variable EC50s (Fig. 1a–c, Extended Data Fig. 1d). We also found that the active form of molnupiravir EIDD-1931 was active across cell types (Extended Data Fig. 3a). We also validated antiviral activity of remdesivir and molnupiravir in Calu-3 cells using a quantitative RT-qPCR assay and found that these nucleoside analogs had strong antiviral activity by this orthogonal assay (Fig. 1d).

Some of the additional antiviral nucleoside analogs we identified are thought to act as antimetabolites by competing with the natural ligands inhibiting enzymatic function and are generally used for cancer or immunosuppression (eg 6-mercaptopurine, Extended Data Fig. 1e)⁴. We found that these drugs are non-toxic in Calu-3 cells at the antiviral concentrations (Fig. 1a–c, Extended Data Fig. 1d–e), at least in part because these cells divide slowly. Moreover, we found that these nucleoside analogs show very divergent cell type specific activity and toxicities (Fig. 1a–d, Extended Data Fig. 1d–e). For example, tuberculosis displays antiviral activity in Calu-3, Caco-2 and Huh7.5 cells with

toxicity in A549-Ace2 and Vero cells. In contrast, Thioguanine and 6-Mercaptopurine are active in Calu-3 and A549-Ace2 cells but not active in Caco-2 or Vero cells. We also monitored the antiviral activity of a subset of these nucleoside analogs in Calu-3 cells by RT-qPCR, and found that they all had significant antiviral activity (Fig. 1d). We also tested a subset of these antiviral nucleosides in primary human nasal air-liquid interface cultures and found that only remdesivir and molnupiravir showed potent antiviral activity as measured by RT-qPCR (Fig. 1e). These studies demonstrate cell-type specific antiviral activities of nucleoside analogs.

Given that the two direct acting antiviral nucleosides are distinct nucleoside derivatives, with remdesivir an adenosine analog and molnupiravir a cytosine analog, we tested whether the combination of these nucleosides would show antiviral synergy. Synergy is defined as having greater than an additive effect. Calu-3 cells were treated with six concentrations of each drug individually and in combination with each other resulting in a matrix of 36 drug-x drug-y concentration pairs monitoring viral infection. We use BLISS analysis to determine if the two drugs interact¹⁹. The null hypothesis in this model is that the drugs are additive, with a positive interaction leading to synergy and a negative interaction leading to antagonism. We found that the co-treatment of Calu-3 cells with remdesivir and molnupiravir during SARS-CoV-2 infection was additive (Fig. 1f, Extended Data Fig. 3b–d).

Antimetabolites are thought to act, at least in part, through inhibition of nucleoside biosynthesis by binding to metabolic enzymes and competing with the natural ligands⁴. Cells have two pathways for nucleoside biogenesis, *de novo* synthesis and salvage pathways that recycle purines and pyrimidines from degradation products. While salvage pathways sustain cell viability, they cannot supply sufficient amounts of NTPs to allow for fast proliferation or support viral replication²⁰. Therefore, in addition to applications in cancer, the inhibition of the *de novo* nucleotide biosynthesis constitutes a broad-spectrum antiviral strategy. In support of this, our screen identified a subset of known inhibitors of host encoded nucleoside biosynthetic enzymes within the pyrimidine and purine biosynthetic pathways shown in Fig. 2a. This included three pyrimidine biosynthesis inhibitors: the two DHODH inhibitors BAY-2402234 and Brequinar, as well as the UMPS inhibitor pyrazofurin. Dose-response studies show that these drugs are active in Calu-3 cells with low toxicity (Fig. 2b). We did not identify the classical IMPDH inhibitors ribavirin or mycophenolic acid in our screens, although ribavirin and mycophenolic acid did inhibit viral infection at higher doses in Calu-3 cells (Extended Data Fig. 4a). We also tested the human respiratory cells A549-ACE2 cells and the panel of human cell lines that are permissive to SARS-CoV-2, and found that the DHODH inhibitors were active while pyrazofurin had modest activity and AVN944 showed toxicity (Extended Data Fig. 4b–c).

We confirmed that the antiviral activity of the pyrimidine biosynthesis inhibitors was through nucleoside metabolism as we could fully block the antiviral activity of BAY-2402234, Brequinar or pyrazofurin by treating Calu-3 cells with the pyrimidine nucleosides cytidine and/or uridine, but not the purine nucleosides adenosine or guanosine (Fig. 2c). IMPDH is required for *de novo* guanosine biosynthesis and thus treatment of cells with the purine guanosine, but not adenosine or the pyrimidine nucleosides, could reverse the antiviral activity of AVN944 (Fig. 2c). In addition, we monitored nucleoside pools upon treatment with the DHODH inhibitor brequinar or IMPDH inhibitor AVN944 and found that treatment with brequinar reduced levels of the pyrimidine triphosphates while treatment with AVN944 reduced guanosine triphosphate levels with increases in the production of ATP which is produced from a common precursor that is shunted into that pathway upon inhibition of IMPDH (Fig. 2d). These data demonstrate that altering nucleoside pools can block viral replication in the absence of cytotoxicity.

Next, we set out to determine if altering pyrimidine or purine pools using host-directed metabolic inhibitors would synergize with the antiviral nucleoside analogs remdesivir or molnupiravir. Using our

microscopy-based assay we performed 2x2 dose response analysis. We found striking synergy between DHODH inhibitors (Brequinar or BAY-2402234) and nucleoside analogs (molnupiravir or remdesivir) (Fig. 3a-b, Extended Data Fig. 5). This synergy was observed in the sub-micromolar range of the DHODH inhibitor Brequinar and low nanomolar for BAY-2402234. We also tested whether the combination of molnupiravir or remdesivir with the pyrazofurin, which inhibits UMPS also shows synergy and found that it does (Extended Data Fig. 6a). In contrast, we observed little interaction between the IMPDH inhibitor AVN944 and either antiviral nucleoside analog, with a modest trend toward antagonism (Fig. 3c, Extended Data Fig. 6b-d).

Given the emergence of SARS-CoV-2 variants and the promise of antivirals to have activity against diverse strains of SARS-CoV-2, we also tested whether these drugs were active against SARS-CoV-2 variants (alpha, beta, gamma and delta) and found that all strains were sensitive to molnupiravir, remdesivir, and the pyrimidine biosynthesis inhibitors (Extended Data Fig. 7a). The ancestral strain (WAI) and delta are also sensitive to AVN944 while the others are not. We focused on molnupiravir as it is an orally bioavailable drug and thus can be potentially used in an outpatient setting and is currently undergoing approvals^{21,22}. We tested whether the combination of molnupiravir and the DHODH inhibitors would show antiviral synergy against SARS-CoV-2 variants. We observed synergy between both DHODH inhibitors (Brequinar or BAY-2402234) and molnupiravir (Extended Data Fig. 7b). This suggests that limiting the pyrimidine pool in combination with the nucleoside analog molnupiravir increases the antiviral activity of nucleoside analogs against diverse strains of SARS-CoV-2. The mechanism of this synergy is unclear; however, may be due to increased uptake or incorporation of the nucleoside analogs when pyrimidines are limiting.

We further explored the activity and interactions between DHODH inhibitors and molnupiravir. We used an orthogonal assay to confirm that molnupiravir shows dose-dependent inhibition in Calu-3 cells using RT-qPCR (Fig. 3d, Extended Data Fig. 8a). As single agents Brequinar and BAY-2402234 showed modest levels of inhibition as measured by RT-qPCR (Fig. 3d). However, when we combined treatments, we observed striking decreases in viral replication upon co-administration of either of these DHODH inhibitors and molnupiravir (Fig. 3d).

We next tested the activity of these inhibitors in ALI respiratory epithelial cell cultures that more closely model the human respiratory epithelium. We used both tracheobronchial and nasal ALI cultures as these represent the two major sites of SARS-CoV-2 infection²³. We performed toxicity studies to identify doses of these drugs that did not impact epithelial barrier function (TEER), cilia beating frequency (CBF), or toxicity (LDH) in our nasal ALI cultures²⁴. We found that treatment with molnupiravir, Brequinar, or BAY-2402234 at concentrations up to 300μM were non-toxic in this system (Extended Data Fig. 8c). Therefore, we used these doses as the maximum in our ALI cultures. In bronchial ALI cultures, we found that molnupiravir shows dose dependent activity and that Brequinar had significant single agent activity while BAY-2402234 had little activity as a single agent in these cells (Fig. 3e). However, we found a significant reduction in viral replication upon co-treatment with either Brequinar or BAY-2402234 with molnupiravir (Fig. 3e). In the nasal cells, we found that molnupiravir shows dose dependent activity and that neither Brequinar nor BAY-2402234 had significant single agent activity (Fig. 3f). Again, we found a significant reduction in viral replication upon co-treatment of molnupiravir with either DHODH inhibitor (Fig. 3f).

Molnupiravir and Brequinar are both orally dosed drugs and molnupiravir has been approved in COVID-19 patients. We observed synergistic antiviral activity with this combination of drugs in diverse model cell systems; therefore, we set out to test whether this combination would also show benefit in the treatment of SARS-CoV-2 infection in vivo. We used a mouse model of infection where wild type Balb/C mice are intranasally inoculated with SARS-CoV-2 Beta strain B.1.351 (1e5 PFU/mouse). In this model we found robust replication 2dpi as

measured by viral titers in the lungs ($\sim 10^8$ PFU/g lung), bronchiolar sloughing of infected epithelial cells, and significant inflammatory cell infiltration including edema with peribronchiolar and perivascular cuffing as measured by histology (Fig. 4). As expected, when we treated the animals with molnupiravir (EIDD-2801) we observed dose-dependent reduction in viral titers (Fig. 4a). In addition, there was only minor mitigation of the lung pathology with significant epithelial cell infection and inflammatory cell infiltration other than at the highest dose of molnupiravir tested (150mg/mL) (Fig. 4b-c). We also treated mice with Brequinar alone, at doses similar to COVID-19 clinical trials (NCT04575038), and observed no impact on viral titers or lung pathology at 2 days post infection (Fig. 4a-c). Next, we combined treatments with multiple dosing of molnupiravir and a single dose of Brequinar (20mg/kg). We found a ~4 Log reduction in viral titers upon co-treatment with Brequinar and molnupiravir at the highest dose of molnupiravir, and a significant reduction in titers at both 150 mg/kg and 50 mg/kg molnupiravir when combined with Brequinar compared to molnupiravir alone (Fig. 4a). Strikingly, we observed strong suppression of inflammation in the lung where even at the lowest combination dose there is reduced peribronchiolar and perivascular cuffing with lessened alveolar and interstitial inflammation and edema (Fig. 4b-c). Moreover, co-treatment led to a clear protection of lung architecture with little alterations to bronchiolar and alveolar cells. These low doses of brequinar are outside the toxic range and show protection. We also performed an experiment where we used a single dose of molnupiravir (50mg/kg) alone, and in combination with increasing doses of Brequinar (20mg/kg and 50mg/kg) (Fig. 4d-f). We again observed significant decreases in viral titers upon co-treatment of molnupiravir and Brequinar over molnupiravir alone (Fig. 4d). Histology analysis revealed that treatment with molnupiravir modestly reduced SARS-CoV-2-induced pathology and that co-administration with Brequinar further reduced the inflammatory response seen with molnupiravir alone (Fig. 4b, c, e, f).

We also performed a study using therapeutic dosing in this model. We infected mice intranasally and on D1 post infection we treated with molnupiravir, brequinar or the combination and quantified viral infection and inflammation in the lungs on D3 post infection. We found that the addition of brequinar to molnupiravir reduced viral titers and inflammation (Extended Data Fig. 9). Altogether, this combination of molnupiravir and Brequinar for COVID-19 treatment shows promise as we observe both reduction in viral replication and decreased pathology when dosed before or after infection.

Given that for many viral infections combinations of antivirals are needed to suppress infection, we suggest that combining nucleoside analogs with DHODH inhibitors would be beneficial, as the combination would both reduce replication and inflammation due to SARS-CoV-2 infection within therapeutic ranges. Due to the recent success of the SARS-CoV-2 protease inhibitor Paxlovid (PF-07321332), we also tested the combination of this drug with molnupiravir or remdesivir against SARS-CoV-2 beta or delta strains. We found that these combinations are additive (Extended Data Fig. 10). Thus there are a number of potential combinations that should be tested in clinical trials that may alter the trajectory of the current pandemic.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-022-04482-x>.

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A. Screened ~19,000 compounds Validated 122 drugs in Calu-3

B.

Nucleoside	Type	EC50	CC50	SI
6-Mercaptopurine	purine	0.05	> 50	> 1005
6-Thio-dG	purine	0.04	1.14	26
6-Thiopurine riboside	purine	0.05	> 50	> 1083
Azaguanine-8	purine	0.28	6.57	23
Azathioprine	purine	0.20	> 50	> 255
BCNA	pyrimidine	0.01	4.83	376
Cloturin	purine	0.64	> 20	31
FLUFYLLINE	purine	0.51	10.23	20
gemcitabine	pyrimidine	0.04	> 20	> 1235
Gs-441524	purine	0.10	> 50	> 524
Mercaptopurine	purine	0.09	> 50	> 582
Molnupiravir	pyrimidine	0.10	> 50	> 500
remdesivir	purine	0.06	> 10	> 181
THIAMIPRINE	purine	0.03	> 50	> 1749
Thioguanine	purine	0.03	> 50	> 1609
Tubercidin	purine	0.05	9.36	190

C.

D.

E.

F.

Fig. 1 | Antiviral nucleosides are highly active in respiratory cells and show cell-type specific activity. A. Pie chart of antivirals validated in Calu-3 cells with Selective Index (EC50/CC50)>3. From ~18,000 compounds, 122 show activity. B. 16 nucleoside analogs validated in Calu-3 cells with the nucleoside type listed along with the EC50, CC50, and SI. C. Calu-3, A549-Ace2, or Caco-2 cells were treated with the indicated nucleosides in dose response showing infection (blue) and toxicity (green). Data are presented as mean $\pm$ SD (n=3 independent biological replicates). D. Calu-3 cells pretreated with vehicle or 10 μ M of the indicated drugs and infected with SARS-CoV-2 for 48h and total RNA was subject to RT-qPCR analysis of viral infection. Data are presented as

mean $\pm$ SEM for reduction compared to vehicle control (n=4 independent biological replicates) (***) $p < 0.001$, one way ANOVA, values listed in Extended Data Fig. 1f). E. RT-qPCR analysis of viral infection shown for the indicated drugs in nasal air-liquid interface cultures; remdesivir (10 μ M), EIDD-2801 (10 μ M); Azathioprine (30 μ M); Mercaptopurine (30 μ M). Data are presented as mean values $\pm$ SEM (n=2 independent biological replicates). F. BLISS analysis of the 2x2 combination of remdesivir and molnupiravir in Calu-3 cells showing additivity. Data are presented as mean values of Excess over BLISS for n=9 independent biological replicates. The statistical significance was determined by a one-sample t-test (* $p < 5 \times 10^{-2}$; ** $p < 10^{-3}$; *** $p < 10^{-4}$).

Fig. 2 | Inhibitors of host nucleoside metabolism are antiviral. A. Simplified schematic of nucleoside metabolism. (B). Dose response analysis of the pyrimidine biosynthesis or purine biosynthesis inhibitors in Calu-3 cells. Infection (blue) and toxicity (green). Data are presented as mean $\pm$ SD (n=3 independent biological replicates). (C) Analysis of Calu-3 cells treated with the indicated inhibitor in the presence of increasing concentrations of the

indicated nucleosides. Data are presented as mean $\pm$ SD (n=3 independent biological replicates). (D) Calu-3 cells treated with the indicated inhibitor for 24h and cell pellets subject to LC/MS to quantify the nucleoside triphosphate levels. Mean $\pm$ SEM shown (n=6 independent biological replicates). *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Dunnett's multiple comparisons test. (p-values are: ATP: p=0.0177 GTP: p=0.0045 CTP: p<0.0001 UTP: p=0.0009).

Fig. 3 | Combination of Molnupiravir or Remdesivir with DHODH inhibitors is synergistically antiviral in vitro. (A-C) BLISS analysis in Calu3 cells with molnupiravir (EIDD-2801) or remdesivir in combination with (A) DHODH inhibitors Brequinar or (B) BAY-2402234; (C) IMPDH inhibitor AVN944. Data are presented as mean values of Excess over BLISS. Number of independent biological replicates (N) is indicated for combination tested. The statistical significance was determined by a one-sample t-test (* $p < 5 \times 10^{-2}$; ** $p < 10^{-3}$; *** $p < 10^{-4}$). (D) Calu-3 cells were treated with the indicated drugs and infected with SARS-CoV-2. 48hpi total RNA was subject to RT-qPCR was analysis of infection. Data are presented as mean $\pm$ SEM for reduction compared to vehicle

control (n=3 independent biological replicates). Black asterisk show p-value relative to vehicle, ** $p < 0.01$, one way ANOVA. Green asterisks show p-value relative to single treatment with molnupiravir, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one way ANOVA. P-values listed in Extended Data Fig. 8b. ALI-bronchial (E), or ALI-nasal (F) cells were treated with the indicated drugs and infected with SARS-CoV-2. 72hpi viral replication was quantified by RT-qPCR and expression (viral RNA/18S) was normalized to vehicle treated cells. Data are presented as mean $\pm$ SE (n=2 independent biological replicates). (D-F) Indicated concentration of drugs is in uM.

Fig. 4 | Combination of molnupiravir and Brequinar reduces SARS-CoV-2 infection and inflammation in vivo. Wild type Balb/C mice were treated with Brequinar (IP) and/or molnupiravir (EIDD-2801) (PO) daily at the indicated concentrations starting 12 hours before infection. Mice (N=5 per group over 2 independent experiments) were intranasally inoculated with 1×10^5 PFU/mouse of SARS-CoV-2/B.1.351. 2 dpi, lungs analyzed for viral titer by (A,D) plaque assay

or (B,C,E,F) fixed in 4% PFA for H&E staining and quantified for interstitial inflammation. N=5 mice per group. Mean±SD shown. *p<0.05, **p<0.01, ***p<0.001, non-parametric one-way ANOVA with Dunn's multiple comparison test. Red compared to vehicle, blue compared to molnupiravir. P-values listed in Extended Data Fig. 8d. Scale bar = 200µm.

Methods

Viruses and cells

SARS-CoV-2 WA-1 was provided by BEI/CDC. SARS-CoV-2 (alpha, beta, gamma and delta) was provided by Dr. Andrew Pekosz (Johns Hopkins University School of Public Health). Virus stocks were amplified using the ARTIC primer set (cite PMID: 32908977) and sequenced using the MinION system (Oxford Nanopore Technologies, Oxford, UK) by the J. Craig Venter Institute (Maryland, USA) to >4000x genome coverage. Stock sequence was verified by aligning reads to the reference genome provided by BEI (GSAID accession: EPI_ISL_890360) using minimap2 version 2.22 with the “map-ont” presets (cite PMID: 29750242), followed by inspection of the consensus sequence and alignment using IGV (cite PMID: 21221095). Stocks had <1% variation. Stock virus was prepared by infection of Vero E6/TMPRSS2 cells in growth media (DMEM (Quality Biological), supplemented with 10% (v/v) fetal bovine serum (Gibco), 1% (v/v) penicillin/streptomycin (Gemini Bio-products) and 1% (v/v) L-glutamine (2 mM final concentration, Gibco) fetal bovine serum plus for two or three days when CPE was visible. Media were collected and clarified by centrifugation prior to being aliquoted for storage at -80 °C. Titer of stock was determined by plaque assay or TCID50 analysis using Vero E6 cells as described previously (5). All work with infectious virus was performed in a Biosafety Level 3 laboratory and approved by University of Pennsylvania Biosafety Committee and The University of Maryland School of Medicine Institutional Biosafety Committee.

Vero (ATCC, CCL81 or E6) and Huh7.5 cells (C. Rice, Rockefeller) were cultured in DMEM, supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, 1% (v/v) L-Glutamax. Calu-3 cells (ATCC, HTB-55) were cultured in MEM, supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, 1% (v/v) L-glutamine, and 1% (v/v) non-essential amino acids. A549-ACE2 cells were cultured in RPMI1640 supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, 1% (v/v) L-Glutamax. Caco-2 (ATCC, HTB-37) were cultured in MEM alpha supplemented with 20% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, 1% (v/v) L-glutamine. All cells were grown at 37°C, 5% CO₂ and 20% O₂. All cell lines are validated and mycoplasma free.

Day 20 air liquid interface EpiAirway tracheobronchial tissues were obtained from MatTek Corporation (AIR-100) and used after Day 30. Tissues were fed twice a week until use. Air liquid interface pooled nasal epithelial cultures from Epithelix were fed twice a week until use. Toxicity studies were performed on nasal air-liquid interface cultures. Daily basolateral exposure of test compounds was evaluated using tissue integrity (TEER); cytotoxicity as basolateral LDH release and cilia beating frequency (CBF) at 72h.

Tissue Integrity (TEER) Tissue integrity is determined by monitoring trans-epithelial electrical resistance (TEER) using the EVOM3 Ohm Meter (World Precision Instruments). Resistance values (Ω) were converted to TEER ($\Omega \cdot \text{cm}^2$) using the following formula: $\text{TEER} (\Omega \cdot \text{cm}^2) = (\text{resistance value} (\Omega) - 100 (\Omega)) \times 0.33 (\text{cm}^2)$, where 100 Ω is the resistance of the membrane and 0.33 cm^2 is the total surface of the epithelium.

Cytotoxicity (LDH release) Basolateral lactate dehydrogenase release was quantified using Cytotoxicity LDH Assay Kit-WST (Dojindo, CK12-20), measuring the absorbance of each sample at 490nm with a microplate reader. To determine the percentage of cytotoxicity, the following equation was used (A = absorbance values): $\text{Cytotoxicity} (\%) = (A (\text{high control}) - A (\text{low control})) / (A (\text{high control}) - A (\text{low control})) \times 100$. The high control value was obtained by 10% Triton X-100 apical treatment (24 hours). Triton X-100 causes a massive LDH release and corresponds to 100% cytotoxicity.

Cilia Beating Frequency (CBF) Cilia beating frequency was measured using the following setup: a Sony XCD V60 camera connected to an Olympus BX51 microscope with a 5x objective and a camera specific software. The cilia beating frequency is expressed as Hz. 256 images were captured at high frequency rate (125 frames per second) at 34°C, cilia beating frequency was then calculated using Cilia-X software (Epithelix).

High-Throughput Screening

Ten-thousand Calu-3 cells were plated per well of 384 well assay plates (Corning) in 20ul of growth medium. For the PENN and NCATS libraries, 50nL of drugs were added at a final concentration of 0.8 μM in 0.2% DMSO. For the ReFrame library, 20ul of MEM was dispensed to compound source plates pre-spotted with 50nL of 10mM compound in DMSO. 5ul of diluted compound was added per well of 384W assay plate yielding a final concentration of 3.6 μM in 0.04% DMSO. The positive control 10uM Remdesivir (n=32) and the negative control 0.2% DMSO (n=32) were spotted on each plate. One hour post drug addition, cells were infected with SARS-CoV-2 (MOI=0.5). Cells were fixed 40-48hpi in 4% formaldehyde/PBS for 15min at room temperature, washed three times with PBS, blocked with 2% BSA/PBST for 60 minutes, and incubated in primary antibody (anti-dsRNA J2, absolute antibody, 1:500) overnight at 4°C. Cells were washed 3x in PBST with an automated plate washer (Biotek) and incubated in secondary antibody (anti-mouse alexa 488, 1:100 and Hoescht 33342) for 1h at room temperature. Cells were washed 3x in PBST with an automated plate washer and imaged using an automated microscope (ImageXpress Micro, Molecular Devices). Cells were imaged with a 10X objective, and four sites per well were captured. The total number of cells and the number of infected (dsRNA+) cells were measured using the cell scoring module (MetaXpress 5.3.3), and the percentage of infected cells was calculated. The aggregated percent infection of the 0.2% DMSO (n=32) and 10uM Remdesivir control wells (n=32) on each assay plate were used to calculate z'-factors, as a measure of assay performance and data quality. Sample well infection was normalized to aggregated DMSO plate control wells and expressed as Percentage of Control [$\text{POC} = (\% \text{Infection}_{\text{sample}} / \text{Average } \% \text{Infection}_{\text{DMSO}}) \times 100$] and Z-score [$Z = (\% \text{Infection}_{\text{sample}} - \text{Average } \% \text{Infection}_{\text{DMSO}}) / \text{Standard Deviation } \% \text{Infection}_{\text{DMSO}}$] in Spotfire (PerkinElmer). Candidate hits were selected as the following: for UPENN screen compounds with POC <40% for infection and viability >80% in either replicate; for NCATS screen POC <40% for infection and viability >80%; For the ReFrame screen, POC <50% for infection and viability >60% viability for the average of the replicates, compared to DMSO control.

Dose response studies

Candidate drugs from the UPENN and NCATS library were purchased as powders from Selleckchem, MedchemExpress, Cayman and MedKoo and suspended in DMSO. Drugs were arrayed in 8-pt dose-response in 384 well plates. For the ReFrame validation, 15ul of MEM was dispensed to compound source plates pre-spotted with 100nL of compound in DMSO arrayed in an 8 pt dose response. 5ul of diluted compound was added per well of 384W assay plate yielding final concentrations of 9.5, 3.1, 1.0, 0.35, 0.12, 0.04, 0.01, and 0.004 μM in 0.1% DMSO. Calu-3 (10,000 cells), A549-ACE2 (3,000), Caco-2 (1,500), Huh7.5 (3,000) or Vero (3,000) were plated in 384 well plates. Twenty-four hours post plating (72 hours for Caco-2), drug additions and infections were performed using the screening conditions. 0.2% DMSO (n=32) and 10 μM Remdesivir (n=32) were included on each plate as controls for normalization. For nucleoside rescue experiments, cells were treated with an EC90 concentration of a given nucleoside biosynthesis inhibitor in combination with a dose response of nucleosides. Infection at each drug concentration was normalized to aggregated DMSO plate control wells and expressed as percentage-of-control ($\text{POC} = \% \text{Infection}_{\text{sample}} / \text{Avg } \% \text{Infection}_{\text{DMSO cont}}$). A non-linear regression curve fit analysis (GraphPad Prism 8) was performed on POC of % Infection and cell viability using log10 transformed concentration values to calculate EC50 values for % Infection and CC50 values for cell viability for each drug/cell line combination. The EC50 and CC50 values represent the average of ≥ 2 independent experimental replicates. Selectivity index (SI) was calculated as a ratio of drug's CC50 and EC50 values ($\text{SI} = \text{CC50} / \text{EC50}$).

Quantitative drug combination analysis

Using our HTS assay conditions, drugs were added to Calu-3 cells in 384W assay plates using a Tecan D300e dispenser in a matrix that combined six concentrations of remdesivir (up to 2µM) or molnupiravir (up to 10µM) with six concentrations of Brequinar (up to 10µM), BAY-2402234 (up to 0.5µM), and AVN944 (up to 2µM), resulting in 36 concentration combinations per drug pair in 0.2% DMSO. Each combination was independently repeated at least 3 times as technical duplicates in each biological replicate. Sample well infection was normalized to aggregated 0.2% DMSO plate control wells (n=32) and expressed as Percentage of Control. Synergy between drug combinations was determined by the Bliss independence model, to quantitatively assess drug interaction patterns within the drug-drug combination matrix (1, 2). The Bliss expectation (E) for a combined response was calculated by $E = (A + B) - (A \times B)$ where A and B are the fractional inhibition of SARS-CoV-2 infection of drug A and B at a given dose. The difference between the Bliss expectation and the observed inhibition of SARS-CoV-2 infection for the combination of drugs A and B at the same dose is the BLISS value. Bliss values between 0-10 indicate that the combination is additive (as expected for independent pathway effects); Bliss values >20 indicates activity greater than additive (synergy); and values <0 indicate the combination is less than additive (antagonism).

RT-qPCR

Calu-3 cells (750,000 cells/well) were plated in 6 well plates. The next day, drugs were added to cells. One hour later cells were infected with SARS-CoV-2 (MOI=0.3). For ALI cultures, the apical surface was washed with OptiMEM, and cells placed into fresh media with drugs added to the basolateral surface. Cells were infected apically with SARS-CoV-2 (MOI=0.2) for one hour and subsequently the virus inoculum was removed. The cells were placed into fresh media daily with the indicated drugs. At the indicated time point, total RNA was purified using Trizol (Invitrogen) followed by RNA Clean and Concentrate kit (Zymo Research) 48 hpi for Calu-3 and ALI cultures. For cDNA synthesis, reverse transcription was performed with random hexamers and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). Synthesized RNA was used as a standard (BEI). Gene specific primers to SARS-CoV-2 (Wuhan v1, NSP14) and SYBR green master mix (Applied Biosystems) were used to amplify viral RNA and 18S rRNA primers were used to amplify cellular RNA using the QuantStudio 6 Flex RT-PCR system (Applied Biosystems). Relative quantities of viral and cellular RNA were calculated using the standard curve method (3). Viral RNA was normalized to 18S RNA for each sample (Wuhan V1/18S) (4).

Metabolomics

Calu-3 cells treated for 24h with the indicated drugs were snap frozen in liquid nitrogen, and thawed cell pellets on ice were homogenized with ice-cold 80% methanol. The samples were vortexed for 10 seconds to lyse cells and the cell homogenate was spiked with isotopically-labelled nucleotide internal standards. The mixture was extracted with ice-cold methanol, vortexed, and centrifuged at 18,100 xg for 5 min at 4 °C. The supernatant was dried under nitrogen at 45 °C and reconstituted in 50% methanol for LC/MS on an Agilent 1290 Infinity UHPLC/6495B triple quadrupole mass spectrometer. A 12-minute linear gradient from 95% B (acetonitrile) to 54% A (10 mM ammonium acetate, pH 9, 0.1% medronic acid) on an Agilent PEEK HILIC-z 2 x 100 mm, 2.7 µm column was used to separate nucleotides. Multiple reaction monitoring (MRM) was used to quantitate a fragment ion of the parent ion of each nucleotide with standard calibration curves. The raw data was normalized from µM to nmol/mg protein using a BCA assay from input samples.

Mouse studies

Mouse challenge studies were approved by The University of Maryland School of Medicine IACUC. 8-10 week old female Balb/C mice were randomized and not blinded. EIDD-2801 (MedChemExpress #HY-135853) was resuspended in corn oil (Sigma #8267) and 10% DMSO (Sigma #2438), with dosing twice a day as oral gavage. Brequinar (MedChemExpress #HY-108325) was resuspended in 10% DMSO and sterile saline, with dosing once a day as intraperitoneal injection. Our PK studies showed a ~10h half life of brequinar, and dosing was either started 12h before infection or 24h after infection as indicated.

Mice were anaesthetized by intraperitoneal injection with 50 µL of a mix of xylazine (0.38 mg/mouse) and ketamine (1.3 mg/mouse) diluted in phosphate buffered saline (PBS). Mice were intranasally inoculated with 1×10^5 pfu of beta variant of SARS-CoV-2 in 50 µL. Challenged mice were weighed on day of infection and daily for 2 days post infection, there were no significant changes in weights observed. For prophylactic dosing, 2-days post infection, or therapeutic dosing 3-days post infection, 5 mice were sacrificed from each treatment and control group, lungs were harvested to determine viral titer by a plaque assay, and fixed in 4% paraformaldehyde for 24 hours before sectioning and staining with hematoxylin and eosin by UMSOM Histology Core(5). Pathological scoring on blinded H&E stained sections was performed for each mouse and analyzed for inflammation.

Statistical analyses

Statistics were performed with GraphPad Prism 9.2 software (La Jolla, CA). Lung titers for the treatment groups were compared to vehicle and statistically analyzed using a non-parametric one-way ANOVA with Dunnett's multiple comparison test. For metabolomics one-way ANOVA with Dunnett's post hoc test for multiple comparisons to determine significance from vehicle control. For RT-qPCR two way ANOVA test. For synergy studies Combenefit software (update 2016-06-01) was used.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

All data in this paper are presented in the main text and supplementary text. Supplementary Information is available for this paper. Source data are provided with this paper.

Acknowledgements We thank members of the Cherry, Lynch, Frieman and Penn Center for Precision Medicine for advice and discussion. We thank Ranawaka Perera for viral preparations. We thank David Nguyen for his data analysis and all members of the High-Throughput Screening Core at University of Pennsylvania for reagents and technical support. We thank the Penn Metabolomics Core in the Cardiovascular Institute for LC/MS quantitation of nucleotides. We thank S. Weiss and Y. Li for sharing SARS-Related Coronavirus 2, Isolate USA-WA1/2020). We thank Andy Pekosz for SARS-CoV-2 variants (alpha, beta, gamma, delta). We thank Xin Hu, Richard Eastman, Matthew Hall, and members of NCATS Compound Management for assembly and preparation of the NCATS compound collections. We thank CALIBR for providing the ReFrame library and validation plates. This work was supported by grants from the National Institutes of Health to S.C. (R01AI074951, R01AI122749, 1R21AI151882, and R01AI140539) as well as funding from the Penn Center for Precision Medicine, Mercatus, and the Bill and Melinda Gates Foundation. S.C. is a recipient of the Burroughs Wellcome Investigators in the Pathogenesis of Infectious Disease Award, the Deans Innovation Fund as well as Linda and Laddy Montague. Work at NCATS was funded by the by the Intramural Research Program of the National Center for Advancing Translational Sciences, National Institutes of Health (ZIA Project # TR000414-01). MBF, RMJ, HLH and SMW supported by funding from Bill and Melinda Gates Foundation (INV-016638), NIH R21AI158134, NIH R21AI153480 and HHS/BARDA ASPR-20-01495.

Author contributions Research design D.C.S., R.M.J., E.L., S.C., M.F., M.B.F. and S.C.; experimentation D.C.S., R.M.J., K.A., J.M., K.W., B.K., M.D., S.W., H.L.H., C.D., L.C., J.S.L., M.L., E.L., S.C., M.F., M.B.F. and S.C.; data analysis D.C.S., R.M.J., K.A., J.M., K.W., B.K., M.D., S.W., H.L.H., C.D., L.C., J.S.L., M.L., E.L., S.C., M.F., M.B.F. and S.C.; writing D.C.S., E.L., S.C., M.F., C.A.T., M.B.F. and S.C.; supervision D.C.S., S.C., M.F., M.B.F. and S.C.

Competing interests The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41586-022-04482-x>.

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Peer review information *Nature* thanks the anonymous reviewers for their contribution to the peer review of this work.

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ACCELERATED ARTICLE PREVIEW

Extended Data Fig. 1 | High-throughput screening in Calu-3 cells to identify antivirals against SARS-CoV-2. A-C. High throughput screening of ~18,000 drugs including the (A) UPENN library, (B) NCATS library and (C) ReFrame library in Calu-3 cells infected with SARS-CoV-2. Percent of Control (POC) for % infection is plotted versus rank of the drug across the primary screens. Yellow circles, DMSO controls; Green circles, remdesivir controls; Blue Circles, sample wells. Solid horizontal line represents 100% Infection of DMSO control wells.

D. Huh7.5 or Vero (CCL81) cells were treated with the indicated nucleosides in dose response showing infection (blue) and toxicity (green). Data are presented as mean $\pm$ SD (n=3 independent biological replicates). E. The indicated cell line was treated with increasing doses of 6-mercaptopurine and dose responses for infection (blue) and toxicity (green) are shown. Data are presented as mean $\pm$ SD (n=3 independent biological replicates). F. p-values from Fig. 1d.

Extended Data Fig. 2 | Structures of antiviral nucleoside analogs shown. Nucleoside-related analog structures shown.

Extended Data Fig. 3 | Combination of remdesivir and molnupiravir is additive. A. The indicated cell line was treated with increasing doses of EIDD-1931 and dose responses for infection (blue) and toxicity (green) are shown. Data are presented as mean $\pm$ SD (n=3 independent biological replicates). B. anti-SARS2 activity of remdesivir and molnupiravir as single

agents. Data are presented as mean $\pm$ SD. C. anti-SARS2 activity of remdesivir in combination with a single concentration of molnupiravir or molnupiravir in combination with a single concentration of remdesivir. D. Two-dimensional representation of dose response interaction matrix for percent of control of infection mean $\pm$ SD, n=9 independent biological replicates.

Extended Data Fig. 4 | Inhibitors of host nucleoside metabolism are antiviral. A. Calu-3 cells were treated with the indicated IMPDH inhibitors in dose response showing infection (blue) and toxicity (green). Data are presented as mean $\pm$ SD (n=4 independent biological replicates). B. A549-Ace2

cells were treated with the indicated inhibitors in dose response showing infection (blue) and toxicity (green). Data are presented as mean $\pm$ SD (n=4 independent biological replicates). C. Table of the EC50, CC50 and SI for the indicated cell line and drug (uM).

Extended Data Fig. 5 | Combination of molnupiravir or remdesivir with DHODH inhibitors are synergistically antiviral in vitro. A. anti-SARS2 activity of molnupiravir, remdesivir, and Brequinar, as single agents. Data are presented as mean $\pm$ SD. B. anti-SARS2 activity of molnupiravir or remdesivir in combination with a single concentration of Brequinar. C. Two-dimensional representation of dose response interaction matrix for percent of control of infection. Data are presented as mean $\pm$ SD (n=6 independent biological

replicates). D. anti-SARS2 activity of molnupiravir, remdesivir, and BAY-2402234, as single agents. Data are presented as mean $\pm$ SD. E. anti-SARS2 activity of molnupiravir or remdesivir in combination with a single concentration of BAY-2402234. F. Two-dimensional representation of dose response interaction matrix for percent of control of infection. Data are presented as mean $\pm$ SD (n=6 for EIDD-2801, n=8 for remdesivir for independent biological replicates).

Extended Data Fig. 6 | Combination of molnupiravir or remdesivir with Pyrazofurin but not AVN944 is synergistically antiviral in vitro. A. BLISS analysis in Calu3 cells with molnupiravir (EIDD-2801) or remdesivir in combination with pyrazofurin. Data are presented as mean values of Excess over BLISS. (n=4 independent biological replicates). The statistical significance was determined by a one-sample t-test (* $p < 5 \times 10^{-2}$; ** $p < 10^{-3}$).

B. anti-SARS2 activity of molnupiravir, remdesivir, and AVN944 as single agents. Data are presented as mean $\pm$ SD. B. anti-SARS2 activity of molnupiravir or remdesivir in combination with a single concentration of AVN944. C. Two-dimensional representation of dose response interaction matrix for percent of control of infection. Data are presented as mean $\pm$ SD (n=10 for EIDD-2801, n=6 for remdesivir for independent biological replicates).

A

	SARS-CoV-2 WA1			SARS-CoV-2 Alpha			SARS-CoV-2 Beta			SARS-CoV-2 Gamma			SARS-CoV-2 Delta		
	EC50	CC50	SI	EC50	CC50	SI	EC50	CC50	SI	EC50	CC50	SI	EC50	CC50	SI
remdesivir	0.123	>4	>32	0.051	>2	>40	0.049	>2	>41	0.101	>2	>20	0.079	>2	>25
molnupiravir	0.708	>50	>71	1.138	>10	>9	1.39	>10	>7	2.263	>10	>4	1.271	>10	>8
BAY-2402234	0.005	>20	>4000	0.006	>0.5	>83	0.009	>0.5	>56	0.011	>0.5	>50	0.006	>0.5	>83
brequinar	0.794	>50	>62	0.144	>10	>69	0.697	>10	>14	0.629	>10	>16	0.379	>10	>26
AVN-944	0.044	>50	>1141	5	>10	>2	>2	>2	1	>10	>10	1	0.271	>10	>37
pyrazofurin	0.185	>50	>270	0.775	>5	>6	0.466	>5	>11	0.602	>5	>8	0.377	>5	>13
PF-07321332	0.173	>5	>29	0.21	>2.5	>12	0.225	50	>222	0.282	>2.5	>9	0.203	>5	>25

Extended Data Fig. 7 | Combination of molnupiravir with DHODH inhibitors is synergistic against SARS-CoV-2 variants. A. Table of EC₅₀, CC₅₀ and SI for the indicated variant and the indicated drugs (uM) in Calu-3 cells. B. BLISS analysis in Calu3 cells with molnupiravir (EIDD-2801) in combination with DHODH inhibitors Brequinar or BAY-2402234 infected with the indicated

variant. Data are presented as mean values of Excess over BLISS. The following number of independent replicates were performed: SARS-CoV2 Alpha; n=8 for BAY2402234, n=6 Brequinar; SARS-CoV2 Beta; n=7, SARS-CoV2 Gamma; n=6 BAY2402234, n=8; SARS-CoV2; Delta: n=8. The statistical significance was determined by a one-sample t-test (* p<5x10⁻²; ** p<10⁻³; *** p<10⁻⁴).

Figure S8

Extended Data Fig. 8 | Nucleoside-related drugs are well-tolerated in air-liquid interface cultures. A. Calu-3 cells were treated with the indicated concentrations of Molnupiravir (EIDD-2801) and infected with SARS-CoV-2. 48hpi viral replication was quantified by RT-qPCR and expression (viral RNA/18S) was normalized to vehicle treated cells. Data are presented as

C Maximal tested tolerated dose in nasal air-liquid interface cultures at 72h post basolateral daily exposure

Compound	TEER	CBF	LDH
Molnupiravir	30 uM	30 uM	30 uM
Remdesivir	30 uM	30 uM	30 uM
Brequinar	30 uM	30 uM	30 uM
BAY2402234	30 uM	30 uM	30 uM
AVN944	30 uM	>10 uM	10 uM

D

Figure	Summary	Adjusted P Value
Figure 4A (red stars)		
B.1.351 Only vs. EIDD-2801 (150 mg/kg)	***	0.0002
B.1.351 Only vs. EIDD-2801 (50 mg/kg)	*	0.0452
B.1.351 Only vs. EIDD (150 mg/kg) +Breq (20 mg/kg)	***	0.0002
B.1.351 Only vs. EIDD (50 mg/kg) +Breq (20 mg/kg)	**	0.0032
(blue stars)		
EIDD-2801 (150 mg/kg) vs. EIDD (150 mg/kg) +Breq (20 mg/kg)	**	0.0087
Figure 4B (red stars)		
B.1.351 Only vs. EIDD (150 mg/kg) +Breq (20 mg/kg)	***	0.0008
B.1.351 Only vs. EIDD (50 mg/kg) +Breq (20 mg/kg)	**	0.0071
B.1.351 Only vs. EIDD (16.6 mg/kg) +Breq (20 mg/kg)	**	0.0018
B.1.351 Only vs. Brequinar (20 mg/kg)	**	0.0029
(blue stars)		
EIDD-2801 (150 mg/kg) vs. EIDD (150 mg/kg) +Breq (20 mg/kg)	**	0.0031
Figure 4D (red stars)		
B.1.351 Only vs. EIDD (50 mg/kg) +Breq (20 mg/kg)	****	<0.0001
B.1.351 Only vs. EIDD (50 mg/kg) +Breq (50 mg/kg)	****	<0.0001
B.1.351 Only vs. EIDD-2801 (50 mg/kg)	****	<0.0001
(blue stars)		
EIDD-2801 (50 mg/kg) vs. EIDD (50 mg/kg) +Breq (20 mg/kg)	*	0.0122
EIDD-2801 (50 mg/kg) vs. EIDD (50 mg/kg) +Breq (50 mg/kg)	**	0.0021
Figure 4E (red stars)		
B.1.351 Only vs. EIDD (50 mg/kg) +Breq (50 mg/kg)	**	0.0059
B.1.351 Only vs. EIDD (50 mg/kg) +Breq (20 mg/kg)	*	0.021

mean±SEM for n=2-3 biological replicates. B. P-values for Fig. 3d. C. Nasal air-liquid interface cells were treated daily on the basolateral side with the indicated concentrations of drug, and at 72h TEER, Cilia beating frequency (CBF) and toxicity (LDH) were measured. D. P-values for Fig. 4.

Extended Data Fig. 9 | Combination of molnupiravir and Brequinar reduces SARS-CoV-2 infection and inflammation in vivo during therapeutic dosing.

Wild type Balb/C mice were treated with Brequinar (IP) and/or molnupiravir (EIDD-2801) (PO) daily at the indicated concentrations starting 24 hours after infection. Mice (N=5 mice per group) were intranasally inoculated with 1x10⁵ PFU/mouse of SARS-CoV-2/B.1.351. (A) 3 dpi, lungs analyzed for viral titer by plaque assay, Mean±SD shown. * p<0.05, ** p<0.01, non-parametric one-way

ANOVA with Dunnett's multiple comparison test. (p-values: B1.135 only vs EIDD-2801 (25 mg/kg) = 0.045, vs EIDD-2801 (50 mg/kg) = 0.016, vs EIDD-2801 (25 mg/kg) + Breq (50 mg/kg) = 0.0017. (B, C) 3 dpi, lungs fixed in 4% PFA for H&E staining (Scale bar = 200μm) and quantified for interstitial inflammation. Mean±SD shown. ** p<0.01, non-parametric one-way ANOVA with Dunnett's multiple comparison test. (p-value = 0.0015).

SARS-CoV-2 Beta

SARS-CoV-2 Delta

Extended Data Fig. 10 | Combination of molnupiravir or remdesivir with the protease inhibitor PF-07321332 is additive against SARS-CoV-2 (Beta, Delta). A. BLISS analysis in Calu-3 cells with molnupiravir (EIDD-2801) or remdesivir in combination with PF-07321332 infected with the indicated

SARS-CoV-2 variant. Data are presented as mean Excess over BLISS for n=2 independent biological replicates (SARS-CoV-2 Beta); n=6 independent biological replicates (SARS-CoV-2 Delta). The statistical significance was determined by a one-sample t-test (* $p < 5 \times 10^{-2}$; ** $p < 10^{-3}$).

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- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
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All data in this paper are presented in the main text and supplementary text. Supplementary Information is available for this paper.

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All studies must disclose on these points even when the disclosure is negative.

Sample size	Use standards in the field for each assay type.
Data exclusions	none
Replication	Every experiment was replicated multiple times as described in the legends.
Randomization	Not applicable, all cell cultures are treated as equal.
Blinding	Performed for histology.

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Methods

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☒	☐ ChIP-seq
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Antibodies

Antibodies used	All listed in methods. Anti J2, Absolute Biosciences.
Validation	All verified by vendors and we verified J2 antibody only detect infected cells.

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Cell line source(s)	All stated in methods. ATCC for all but Huh7.5 (C. Rice), A549 ACE2, (S. Weiss)
Authentication	ATCC , and express lineage specific markers.
Mycoplasma contamination	We perform every 3 months. All cultures were negative.
Commonly misidentified lines (See ICLAC register)	Not used.

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals	All specified in methods. 8-10 week old female Balb/C.
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Wild animals	<div>None</div>
Field-collected samples	<div>None</div>
Ethics oversight	<div>University of Maryland School of Medicine IACUC</div>

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