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Orally bioavailable RORγ/DHODH dual host-targeting small molecules with broad-spectrum antiviral activity

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ABSTRACT

Host-directed antivirals (HDAs) represent an attractive treatment option and a strategy for pandemic preparedness, especially due to their potential broad-spectrum antiviral activity and high barrier to resistance development. Particularly, dual-targeting HDAs offer a promising approach for antiviral therapy by simultaneously disrupting multiple pathways essential for viral replication.

Izumerogant (IMU-935) targets two host proteins, (i) the retinoic acid receptor-related orphan receptor γ isoform 1 (RORγ1), which modulates cellular cholesterol metabolism, and (ii) the enzyme dihydroorotate dehydrogenase (DHODH), which is involved in *de novo* pyrimidine synthesis. Here, we synthesized optimized derivatives of izumerogant and characterized their antiviral activity in comparison to a recently described structurally distinct RORγ/DHODH dual inhibitor. Cell culture-based infection models for enveloped and non-enveloped DNA and RNA viruses, as well as a retrovirus, demonstrated high potency and broad-spectrum activity against human viral pathogens for RORγ/DHODH dual inhibitors at nanomolar concentrations. Comparative analyses with equipotent single-target inhibitors in metabolite supplementation approaches revealed that

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the dual-targeting mode represents the mechanistic basis for the potent antiviral activity. For SARS-CoV-2, an optimized dual inhibitor completely blocked viral replication in human airway epithelial cells at 5 nM and displayed a synergistic drug interaction with the nucleoside analog molnupiravir. In a SARS-CoV-2 mouse model, treatment with a dual inhibitor alone, or in combination with molnupiravir, reduced the viral load by 7- and 58-fold, respectively.

Considering the clinical safety, oral bioavailability, and tolerability of izumerogant in a recent Phase I study, izumerogant-like drugs represent potent dual-targeting antiviral HDAs with pronounced broad-spectrum activity for further clinical development.

1. Introduction

Despite great progress in the development of antiviral medication over the past decades, the repertoire of clinically approved drugs is still limited to a few human pathogenic viruses including immunodeficiency virus type 1 (HIV-1), certain human herpesviruses, influenza A and B viruses, hepatitis B (HBV) and C viruses (HCV), as well as the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2). Most approved therapeutics are direct-acting antivirals (DAAs) that are often associated with adverse events such as toxicity, limited therapeutic efficacy, or the development of viral drug resistance. Although combination treatment might address these issues at least in part, there is still an urgent need for the development of safe and effective antiviral drugs. Host-directed antivirals (HDAs) that target essential steps within host cells during the virus replication cycle are an appealing alternative approach (Kumar et al., 2020). Since viruses often exploit common cellular pathways, modulating those pathways is a promising broad-spectrum antiviral strategy with a higher barrier of viral resistance. Moreover, it is possible to develop such molecules before the emergence of new viruses or variants, making HDAs an attractive approach for pandemic preparedness. Despite the advantages of this concept, the number of HDAs approved for antiviral treatment is limited, thus representing an attractive opportunity for future drug development (Kaufmann et al., 2018; Ma et al., 2021).

We and others recently validated two host targets that are promising candidates for the development of antiviral HDAs, namely the nuclear transcription factor retinoic-acid related orphan receptor gamma (RORγ) (Wangen et al., 2024) and the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH) (Hahn et al., 2020; Gong et al., 2019; Purificação et al., 2024; Stegmann et al., 2022; Xiong et al., 2020; Zheng et al., 2022). While the involvement of ubiquitously expressed RORγ isoform 1 (RORγ1) in gluconeogenesis, lipid homeostasis, and sterol metabolism has been well established (Raichur et al., 2007; Zou et al., 2022), its virus-supportive role has been described only recently (Wangen et al., 2024). The activity of RORγ can be regulated by two types of small molecule modulators. Agonists mediate the recruitment of transcriptional coactivators or prevent the recruitment of transcriptional corepressors, thereby enhancing the transcriptional activity of RORγ. Conversely, inverse agonists decrease the transcriptional activity by recruiting corepressors or displacing coactivators (Gege, 2021). By applying different RORγ modulators like the inverse agonist cedirogant on viral replication assays, we previously demonstrated their *in vitro* antiviral activity against the major human pathogenic viruses SARS-CoV-2, human cytomegalovirus (HCMV), varicella zoster virus (VZV), and partially against HIV-1 (Wangen et al., 2024).

DHODH catalyzes the rate-limiting step in the *de novo* synthesis of pyrimidines by conversion of dihydroorotate to orotate. Inhibition of DHODH, and thus a block of nucleotide biosynthesis, results in the depletion of essential DNA and RNA building blocks. The activity of DHODH inhibitors is limited to highly metabolically active cells like cancer cells or hyperactivated lymphocytes (Klotz et al., 2019). As a result, DHODH inhibitors are approved for the treatment of autoimmune diseases like rheumatoid arthritis and multiple sclerosis (Oh and O'Connor, 2013a, b) and are actively investigated in cancer and anti-infective research (Boschi et al., 2019; Madak et al., 2019; Zhou et al., 2021). In contrast to normal cell division, virus replication requires elevated amounts of (deoxy)nucleotides, rendering infected cells sensitive towards DHODH inhibition (Bonavia et al., 2011). Despite promising *in vitro* antiviral data, clinical evidence for efficacy of monotherapy with DHODH inhibitors is currently lacking. However, recent results from the Phase 2 clinical trial (NCT04379271) with vidofludimus calcium (VidoCa), a potent DHODH inhibitor currently in clinical development for the treatment of various forms of multiple sclerosis, indicated clinical benefits in the treatment of COVID-19 patients. Analyses revealed a shorter time to clinical improvement of COVID-19 patients by approximately one day compared to the placebo control group which correlated with VidoCa serum levels (Vehreschild et al., 2022).

Izumerogant (IMU-935) is an orally bioavailable dual RORγ inverse agonist and DHODH inhibitor with the potential for simultaneous targeting of two virus-supportive pathways (Kohlhof et al., 2019). The compound class was discovered in a phenotypic screen in human peripheral blood mononuclear cells (PBMCs) for activity against interleukin-17A and −17F (IL-17A/F) and interferon gamma (IFNγ). The two targets were deconvoluted and validated. The compound class was then optimized towards the oral drug candidate izumerugant. As izumerogant has already been demonstrated to be safe in healthy subjects with a pharmacokinetic profile that supports once-daily oral application (Polasek et al., 2023), a drug repurposing approach may represent a promising strategy for antiviral treatment. In this study, we describe the potent broad-spectrum antiviral activity of izumerogant and related molecules and establish the dual targeting mode as the causative mechanistic basis.

2. Materials and methods

2.1. Cultivation of eukaryotic cells

Eukaryotic cells were cultivated at 37 °C, 5% CO_2 and 80% humidity in media supplemented with 10% FCS (anprotec, Bruckberg, Germany), 1% GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA) and 10 μg/mL gentamicin. Specifically, Caco-2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific) containing 1% MEM Non-Essential Amino Acids Solution (Thermo Fisher Scientific), A549 cells in DMEM and human primary foreskin fibroblasts (HFFs) in Minimum Essential Medium (MEM, Thermo Fisher Scientific).

2.2. Small molecules

Cedirogant (WO2016/200851), izumerogant (WO2019/048541), derivatives **1311**, **1404**, **1514** (WO2023/198873), **1414** (Cpd. 16 in (Vietor et al., 2023)) and **1797** (Cpd. 14d in (Chen et al., 2022)) were synthesized as described and provided by Immunic AG. Other compounds were obtained from commercial sources: EIDD-1931 (active metabolite of molnupiravir, MCE, Monmouth Junction, NJ, USA), molnupiravir (Synnovator, Cary, USA), ganciclovir (GCV, Sigma-Aldrich, Steinheim, Germany), and brincidofovir (BCV, Biomol, Hamburg, Germany). The stocks were prepared as 10 mM solutions in dimethyl sulfoxide, with the exception of BCV, which was prepared at 1 mM. All compounds used within this study have a purity of ≥95%.

2.3. Determination of RORγ/DHODH single target inhibition in vitro

The *in vitro* inhibition of DHODH was determined as described earlier using an N-terminally truncated recombinant human DHODH (Zeng et al., 2019). Final assay conditions contained 60 μM 2,6-dichloroindophenol, 50 μM decylubiquinone, 100 μM dihydroorotate in a buffer with 150 mM KCl, 0.1% Triton X-100 and 50 mM TrisHCl pH 8.0. The DHODH protein amount used was previously titrated to generate an average slope of approximately 0.2 AU/min. Each compound was analyzed in serial dilutions relative to the no inhibitor control. The reaction was started by the addition of dihydroorotate followed by measurement of the absorption at 600 nm for 2 min at 30 °C. Each compound concentration included for the determination of the IC_{50} was analyzed in three or more independent experiments.

To determine the *in vitro* inhibition of RORγ, the Human RORγ Reporter Assay Kit (IB04001, Indigo Biosciences, State College, PA, USA) was used according to the manufacturer's protocol.

2.4. Primary cell culture-based virus infection models

Viral replication of SARS-CoV-2, HCMV and human adenovirus C5 (HAdV5) was performed using quadruplicate determinations as described in detail earlier (Wangen et al., 2024). For SARS-CoV-2, Caco-2 cells were infected with the recombinant SARS-CoV-2 that expresses YFP instead of the viral ORF6 protein (d6-YFP) at an MOI of 0.003 (Hahn et al., 2021; Herrmann et al., 2021). For the HCMV GFP-based replication assay, HFFs were infected with the HCMV AD169-GFP reporter virus at an MOI of 0.001 (Marschall et al., 2000). For analyzing the replication of HAdV5, a mNeonGreen-expressing reporter virus (HAdV5 neongreen) has been newly established (see supplementary methods). The anti-HAdV5 activity was determined by infection A549 cells with HAdV5 neongreen and subsequently treating with antiviral compounds. The viral dilution used for infection has been previously determined to yield 25–50% of the maximum fluorescence yield. Cells were fixed at 30 h (SARS-CoV-2), 3 d p.i. (HAdV5) or 7 d p.i. (HCMV) with 10% formalin. Virus replication was determined by quantitation of the intracellular YFP/GFP/mNeonGreen fluorescence in a PerkinElmer Victor X4 Multimode Plate Reader. The 50% effective antiviral concentrations (EC_{50}) were determined by fitting two parameter logistic dose-response curves to the experimentally determined values and are presented as arithmetic means rounded to two significant figures ± standard deviation (SD) derived from the number of independent biological replicates indicated. Certain compounds served as internal references to check the assay consistency and were therefore analyzed in more than three replicates. All available experimental data were used as the basis for the final EC_{50} calculation. To assess the potential drug-induced cytotoxicity, the cell viability was determined by the neutral red uptake assay in parallel uninfected cells as previously described (Repetto et al., 2008). The 50% cytotoxic concentration (CC_{50}), i.e. the concentration inducing a 50% reduction of cell viability, was calculated analogously to the EC_{50} values. The selectivity/specificity index (SI) was calculated as the ratio of CC_{50} to EC_{50}.

2.5. Metabolic rescue of virus replication by supplementation of cholesterol or uridine

The water-soluble cholesterol ester cholesterol-PEG600 (cholesteryl-polyethylene glycol 600 sebacate, Sigma-Aldrich) was dissolved in H_2O at 60 mg/mL equivalent to 30 mM free cholesterol after complete hydrolysis. Stocks of uridine (Sigma) were prepared in H_2O at 10 mM. The supplementation-mediated metabolic rescue efficiency was defined as the consequent increase in EC_{50} value when compared to solvent conditions.

2.6. Assessment of drug interaction by the Loewe additivity fixed-dose ratio method

Drug interaction analyses using the Loewe additivity method were performed as described previously (Chou, 2006; Hahn et al., 2021). Briefly, SARS-CoV-2-infected Caco-2 cells were treated with either the single drugs or a combination thereof starting at a concentration corresponding to the 4-fold EC_{50} value with seven subsequent 2-fold dilution steps. The resulting dose responses were used as input for CompuSyn software (Version 1.0; Chou, T.C.; Martin, N. 2005, CompuSyn for drug combinations; ComboSyn, Inc., Paramus, NJ, USA). Combination index (CI) values at 50, 75, 90 and 95% inhibition of virus replication were used to calculate the weighted CI value (CI_{wt}).

2.7. K18-hACE2 mouse SARS-CoV-2 in vivo model

The experiment was performed in transgenic K18-hACE2 mice (Jackson Laboratory, USA) according to the German Regulations for Animal Welfare after obtaining the necessary approval from the authorized ethics committee of the State Saxony and conducted as described previously (Gege et al., 2024). Mice received either 25 mg/kg twice per day (bid) molnupiravir, 50 mg/kg bid compound **1311** or a combination of both (same doses) bid via oral gavage. The control group received 2.5 mL/kg of the vehicle solution PEG400 (Merck, Darmstadt, Germany). Mice were infected intranasally with 300 FFU of SARS-CoV-2 (Wuhan strain) which consistently produces robust infections. After 4 days, mice were euthanized, and lung tissue was collected, homogenized, and viral RNA was isolated. RT-qPCR was performed using TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher, USA) and 5 μL of isolated RNA as a template, as previously described (Groβ et al., 2020). Synthetic SARS-CoV-2-RNA (Twist Bioscience, South San Francisco, CA, USA) was used as a quantitative standard. For the detection of infectious virus in lungs, Vero-E6 cells were infected with 100 μL of lung homogenates diluted in DMEM for 3 h. After replacing the supernatant with overlay medium (DMEM with 1% methylcellulose, 2% FCS and 1x penicillin/streptomycin), cells were incubated for 27 h. SARS-CoV-2 infected cells were visualized using SARS-CoV-2 S protein-specific immunochemistry staining with anti-SARS-CoV-2 spike glycoprotein S1 antibody (Abcam, Cambridge, Great Britain) as described previously (Case et al., 2020).

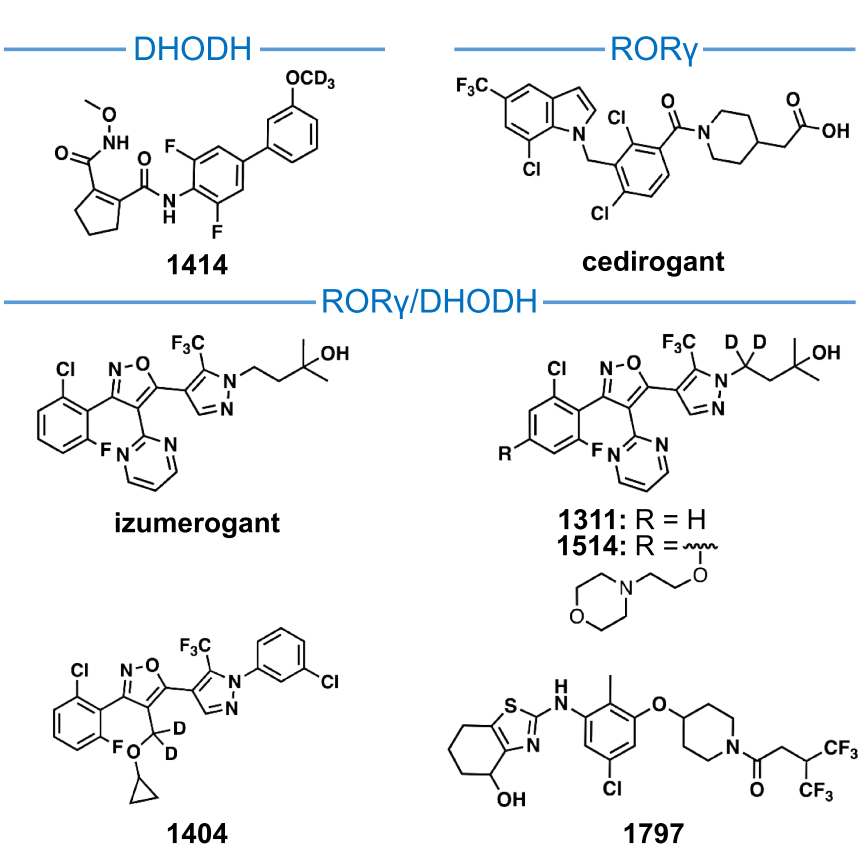
3. Results

3.1. Single target activity of RORγ/DHODH inhibitors

During a structure-activity relationship (SAR) optimization process, we generated a variety of izumerogant analogs with the focus on increased *in vivo* metabolic stability and oral exposure in rodents by selective deuteration of the metabolically labile positions (Pirali et al., 2019) (Fig. 1A). A metabolite identification experiment showed that the alpha position to the pyrazole moiety in izumerogant was preferentially oxidized and replacement of these hydrogen by deuterium atoms resulted in the metabolically more stable derivative **1311** (WO2023/198873). To increase the solubility of the generally quite lipophilic derivatives, the ionizable polar group morpholine was attached via a linker element at a suitable position and furnished compound **1514**, with the knowledge that this might negatively affect its activity. In the same way, the metabolically labile position of a structurally related molecule was deuterated, yielding **1404**.

First, we determined the single target activity as half-maximal inhibitory concentrations (IC_{50}) of izumerogant and three of its derivatives for RORγ as well as DHODH (Fig. 1B, Fig. S1). For comparison, the clinical Phase II RORγ inverse agonist cedirogant (Tyring et al., 2024), the DHODH inhibitor **1414** (Vietor et al., 2023), and **1797**, a recently described structurally distinct dual-acting compound (Chen et al., 2022), were analyzed in parallel (Fig. 1, Fig. S1). Regarding RORγ

A



B

	RORγ (nM)	DHODH (nM)
1414	3310 ± 822	150 ± 30
cedirogant	19.6 ± 7.4	inactive (>100 μM)
izumerogant	10.0 ± 2.8	98 ± 9
1311	11.9 ± 2.9	90 ± 6
1514	952 ± 693	2200 ± 260
1404	9.7 ± 3.6	100 ± 4
1797	33.4 ± 8.1	520 ± 40

Fig. 1. Chemical structures and single target activities of selected compounds. **(A)** Chemical structures of the RORγ inverse agonist cedirogant, the DHODH inhibitor **1414**, and RORγ/DHODH dual inhibitors izumerogant, **1797**, **1514**, **1311** and **1404**. (**B**) Single target IC_{50} values for DHODH inhibition measured by a cell-free enzyme activity assay and for RORγ determined by a reporter cell-based luciferase assay. Mean values ± standard deviation from at least three independent experiments are depicted.

inhibition, compounds **1311** and **1404** yielded similar IC_{50} values compared to izumerogant of approximately 10 nM. Notably, this activity is comparable to the reference compound cedirogant, which displays an IC_{50} of 20 nM (Fig. 1B, Fig. S1A), while compound **1797** was slightly less active (IC_{50} of 33.4 nM). As expected, **1514** displayed a 22-fold reduced target inhibition associated with the introduction of the morpholine group (Fig. 1B, Fig. S1A). Of note, for the single targeting DHODH inhibitor **1414**, we obtained an IC_{50} above 3 μM, suggesting that it is incapable of inhibiting RORγ (Fig. 1B, Fig. S1A). For DHODH inhibition, izumerogant and the derivatives **1311** and **1404** displayed IC_{50} values of around 100 nM, comparable to reference inhibitor **1414**, which did not modulate RORγ (Fig. 1B, Fig. S1B). As already assumed during the SAR process, compound **1514** showed a weak activity against DHODH in the micromolar range. The moderate DHODH inhibition by **1797** and the lack of activity of cedirogant on DHODH activity were likewise confirmed (Fig. 1B, Fig. S1B).

3.2. RORγ/DHODH inhibitors potently restrict SARS-CoV-2, HCMV, and HAdV5 replication in cell culture infection models

The antiviral activity of dual RORγ/DHODH inhibitors was evaluated against a representative enveloped RNA virus (SARS-CoV-2), an enveloped DNA virus (HCMV), and a non-enveloped DNA virus (HAdV5) using recombinant reporter viruses in appropriate cell culture models. The DHODH inhibitor **1414** and the RORγ inverse agonist cedirogant were analyzed in parallel as selective single target reference compounds (Fig. 2). Nucleoside analogs, namely the active metabolite of molnupiravir (EIDD-1931), ganciclovir (GCV) or brincidofovir (BCV), were employed as internal controls for SARS-CoV-2, for HCMV and HAdV5, respectively. EIDD-1931, GCV and BCV exhibited half maximal effective

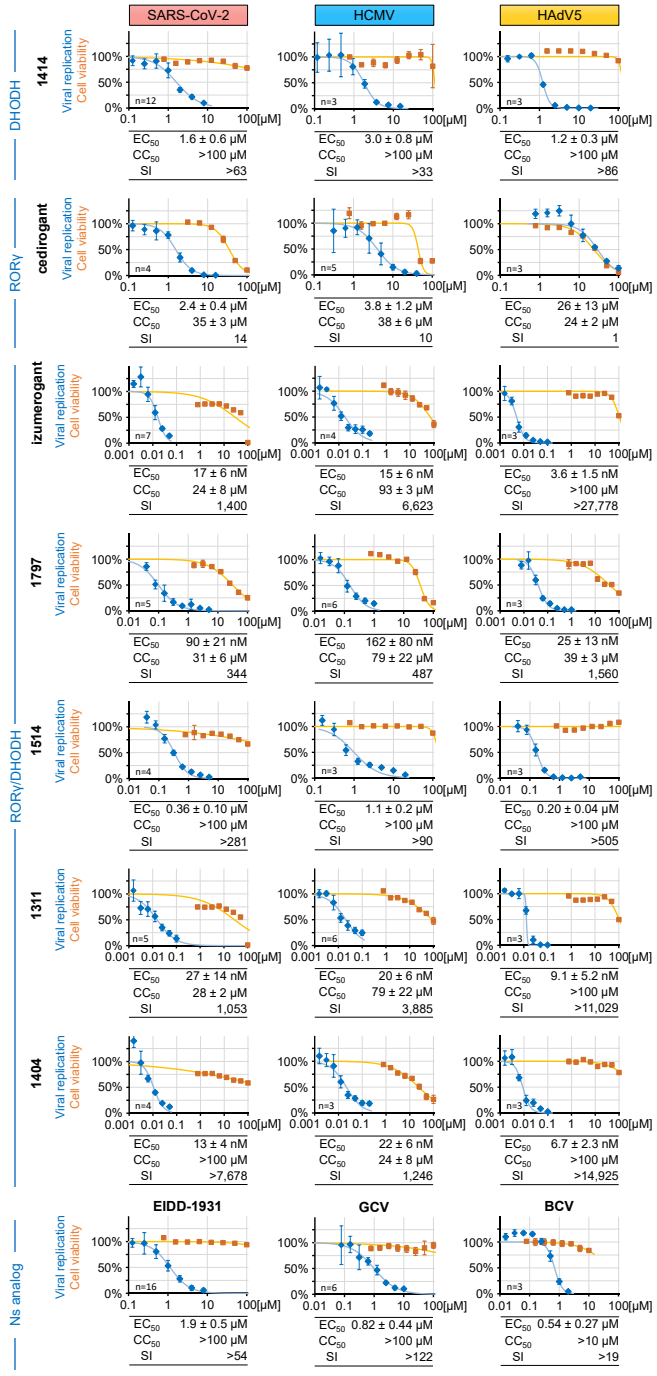


Fig. 2. Antiviral activity of DHODH- and RORγ-modulating small molecules. Caco-2 cells infected with SARS-CoV-2 d6-YFP, or HFFs infected with HCMV AD169-GFP or A549 cells infected with HAdV5 mneongreen. Cells were subsequently treated with descending concentrations of the DHODH inhibitor **1414**, the RORγ inverse agonist cedirogant, indicated RORγ/DHODH dual inhibitors or an appropriate antiviral nucleoside (Ns) analog, i.e. the active metabolite of molnupiravir (EIDD-1931), ganciclovir (GCV) and brincidofovir (BCV). Viral replication was measured by quantitation of the cell-associated YFP/GFP/mNeonGreen fluorescence at 30 h p.i., 7 d p.i. or 3 d p.i. Cell viability was determined in parallel uninfected cell cultures for the equivalent duration of exposure to the compounds. Shown is one representative dataset of the total number of experimental replicates stated in the lower left of each diagram.

antiviral concentrations (EC_{50}) in the range of 0.54–1.9 µM and confirmed the functionality of the replication assays. The single-targeting DHODH inhibitor **1414** showed EC_{50} values between 1.2 and 3.0 µM for all three viruses without any signs of cytotoxicity up to 100 µM. In contrast, the RORγ inverse agonist cedirogant was active against SARS-CoV-2 and HCMV with comparable EC_{50} values but did not exhibit any specific antiviral activity against HAdV5 (Fig. 2). The selectivity indices (SI) for SARS-CoV-2 and HCMV of 14 and 10, respectively, indicated a genuine antiviral activity consistent with our previous study (Wangen et al., 2024). Remarkably, the dual RORγ/DHODH inhibitor izumerogant efficiently blocked the replication of all three viruses with EC_{50} values between 3.6 and 17 nM. Some cytotoxicity was observed at higher concentrations, which differed between the cell types as illustrated by SI values ranging from 1400 for SARS-CoV-2 to 6623 for HCMV and >27,778 for HAdV5. To rank the inhibitory effect of izumerogant, we compared its antiviral activity to dual-targeting **1797**, which showed *in vivo* effects in the treatment of refractory inflammatory bowel disease (Chen et al., 2022). In agreement with izumerogant, **1797** reduced the replication of SARS-CoV-2, HCMV, and HAdV5 (Fig. 2), however with EC_{50} values (25–160 nM) higher than for izumerogant (3.6–17 nM) concomitant with lower CC_{50} values. To investigate the antiviral potential of the novel izumerogant derivatives, three molecules with different chemical properties and different levels of single target inhibition were selected: **1514**, **1311**, and **1404** (Fig. 1B). Compound **1514** exhibited EC_{50} values in the low to submicromolar concentration range, which is likely a consequence of reduced target inhibition for both RORγ and DHODH (Fig. 2). In agreement with previous reports, compound **1311**, which only differs from the drug candidate izumerogant by deuteration (WO2023/198873), showed similar inhibition of the three viruses compared to izumerogant with EC_{50} values varying between 9.1 and 27 nM. Similarly, compound **1404** blocked the replication of SARS-CoV-2, HCMV, and HAdV5 at comparable concentrations as izumerogant and **1311**. In all cases, the antiviral activity of dual host-targeting molecules was approximately 3- to 100-fold more pronounced compared to the reference drugs **1414** and cedirogant.

Additionally, we confirmed the antiviral activity of the dual RORγ/DHODH inhibitor **1404** against SARS-CoV-2 in a physiologically relevant system. Primary airway epithelial cells (hAECs) authentically reproduce distinct parts of the respiratory system and represent a major target for SARS-CoV-2 infection (Heinen et al., 2021). Derivative **1404** potently inhibited viral replication at concentrations as low as 5 nM to the detection limit (Fig. S2). Taken together, RORγ/DHODH dual compounds exhibit a profound antiviral activity against SARS-CoV-2, HCMV, and HAdV5 in cell culture and against SARS-CoV-2 in a primary *ex vivo* viral replication model.

3.3. The antiviral effect of dual inhibitors is dependent on the targeting of both RORγ and DHODH

To determine the individual contribution of both host targets on virus inhibition, we performed supplementation experiments with uridine, cholesterol, or both in SARS-CoV-2-infected Caco-2 cells. Basically, inhibition of DHODH prevents production of the ribonucleotide uridine monophosphate (UMP), which is the precursor of all pyrimidines (Fox et al., 1999). Thus, addition of external uridine was expected to restore nucleotide levels, thereby antagonizing the effect of DHODH inhibitors. We recently reported that cholesterol supplementation rescues RORγ inhibition (Wangen et al., 2024). As expected, the single targeting DHODH inhibitor **1414** and the single targeting RORγ inverse agonist cedirogant were affected by the addition of uridine or cholesterol, respectively, thereby confirming the target selectivity (Fig. 3). In the case of the dual inhibitors, the addition of uridine increased the EC_{50} values in a range from 2.7 to 10 µM for all compounds. The rescue efficiency for cholesterol differed between the various compounds. While for **1797** and **1514** a 1.6- or 1.8-fold increase was observed, **1311** and izumerogant displayed a 3.9- or 3.3-fold change. The most pronounced

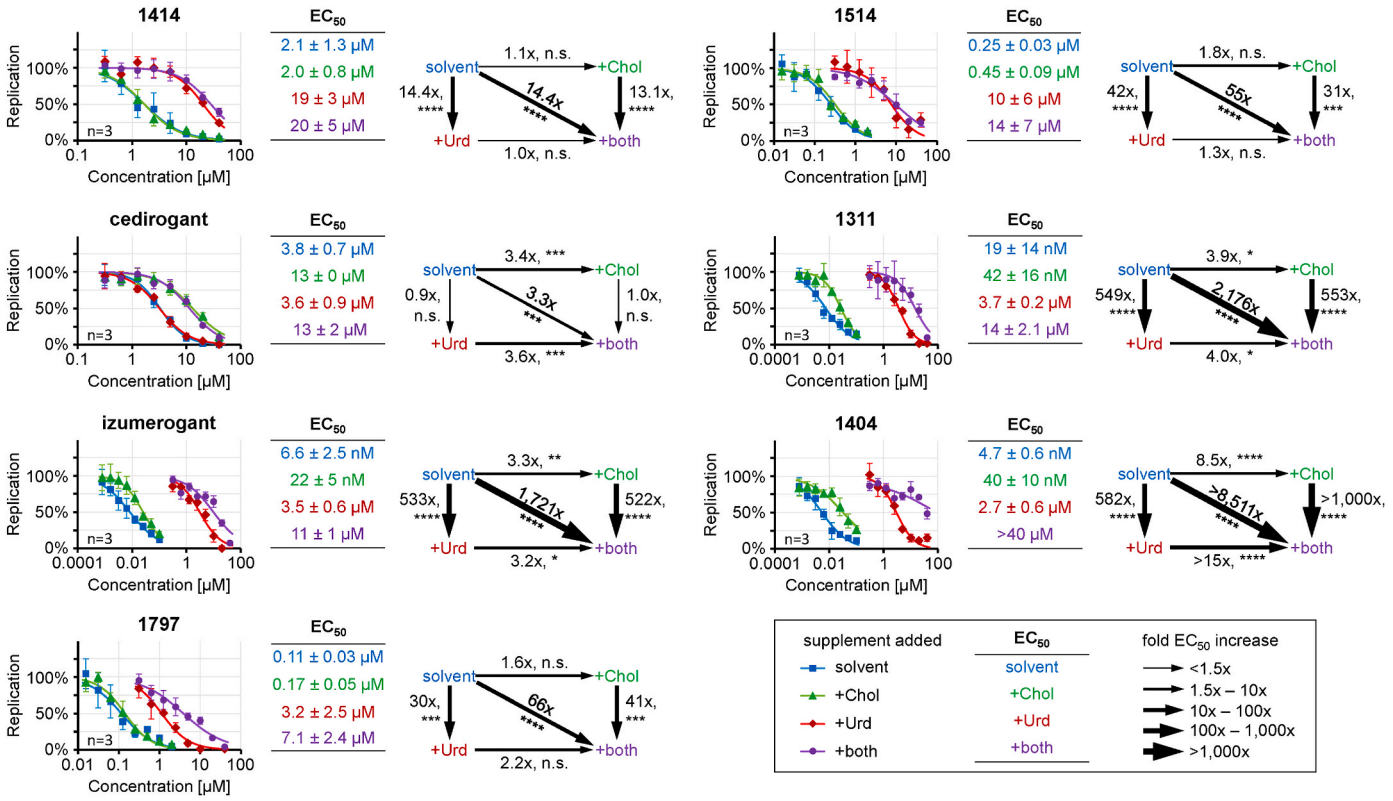


Fig. 3. Partial reversal of the antiviral effects by the supplementation with exogenously added cholesterol or uridine. Caco-2 cells infected with SARS-CoV-2 d6-YFP were treated with descending concentrations of the DHODH inhibitor **1414**, the RORγ inverse agonist cedirogant or indicated RORγ/DHODH dual inhibitors. For each setting, one of four parallel titrations was either supplemented with solvent (blue), cholesterol-PEG600 equivalent to 300 µM free cholesterol (+Chol, green), 100 µM uridine (+Urd, red) or a combination of Chol and Urd (+both, violet). EC_{50} values were determined from the dose responses for three independent experiments. The mean increases of EC_{50} in response to metabolite supplementation were determined and graphed in an arrow diagram. The direction of the arrow indicates the nature of the change. An arrow pointing right indicates an increase mediated by the addition of cholesterol, whereas an arrow pointing downwards indicates the effect of uridine. The diagonal arrow indicates the change induced by both supplements. The fold change values are depicted adjacent to the arrows and are additionally represented by the line thickness of the arrows. Statistical evaluation was performed using the one-way ANOVA with the Tukey's multiple comparison post-hoc test on the log_{10}-transformed EC_{50} values. n.s., not significant; *, $p < 0.05$; **$p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

effect was observed for **1404**, which achieved an 8.7-fold increase in EC_{50}. Taken together, these data demonstrate the inhibition of DHODH and RORy as the mechanistic basis of the antiviral activity of the dual-targeting compounds.

3.4. RORγ/DHODH inhibitors synergize with EIDD-1931 in cell culture

DHODH inhibitors act synergistically with nucleoside analogs, both in cell culture and in animal models (Hahn et al., 2020; Min and Sun, 2022; Schultz et al., 2022; Stegmann et al., 2022). To address whether the dual targeting RORγ/DHODH inhibitors maintain their synergistic relationship with nucleoside analogs, we performed combination experiments using **1311** in the SARS-CoV-2 cell culture model. Drug interactions between the **1311** and the active metabolite of molnupiravir (EIDD-1931) were analyzed in a 1:100 ratio by Loewe additivity fixed-dose ratio assay as described previously (Hahn et al., 2021) (Fig. 4A). Based on dose-response curves determined for single drugs and a combination thereof, this method mathematically determines combination indices (CI) for 50%, 75%, 90%, and 95% inhibition of virus replication (Chou, 2006). CI values between 0.9 and 1.1 indicate additive effects, and values below or above synergism or antagonism, respectively. The weighted CI value (CI_{wt}) describes the overall drug interaction and favors the desirable near-complete inhibition of virus replication. The combination of **1311** and EIDD-1931 revealed highly synergistic to synergistic effects for SARS-CoV-2 with a mean CI_{wt} of 0.29 ± 0.08. The synergistic interaction increased when approaching 95% of virus inhibition. In contrast, combination experiments of cedirogant with EIDD-1931 in a 1:1 ratio yielded only additive effects ($CI_{wt} = 0.90 \pm 0.10$) (Fig. 4B). Taken together, the synergistic potential of RORγ/DHODH dual compounds with nucleoside analogs was confirmed and agreed with previous reports for selective DHODH inhibitors. In the case of dual-targeting molecules, DHODH represents the primary component of this synergistic activity.

3.5. The anti-SARS-CoV-2 activity of RORγ/DHODH inhibitors is enhanced after co-treatment with molnupiravir in a mouse model

Next, we investigated the combinatorial effect of dual RORγ/DHODH-targeting compounds with nucleoside analogs against SARS-CoV-2 *in vivo*. Therefore, we selected compound **1311** due to its superior pharmacokinetic profile compared to izumerogant in mice (Table S1). During a 7-day tolerability study, no relevant changes in body weight, hematology, or clinical chemistry parameters were observed (Table S1). Subsequently, mice were pre-treated by oral gavage with either **1311**, molnupiravir, or a combination of both. Two hours post-treatment, mice were intranasally infected with SARS-CoV-2 and sacrificed after four days. One lung lobe was fixed for histopathological analysis, whereas the other lobe was homogenized. Quantification of viral load via RT-qPCR revealed a non-significant 7-fold reduction of viral genomes after treatment with **1311** and a 22-fold reduction upon administration of molnupiravir. Notably, the combination of both drugs yielded a 58-fold decrease in viral load (Fig. 5A). Determination of the viral titer further confirmed these results (Fig. 5B). **1311** alone reduced SARS-CoV-2 replication by 14-fold albeit non-

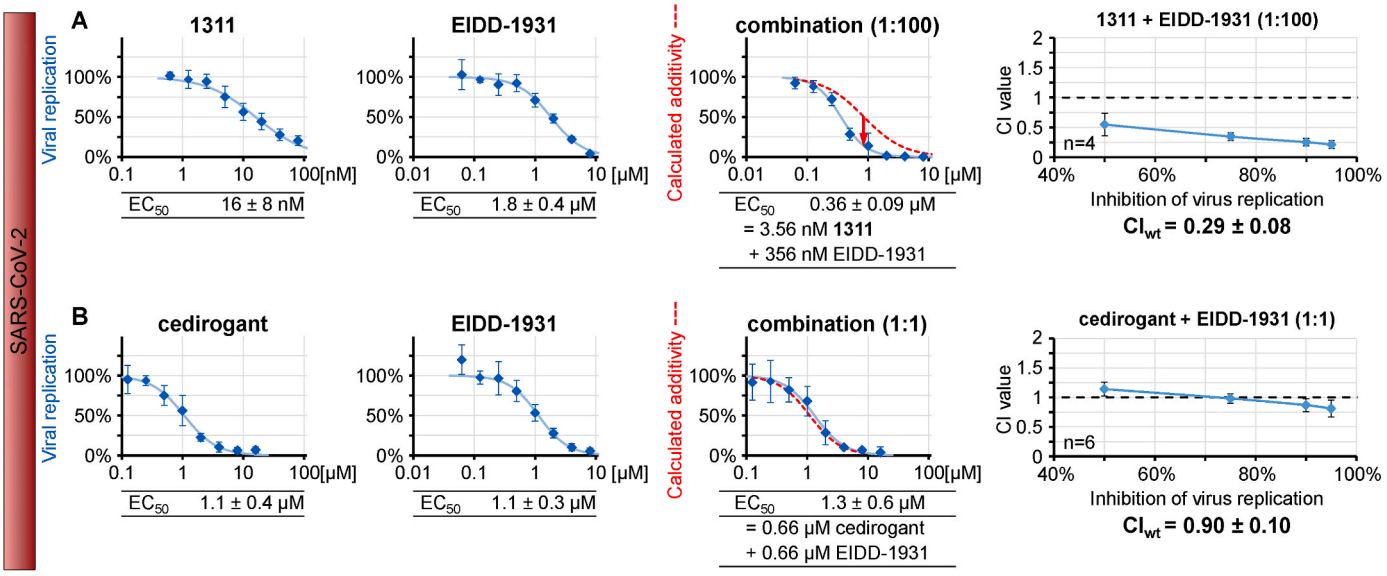


Fig. 4. The RORγ/DHODH dual inhibitor 1311 exhibits a synergistic drug interaction with EIDD-1931. (**A**) Caco-2 cells were infected with SARS-CoV-2 d6-YFP and treated with the dual RORγ/DHODH inhibitor **1311**, EIDD-1931 or a combination of both in a 1:100 ratio. (**B**) In parallel, cells were treated with either the RORγ inverse agonist cedirogant, EIDD-1931, or a combination of both drugs in a 1:1 ratio. Combination indices (CI) were calculated for 50, 75, 90, and 95% virus inhibition using the CompuSyn algorithm. Values represent mean ± SD for independent biological replicates. CI_{wt} < 0.3, strongly synergistic; 0.3–0.7, synergistic; 0.7–0.85, moderately synergistic; 0.85–0.9, slightly synergistic; 0.90–1.10, (nearly) additive; 1.10–1.20, slightly antagonistic; 1.20–1.45, moderately antagonistic; 1.45–3.3, antagonistic; >3.3, strongly antagonistic (Chou, 2006).

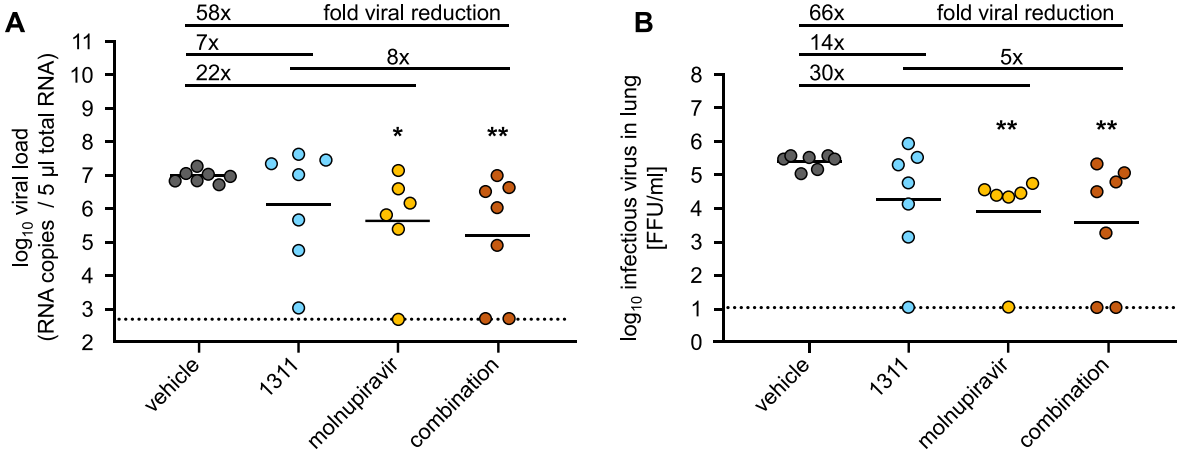


Fig. 5. The RORγ/DHODH dual inhibitor 1311 enhances the antiviral activity of molnupiravir *in vivo*. Female K18-hACE-2 mice received either vehicle (grey), **1311** (blue), molnupiravir (orange) or a combination of **1311** and molnupiravir (red) via oral gavage and were infected with SARS-CoV-2 (Wuhan strain). At day 4 after virus inoculation, the lungs were removed. (**A**) RNA was isolated from lung homogenates and viral genome copies were quantified via RT-qPCR. Data points shown represent viral copy numbers with the geometric mean of each group. The fold reduction in viral load of the vehicle control to the three compound-treated groups as well as **1311** to the combination treatment group is indicated. Statistical evaluation of the data was performed by Mann-Whitney *U* test in comparison to vehicle control (*: $p \leq 0.05$; **: $p \leq 0.01$). (**B**) Lung homogenate was titrated on Vero cells and SARS-CoV-2 infected cells were visualized after 30 h using spike-specific immunochemistry staining. Data points shown represent infectious units/mL of each animal with geometric mean of each group. Each point represents one mouse. Calculated reduction is shown in comparison to vehicle control. Statistical evaluation of the data was performed by Mann-Whitney *U* test in comparison to vehicle control (**, $p \leq 0.01$).

significantly, while treatment with molnupiravir reduced replication significantly by 30-fold. Again, the combinatorial treatment enhanced the antiviral activity, resulting in 66-times less infectious virus. As an indicator for tissue damage, we determined infiltration of lymphocytes into the lung of SARS-CoV-2-infected mice by histopathology (Fig. S3). While treatment with **1311** alone displayed no effect, molnupiravir reduced the median histopathology score of perivascular lymphocytes from 2 to 1.5. Notably, the combinatorial treatment of **1311** and molnupiravir further diminished the infiltration of perivascular lymphocytes, reducing the median score to 1 (Fig. S3). In contrast, no beneficial effects on interstitial lymphocytes were observed neither with **1311** or molnupiravir alone, nor with the combination treatment (Fig. S3). Taken together, the combination of a dual-targeting inhibitor with molnupiravir enhanced its antiviral efficacy and reduced lung tissue damage caused by a SARS-CoV-2 infection *in vivo*.

3.6. RORγ/DHODH inhibitors display a potent broad-spectrum antiviral activity

The pronounced inhibitory activity of izumerogant and related compounds against SARS-CoV-2, HCMV, and HAdV5 (Fig. 2) as well as the uridine- and cholesterol-dependent mechanisms suggests that these

molecules may display a broad-spectrum antiviral activity. To confirm this hypothesis, we performed cell culture replication assays using nine different human pathogenic viruses. To cover a broad spectrum, we included viruses from the Baltimore groups I [HAdV5, HCMV, monkeypox virus (MPXV)], IV [human rhinovirus type 14 (HRV-14), SARS-CoV-2, human coronavirus (hCoV) 229E and OC43, Zika virus (ZIKV)], V [influenza A virus (IAV), lassa mammarenavirus (LASV (Kim et al., 2020),), measles virus (MeV), respiratory syncytial virus (RSV)], and VI (HIV-1) in our analyses (Table 1, Fig. S4). Izumerogant and its close derivatives **1311** and **1404** were active against all viruses tested. In agreement with this finding, the dual-targeting compound **1797** also displayed a similar broad antiviral potential, however with a lower antiviral activity (Table 1, Fig. S4). These results indicate a broad-spectrum activity of the izumerogant compound class, thereby possibly paving the way towards the development of novel, highly potent antiviral drugs.

4. Discussion

As obligate intracellular pathogens, viruses rely on the host cell metabolism to replicate, and many viruses exploit similar cellular resources. Consequently, inhibitors of dependency factors are an appealing strategy for achieving a broad antiviral activity (Kumar et al., 2020; Tripathi et al., 2021). The *de novo* pyrimidine synthesis is widely recognized as a prerequisite for efficient viral replication (Zheng et al., 2022). As a consequence, we and others have extensively demonstrated the antiviral effect of DHODH inhibitors (Hahn et al., 2020; Gong et al., 2019; Purificação et al., 2024; Stegmann et al., 2022; Xiong et al., 2020; Zheng et al., 2022). In addition, we described the antiviral activity of RORγ inverse agonists against HCMV, VZV, SARS-CoV-2, and HIV-1 and proposed the depletion of cellular cholesterol as a mechanistic basis (Wangen et al., 2024). In the present study, we analyzed a panel of selected RORγ/DHODH dual-targeting inhibitors for antiviral activity. Surprisingly, the most active dual inhibitors exhibited EC_{50} values in the lower nanomolar range. This is an improvement of approximately two orders of magnitude compared to the selective DHODH or RORγ inhibitory reference compounds used as controls. It is notable that the single-target inhibition of izumerogant, **1311**, and **1404** was determined in similar concentration ranges to those of the references cedirogant and **1414**. This suggests that the simultaneous inhibition of both cellular activities by a single small molecule may be particularly potent presumably by targeting two crucial steps of viral replication, i.e. viral genome replication as well as membrane remodeling events (Fig. 6). On the one hand, supplementation assays demonstrated the contribution of both cellular targets to the overall antiviral effect, and on the other hand revealed differences in their rescue efficiency. Direct comparison of the three most potent dual-targeting compounds, i.e., izumerogant, **1311** and **1404**, to the less active **1514** and **1797**, revealed a correlation of their rescue efficiency by cholesterol supplementation to the potency of the antiviral activity. This notion was also supported by the fact that izumerogant, **1311** and **1404** likewise exhibited the highest single target RORγ inhibition. This suggests that further fine-tuning the inhibition of both targets might yield even better RORγ/DHODH dual-targeting antivirals in the future. Moreover, the simultaneous inhibition of two cellular targets will likely further increase the already high barrier for development of viral resistance proposed for HDAs.

The EC_{50} values obtained for the individual compounds were comparable between the diverse viral infection models. While for HCMV and IAV apparently lower sensitivities were observed, other replication systems, e.g. MPXV, HIV-1 or SARS-CoV-2, display a seemingly strong response. The observed differences could be attributed to the distinct cell types employed for infection or the various viruses but are nevertheless consistent with the HDA-specific antiviral mode of action. Unexpectedly, a comparable antiviral activity of dual-targeting compounds was detected for HAdV5 and HRV-14 replication as non-enveloped viruses, which was even more surprising when considering the lack of any specific antiviral activity of cedirogant against HAdV5. This potency of dual-targeting compounds against HAdV5 was particularly obvious for **1404** with an EC_{50} of 6.7 nM, but without the cytotoxic effect observed for the RORγ reference compounds cedirogant. This indicates a beneficial activity of dual-targeting compounds compared to pure RORγ modulators also against non-enveloped viruses. The validity of the candidate compounds is also reflected by the excellent SI values from approximately 1000 to >30,000, depending on the compound and the viral replication model used. In general, we demonstrated a pronounced inhibitory activity against several viruses from the Baltimore groups I, IV, V, and VI including enveloped, non-enveloped, DNA, RNA, and a retrovirus with comparable EC_{50} values. This highlights the broad-spectrum antiviral activity of the izumerogant compound class and its potential for further clinical development as an antiviral medication.

We show that the synergistic activity of the RORγ/DHODH dual-targeting compounds and nucleoside analogs is likely due to the DHODH inhibitory activity. Mechanistically, it has been proposed that depletion of pyrimidine nucleotides caused by DHODH inhibition promotes the incorporation of nucleoside analogs into nascent viral genomes (Stegmann et al., 2022). This increase in antiviral activity upon combination was similarly observed in an *in vivo* mouse model of SARS-CoV-2 infection (Stegmann et al., 2022). In the present study, the izumerogant-derived molecule **1311** reduced the viral load in lungs of SARS-CoV-2-infected mice, especially when combined with molnupiravir, thereby confirming the benefit of combination treatment also for RORγ/DHODH dual-targeting compounds. A similar advantageous effect of combination treatment was also observed at the level of tissue damage induction indicated by reduced infiltration of lymphocytes into the lung. Therefore, these results highlight the potential of RORγ/DHODH dual-targeting compounds especially in the context of a combination therapy with nucleoside analogs which are chosen specific to virus type. Since no significant off-target activities on common safety receptors, kinases, or nuclear hormone receptors were observed during preclinical characterization prior to its clinical Phase I trial, izumerogant and its close derivatives are promising small molecules for drug repurposing for antiviral therapy. Moreover, izumerogant displayed a favorable safety profile in a clinical trial. Although the complete tissue distribution has not been analyzed so far, blood serum levels exceeded

Table 1
The broad spectrum antiviral activity of RORγ/DHODH dual inhibitors. HAdV5: human adenovirus C5, HCMV: human cytomegalovirus, MPXV: monkeypox virus, HRV-14: human rhinovirus type 14, SARS-CoV-2: severe acute respiratory syndrome virus type 2, CoV: human coronavirus, ZIKV: Zika virus, IAV: influenza A virus, LASV: lassa mammarenavirus; MeV: measles virus, RSV: respiratory syncytial virus, HIV-1: human immunodeficiency virus type 1.

	Baltimore	Izumerogant (nM)	1311 (nM)	1404 (nM)	1514 (nM)	1797 (nM)
non-enveloped						
HAdV5	I	3.6	9.1	6.2	200	25
HRV-14	IV	–	–	1.0	–	–
enveloped						
HCMV	I	15	20	22	1100	160
MPXV	I	–	1.8	3.2	–	2.5
SARS-CoV-2	IV	17	27	13	360	90
hCoV-229E	IV	–	–	6.5	–	22
hCoV-OC43	IV	–	–	7.5	–	68
ZIKV	IV	–	–	18	–	35
IAV	V	75	–	110	–	1200
LASV[a]	V	–	–	17	–	–
MeV	V	–	–	7.5	–	67
RSV	V	–	–	3.3	–	75
HIV-1	VI	–	–	1.2	–	11

[a] Data previously reported in (Kim et al., 2020); -, not determined.

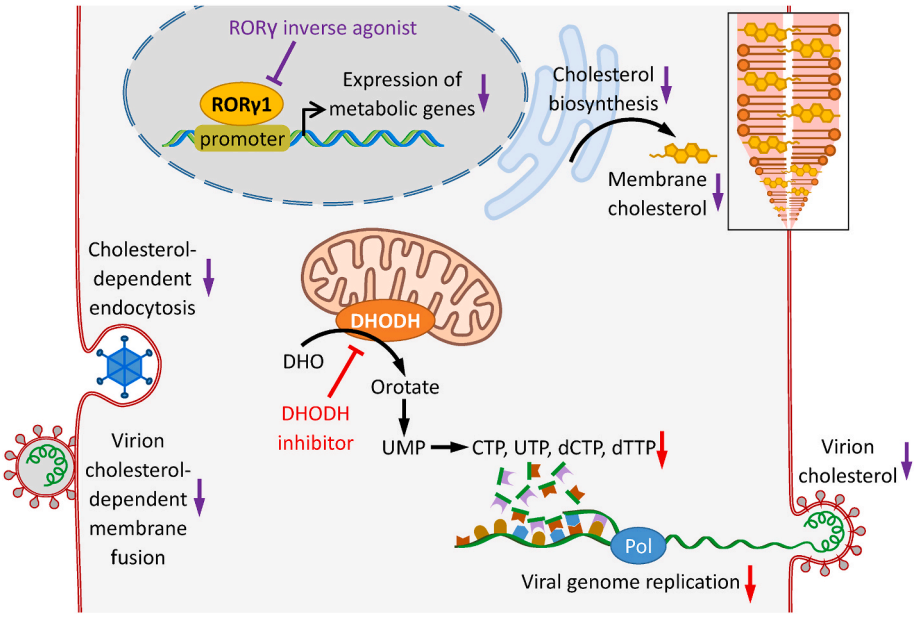


Fig. 6. Schematic representation of the antiviral targets RORγ and DHODH, the associated metabolic pathways and the proposed antiviral mechanism imposed by their inhibition. The inhibition of DHODH reduces the cellular pyrimidine *de novo* synthesis consequently interfering with the viral genome replication (as indicated in red). In the case of RORγ inhibition, the expression of multiple cholesterol synthesis enzymes is reduced by targeting RORγ as the responsible transcription factor (as indicated in violet). The proposed antiviral mechanism is presented by membrane remodeling events, e.g. viral membrane fusion with target cells or viral uptake by endocytosis. DHO, dihydroorotate; UMP, uridine monophosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; dCTP, deoxycytidine triphosphate; dTTP, deoxythymidine triphosphate.

the EC_{50} concentrations determined in the cell culture-based infection models investigated (Polasek et al., 2023).

Despite the promising results represented in this study, several limitations exist, particularly regarding the translational aspects of the dual inhibitors. Due to practical considerations, our study only included female mice, as they exhibit more comparable body weights at the same age, reducing experimental variability. This consistency is particularly important in high-containment studies where the number of animals used must be carefully controlled due to space, cost, and ethical considerations. However, it is important to acknowledge that sex-specific differences can significantly affect how organisms respond to medical treatments. While it seems unlikely, potential sex-related differences in response to certain inhibitors cannot be entirely excluded at this stage. Biological differences between males and females, including hormonal fluctuations, immune responses, and differences in metabolism, can alter the pharmacokinetics and pharmacodynamics of medications. Additionally, sex-related variations in the expression of enzymes that metabolize drugs, or even the distribution of certain drug receptors, could lead to differences in both efficacy and side effects between males and females. Furthermore, the treated cohorts showed variable degrees of reduction in viral loads, with some mice exhibiting undetectable virus RNA. Conversely, vehicle-treated mice demonstrated remarkably consistent viral loads, demonstrating comparable infection efficiency. This interpretation is also corroborated by the rigorous experimental procedures and the use of a highly susceptible mouse line expressing the ACE2 receptor in a multitude of tissues. Therefore, the observed differences are likely attributable to variable responses to the treatment rather than inconsistent infection. **1311** mediated a 7- or 14-fold reduction of virus titer, depending on the readout, which did not reach statistical significance, likely due to the aforementioned variations. Despite this, the overall effect was only 2 to 3 times weaker than that of molnupiravir, a drug that has been successfully used in clinical settings (Benaicha et al., 2023). Notably, this was achieved despite the fact that **1311** was exclusively optimized for human targets. A further limitation is the translation of the drug interaction approaches from *in vitro* to *in vivo* scenarios. While the combination of **1311** and molnupiravir revealed synergistic effects in cell culture experiments, no formal synergism was observed in mice. Nevertheless, the combination treatment demonstrated an overall stronger suppression of viral replication compared to single-drug treatments, suggesting that combination therapies could offer enhanced benefits, even if the expected synergy is not as pronounced in living organisms.

Taken together, RORγ/DHODH dual-targeting inhibitors represent promising drug candidates for broad-spectrum antiviral therapy. Our compounds may be the basis for future broad-acting antiviral therapies, enabling control of seasonal virus outbreaks and promoting pandemic preparedness.

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Wagner: Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis. **Melanie Kögler:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis. **Arne Cordsmeier:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Pascal Irrgang:** Methodology, Investigation, Formal analysis. **Wing-Hang Ip:** Resources, Methodology, Investigation. **Tatjana Weil:** Writing – review & editing, Methodology, Investigation. **Victoria Hunszinger:** Methodology, Investigation. **Rüdiger Groß:** Methodology, Investigation. **Natalie Heinen:** Methodology, Investigation. **Stephanie Pfaender:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Sebastian Reuter:** Resources, Methodology. **Robert Klopfleisch:** Investigation, Funding acquisition. **Nadja Uhlig:** Writing – review & editing, Investigation. **Valentina Eberlein:** Writing – review & editing, Investigation. **Leila Issmail:** Investigation. **Thomas Grunwald:** Writing – review & editing, Supervision, Investigation, Funding acquisition. **Benjamin Hietel:** Investigation. **Holger Cynis:** Writing – review & editing, Supervision, Funding acquisition. **Jan Münch:** Writing – review & editing, Supervision, Funding acquisition. **Konstantin M.J. Sparrer:** Writing – review & editing, Supervision, Funding acquisition. **Armin Ensser:** Supervision, Funding acquisition. **Matthias Tenbusch:** Supervision, Funding acquisition. **Thomas Dobner:** Supervision, Resources, Funding acquisition. **Daniel Vitt:** Supervision, Resources, Project administration, Funding acquisition. **Hella Kohlhof:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Friedrich Hahn:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: C. G., D.V. and H.K. have patent application WO2023/232870 filed on this topic. All other authors declare no competing interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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