Master's thesis

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Department of Clinical and Molecular Medicine

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Antiviral Combinations for Treatment of Enteroviral Infections

Broad-Spectrum Antiviral Triple Combinations Inhibits Enterovirus Replication In Vitro, and Delay Development of Resistant Viral Strains.

Master's thesis in Molecular Medicine
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Abstract

Enteroviruses are the leading cause of the common colds but can also induce to more severe conditions such as diabetes, meningitis, hand-foot-and-mouth disease, and paralysis. Despite the enteroviruses' significant impact on public health, no treatment has yet been approved due to the drugs' cytotoxic effects on host cells and rapid emergence of resistant strains. A potential treatment option with promising results involves combining antiviral drugs with different mechanisms of action against enteroviruses to achieve synergistic activity and thereby enhance therapeutic efficacy.

In our study, we investigated triple combinations consisting of pleconaril, rupintrivir, and either remdesivir or vapendavir against EV1, EV6, EV11, or CVB5 infections in A549 cells. Both the remdesivir-pleconaril-rupintrivir and vapendavir-pleconaril-rupintrivir combinations proved to be effective against enteroviruses, enabling a reduction in drug concentrations compared to single or double drug treatments. A reduction in drug concentration led to decreased toxicity towards host cells. Furthermore, these triple combinations successfully delayed the development of resistant viral strains compared to treatments with single or double drugs, particularly the vapendavir-pleconaril-rupintrivir combination. Sequencing of viral RNA-samples that turned resistant to the remdesivir-pleconaril-rupintrivir combination through passaging identified mutations potentially linked to drug-resistance. Our findings also demonstrated the effectiveness of the two triple combinations against EV1 in human lung organoids and EV-A71 in intestinal organoids.

The development and approval of triple drug BCC therapies represent a promising approach to improving treatment outcomes for enteroviral infections, with the potential to significantly improve patient management and prognosis. However, more research is required, and the drugs must be further evaluated, both experimentally and clinically, to ensure the safest and most efficient treatment options. The update of the drugvirus.info database with previously published triple combinations against viral infections offers easily accessible information about combinatorial treatments for future research, making the process more efficient.

Sammendrag

Enterovirus er den vanligste årsaken til forkjølelsessykdom, men kan også ligge til grunn for mer alvorlige sykdommer, som diabetes, meningitt, munn- og klovsyke, og paralyse. Til tross for deres store utbredelse og relevans, finnes det fremdeles ingen godkjente behandlinger utenom symptomatisk lindring. Dette er hovedsakelig på grunn av at medikamentene ofte har skadelige effekter på humane celler, men også fordi resistente stammer av virusene raskt oppstår som en konsekvens av behandlingen. Det mest lovende alternativet virker å være en kombinasjon av tre, eller flere, antivirale midler som påvirker ulike trinn av replikasjonssyklusen til virusene og som sammen oppnår synergistisk aktivitet slik at den terapeutiske effekten blir forsterket.

Våre studier undersøkte effekten av trippelkombinasjoner bestående av pleconaril, rupintrivir, og remdesivir eller vapendavir mot EV1, EV6, EV11, og CVB5 infiserte A549 celler. Både remdesivir-pleconaril-rupintrivir og vapendavir-pleconaril-rupintrivir hadde god effekt mot enterovirus, og konsentrasjonen av de ulike komponentene kunne reduseres sammenlignet med bruk av substansene alene eller i dobbelkombinasjoner. Ved å redusere konsentrasjonene viste de seg også å være mindre toksiske mot cellene. I tillegg bidro trippelkombinasjonene til å utsette utviklingen av resistente virusstammer sammenlignet med enkel- eller dobbelkombinasjoner. Sekvensering av viralt RNA fra prøver hvor effekten av remdesivir-pleconaril-rupintrivir ble svekket over tid, identifiserte flere mutasjoner som mulig bidrar til resistens hos EV1. I tillegg viste de to trippelkombinasjonene seg å være effektive mot EV1 i humane lungeorganoider og mot EV-A71 i intestinale organoider.

Utvikling og godkjenning av trippelkombinasjoner av antiviral midler viser seg å være en lovende fremgangsmåte for å forbedre behandling av infeksjoner forårsaket av enterovirus, med mål om å gjøre pasientbehandlingen og prognosen bedre. Det er likevel nødvendig med videre forskning for å nærmere evaluere medikamentene både eksperimentelt og klinisk for å sikre de tryggeste og mest effektive behandlingene. Oppdateringen av drugvirus.info databasen med trippelkombinasjoner er et viktig virkemiddel for å effektivisere videre forskning innen dette feltet.

Acknowledgement

First and foremost, I would like to thank my supervisor Professor Denis Kainov for his support and assistance throughout this project. His passion for virology and antiviral drugs has inspired me and helped me understand the importance of the project, which has been a big motivation.

I also want to thank the other colleagues at the institute who have helped me, especially Hilde Lysvand and Erlend Ravlo regarding the practical side of the project, and Aleksandr Ianevski for his help with figures. Thank you to Carlemi Calitz PhD, and dr. Qiuwei Abdullah Pan for their contribution with the organoid experiments. Their methods, results and figures are added in this thesis.

Publications

During my MSc, I have had the honor to contribute some of my methodology and results in an article published in Antiviral Research:

Ianevski A, Frøysa IT, Lysvand H, Calitz C, Smura T, Schjelderup Nilsen HJ, et al (2024). The combination of pleconaril, rupintrivir, and remdesivir efficiently inhibits enterovirus infections in vitro, delaying the development of drug-resistant virus variants. *Antiviral Res, 224*. doi:10.1016/j.antiviral.2024.105842

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Abbreviations

A549 cells	Human Adenocarcinoma Alveolar Basal Epithelial Cells
ATP	Adenosine Triphosphate
BCC	BSA-Containing Drug Cocktail
BME	Basal Medium Eagle
BSA	Broad-Spectrum Antiviral
CC50	Half-maximal cytotoxic concentrations
CTG	Cell-Titer Glo
CV	Coxsackievirus
CVB5	Coxsackievirus B5
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EC50	Half-maximal effective concentration
EV1	Echovirus 1
EV6	Echovirus 6
EV11	Echovirus 11
EV-A71	Enterovirus A71
FBS	Fetal Bovine Serum
MoA	Mechanism of Action
moi	Multiplicity of Infection
HAO	Human Air-Way Organoids
HE cells	Human Embryonic Lung Fibroblasts
HRV	Human Rhinovirus
Pen-Strep	Penicillin-Streptomycin
PV	Poliovirus
RdRp	RNA-dependent RNA polymerase
RPE cells	Retinal Pigment Epithelial
SI	Selectivity Index
T1D	Type 1 diabetes

1 Introduction

1.1 Literature review

Viral infections pose significant global health and financial challenges due to their ability to emerge and re-emerge from natural reservoirs making them unpredictable and difficult to treat (1). Despite extensive research has been performed to understand the biology of the viruses and host interactions, much remains unknown, and effective treatment methods are lacking (1, 2). While vaccines are currently utilized in efforts to combat viral diseases, their development is time consuming and detailed information about the specific strain causing the disease is required, making them inefficient against newly emerging strains (1, 3). Consequently, antiviral treatments offer a more promising option as they can effectively target novel viruses, thereby reducing the risk of severe disease and transmission of the virus. Despite the increasing demand for treatments, more than 200 human viral diseases still lack an approved antiviral drug (1). Currently, only 90 unique antiviral drugs have approved, with many more in various stages of development, reflecting the lengthy and thorough drug development process (Fig. 1).

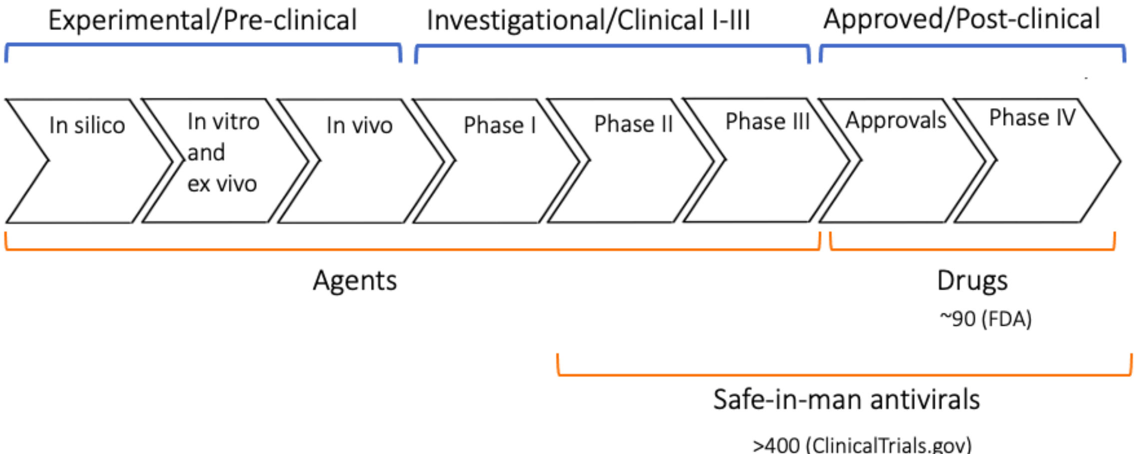


Figure 1. *Illustration of the drug development process, starting from the experimental stage to post-clinical monitoring. The pre-clinical phase begins with investigation and research, followed by testing compounds in vitro to assess potency, selectivity, and pharmacological properties. The pre-clinical phase end with pharmacodynamic, pharmacokinetic, and toxicity testing, in vitro and in vivo. The clinical phases involve administration of the drugs to humans through three phases to evaluate their safety and efficacy, and to confirm clinical doses and administration protocols. Once a drug has been approved by regulatory agencies, its safety continues to be monitored, and it may be withdrawn if unwanted effects are discovered (4).*

The rapid development of drug-resistance and the potential for drug toxicity complicate the approval processes. A promising approach is to use a combination of different drugs, particularly drugs with BSA activity (5, 6). BSA drugs have the ability to inhibit replication of

several viruses, either from the same or different families, and can be categorized as host-cell directed or virus directed (7). Host-cell directed antivirals target cellular components that are essential for viral replication or host response to infection. However, because of their cellular targets, they are often more cytotoxic than the virus directed options. Virus directed antivirals inhibit the formation of new viral particles by targeting crucial steps of the viral replication cycle (5). Side effects of BSA drugs vary depending on the specific drug and dosage, but the most common include nausea, fatigue, headaches, skin rash and diarrhea (8). By combining BSAs into BCCs (BSA-Containing Drug Cocktail), synergistic effects may be obtained, where the drugs together produce a greater effect than the total additive effect of the drugs on their own (3). This enables the drug concentrations to be lowered, consequently reducing the risk of cytotoxic effects (7). It has been suggested that combining antiviral drugs of different classes, different MoA, or drugs that target different stages of the viral replication cycle enhances the likelihood of synergistic activity (9). Figure 2 presents a selection of BSAs.

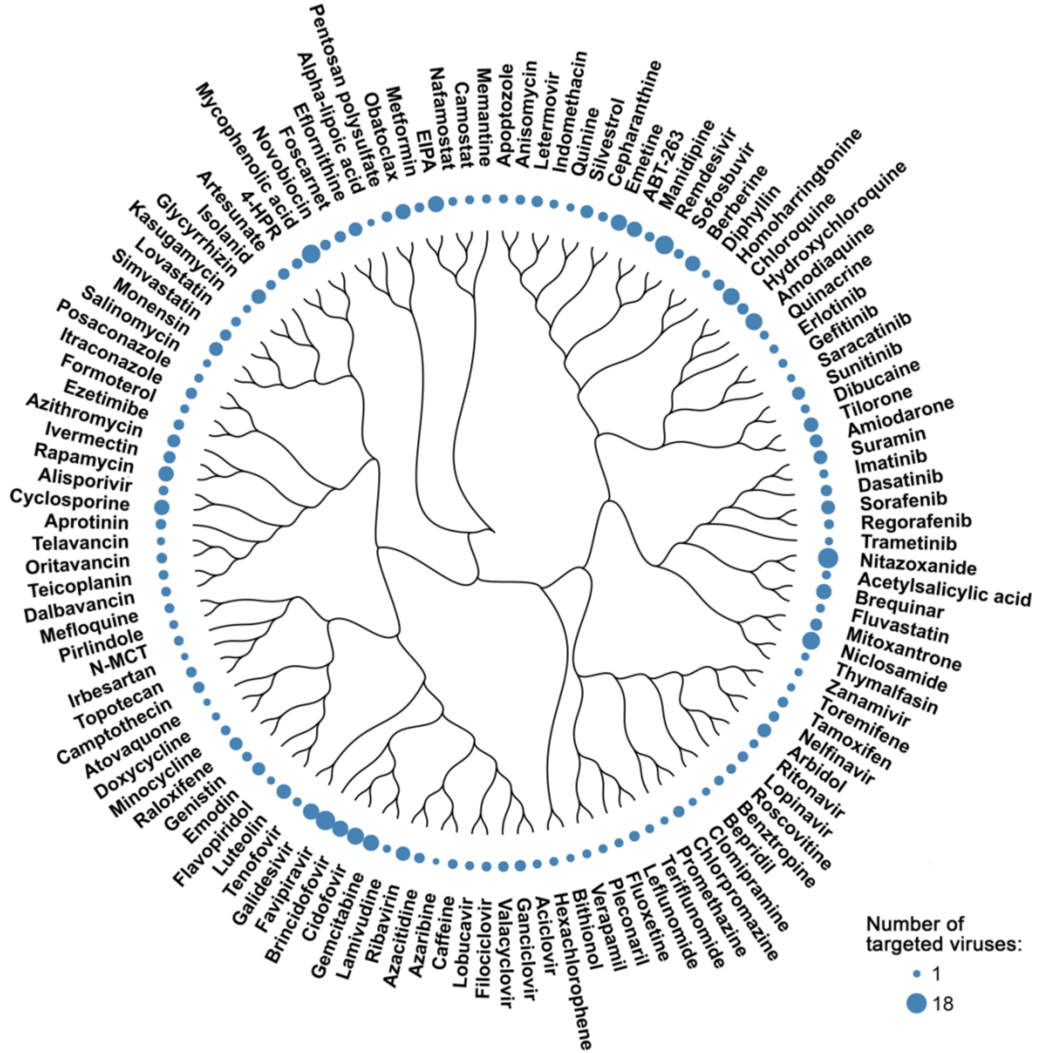


Figure 2. *Examples of BSAs and their structure-antiviral activity relation (adapted from Andersen PI, et al. Int J Infect Dis. 2020 (10))*

Enterovirus is a group of viruses that lack approved drugs, despite their significant contribution to total viral infections and their ability to cause severe illnesses, particularly in newborns and young children. Enteroviruses are small viruses belonging to the *Picornaviridae* family and range from 15 to 30 nm in size. They are non-enveloped with an icosahedral capsid, and their genome is positive-sense single-stranded RNA (+ssRNA) consisting of approximately 7400 nucleotides (11). The *Enterovirus* genus comprises five primary viruses; enterovirus, coxsackievirus, rhinovirus, poliovirus, and echovirus, which are further divided into more than 200 serotypes (12, 13). These viruses can lead to various diseases of differing severity, including meningitis, upper respiratory infections, encephalitis, myocarditis, paralysis and hand-foot-and-mouth disease (Fig. 3), and rhinoviruses are the most common causative agents of regular colds (14, 15). Moreover, studies have identified enterovirus infections, particularly CVB, in the pancreatic islets in newly diagnosed T1D patients, suggesting it being involved the pathogenesis of the disease (16).

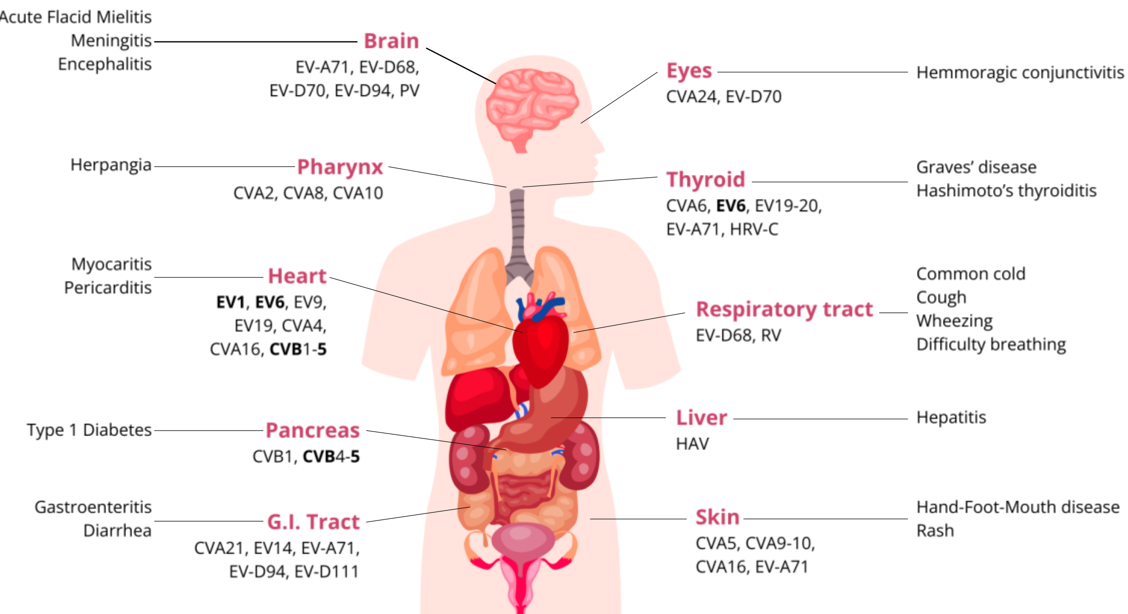


Figure 3. *Illustration of some of the most common tropism of picornavirus infections and the symptoms or diseases they can cause. Viruses used in this thesis are written in bold letters. (Adapted from Filipe IC et al. Enterovirus D: A Small but Versatile Species 2021) (17)*

Transmission of enteroviruses between individuals typically occur through fecal-oral or fecal-hand-oral route. The susceptibility to infection, clinical manifestation, severity, and outcome are largely determined by age, with young children being at the highest risk for infection and are the primary transmitter of the virus. Conversely, once infected with an enterovirus, the more severe cases of disease are often observed in older children and adults, except for CVB, which

can cause critical infection in newborns (11). Approximately 50% of all cases of aseptic meningitis in children are caused by non-polio enteroviruses, particularly echoviruses and coxsackieviruses (18). In the rare instances of central nervous system (CNS) infection following an enteroviral infection, the viruses are assumed to enter the cerebrospinal fluid (CSF) through the choroid plexus after viremia. However, in some cases, the virus can enter the CNS through infection of the muscle and neuromuscular junction, resulting in the spread to terminal axons (11). Various diseases that may result from CNS infection include aseptic meningitis, encephalitis, paralysis and ataxia (18). Young patients who recover from mild CNS enterovirus infection may experience intellectual impairment, and in cases of paralytic disease, permanent and severe disability may occur. Several enteroviruses have caused large epidemics, including echoviruses, coxsackieviruses and enterovirus D70 and A71 (11). The clinical manifestations and potential severe consequences underscore the necessity for approved drugs against enteroviral infections as the current treatments primarily aim to reduce and shorten symptoms (13). For the more severe cases with neurologic syndromes and diseases, fluids and electrolytes are often necessary, along with treatment for cardiovascular and autonomic abnormalities and respiratory failure. Other syndromes and diseases following an enteroviral infection may also require specific treatments (11).

Enteroviruses generally enter the host cells through receptor-mediated endocytosis when attached to one or multiple cell surface receptors, although the specific entry route varies depending on the virus serotype and the type of host cell (19). Replication usually occurs in the cells of the oropharynx and lower gastrointestinal tract (11). Within the endosome, receptor binding and/or pH changes induce uncoating of the virus, releasing the genome into the cytoplasm via endosomal pores (Fig. 4). The +ssRNA is then directly translated into a polyprotein, which undergoes further proteolytic processing by proteases $2A^{pro}$, $3A^{pro}$ and $3CD^{pro}$ into capsid proteins VP0, VP1 and VP3, as well as replication proteins 2A-2C and 3A-3D. Additionally, $2A^{pro}$ and $3C^{pro}$ cleave various host proteins to enhance the efficiency of translation, replication, and spread, while also inhibiting cellular responses to antivirals. Genome replication occurs on replication organelles and is facilitated by the RdRp $3D^{pol}$. The newly synthesized RNA is encapsulated by assembly of structural proteins VP0, VP1 and VP3 into pentamers, along with other processed proteins, to form new enterovirus particles. These new viral particles are released from the host cell through either cell lysis or exocytosis (19).

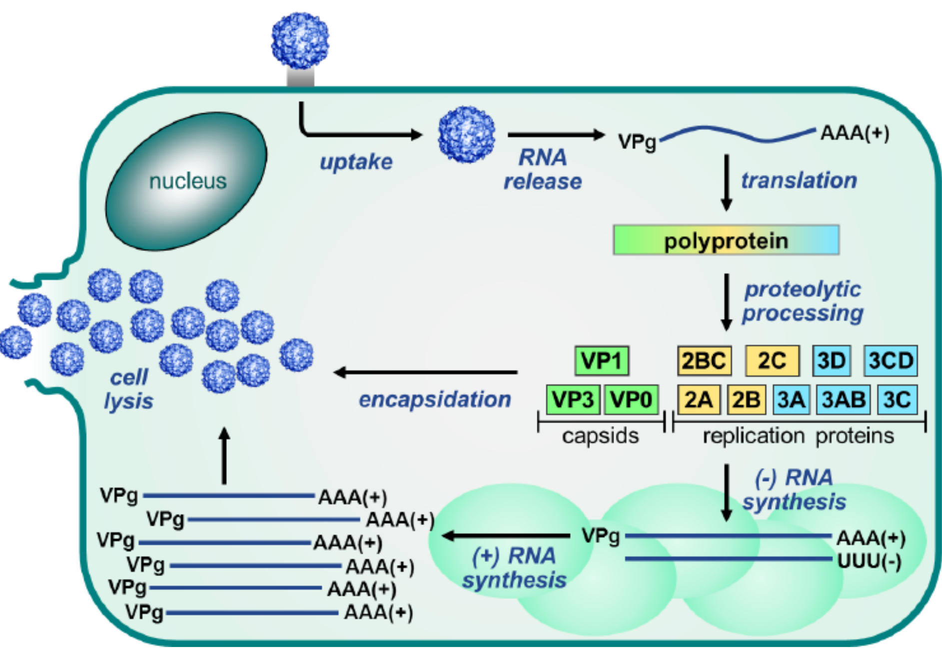


Figure 4*. Illustration of enterovirus replication cycle within host cell, from entry to release of new viral particles (20).*

Due to the vast number of serotypes of non-polio enteroviruses, vaccine development is complex, leading to increased emphasis on drug development. However, the high number of serotypes also presents challenges for antiviral discovery, highlighting the need for BSA development to ensure effectiveness against multiple enterovirus serotypes. Drug targets should encompass components that are highly conserved among the serotypes, including cellular receptors like ICAM-1 for human rhinoviruses, as well as various viral proteins such as proteases, polymerases, and VP1 (13). Despite numerous antiviral candidates, including small molecules, antibodies, and natural compounds, none have yet been approved, largely due to the emergence of resistant strains (21). Since enteroviruses are single-stranded RNA viruses, they are among the most rapidly evolving viruses. Compared to DNA viruses, which have a mutation rate of 10^{-8} to 10^{-6} substitutions per nucleotide site per cell infection (s/n/c), RNA viruses mutate at a rate of 10^{-6} to 10^{-4} s/n/c. The high mutation rate is primarily attributed to the RdRp, which, with some exceptions, lacks proofreading activity. In addition, single-stranded viruses mutate at higher rates than double-stranded viruses (22).

Some of the most promising drugs tested against enteroviruses include pleconaril, remdesivir, rupintrivir, and vapendavir. These are all virus directed drugs targeting different steps of the viral life cycle (see Table S1). Remdesivir is metabolized by host cells into a nucleoside triphosphate, which can serve as an ATP analogue competing with the natural ATP substrate.

This competition may result in the analogue being incorporated into viral RNA chains by the RdRp instead of natural ATP, leading to premature termination of RNA synthesis. Originally developed against Ebola viruses, remdesivir exhibits BSA activity and has shown to be effective against numerous viruses (23).

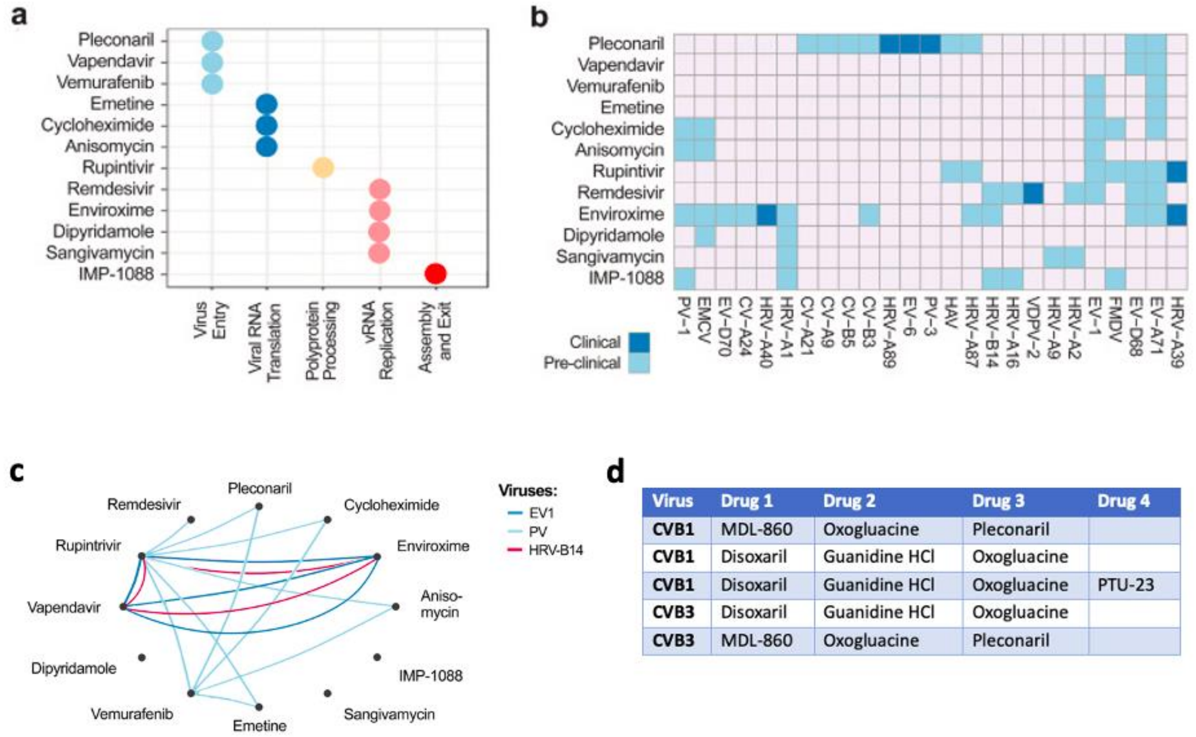
Pleconaril, another BSA, functions as a capsid binder that attaches to hydrophobic pockets containing amino acid residues from VP1 (24-26). When pleconaril binds, the capsid is compressed in a way that prevents host-cell attachment and uncoating of the RNA (27). Pleconaril is effective against most rhino- and coxsackievirus strains but can also be inactive against other strains of rhino- and enteroviruses (26, 28). Despite being one of the most effective drugs against enteroviruses along with vapendavir, pleconaril has never been approved due to limited efficacy, development of resistant strains, and interference with other drugs leading to severe side effects (13). Vapendavir is structurally different to pleconaril, but functions similarly as a BSA capsid binder, attaching to the hydrophobic pocket in VP1 and thereby prevent attachment and entry into the host cell (15). Rupintrivir, the final drug discussed, is developed as an inhibitor of the chymotrypsin-like (3C) protease of enteroviruses (29, 30). The drug contains specific structures that allow the formation of covalent bonds with cysteine residues in the active site of the 3C protease. When rupintrivir binds to the protease, it prevents the protease from exerting its role in the viral replication by inhibiting the proteolytic cleavage of large polyproteins (30).

Vapendavir, pleconaril, remdesivir, and rupintrivir have all undergone clinical trials as single treatments and have demonstrated safety in human use. Their positive results when used individually make them highly relevant for further exploration as part of BCCs to delay or prevent development of resistant strains. Figure 5 provides an example study for each of the four drugs, showing promising results.

DRUG	REFERENCE	STUDY TITLE	STATUS	DOSE
Pleconaril	NCT00031512	Pleconaril Enteroviral Sepsis Syndrome	PHASE2 Completed	5 or 8.5 mg/kg/dose oral every 8 h for 7 days (21 doses) of a 40 mg/mL oral liquid formulation.
Remdesivir	EMA/791331/2022	Vaklury (remdesivir) is used in adults and children, from at least 4 weeks of age with pneumonia requiring supplemental oxygen	EMA approved	Children: 5 mg/kg on the first day, followed by 2.5 mg/kg once a day. Adult: 200 mg infusion on the first day, followed by 100 mg once a day.
Rupintrivir	PMID: 14638501	Phase II, randomized, double-blind, placebo-controlled studies of rupintrivir nasal spray 2-percent suspension for prevention and treatment of experimentally induced rhinovirus colds in healthy volunteers	PHASE2 Completed	Intranasal (8 mg) as prophylaxis (two or five times daily for 5 days)
Vapendavir	NCT06149494	RCT of Vapendavir in Patients With COPD and Human Rhinovirus/Enterovirs Upper Respiratory Infection	PHASE2 Recruiting	500 mg tablets

Figure 5. *Studies including vapendavir, pleconaril, remdesivir, and rupintrivir as single treatment, their status in development, and dose of administration.*

Numerous drugs have been tested against enteroviruses, and some of these, along with published double- and triple combinations, are presented in Figure 6 (31). Based on literature review, twelve drugs were found to be promising as potential treatment options. The drugs of interest were pleconaril, rupintrivir, remdesivir, vapendavir, IMP 1088, anisomycin, emetine, enviroxime, cycloheximide, vemurafenib, dipyridamole, and sangivamycin. The MoA of the drugs are presented in Figure 6a. Figure 6c presents the double-combinations of the mentioned drugs that have been tested against EV1, PV-1 or HRV-B14. The combinations involve nine out of the twelve antivirals tested as monotherapy. Further research of these drugs is of high interest to come closer to an approved treatment of enteroviruses. Today, only a few triple combinations have been published, and they are all tested against CVB (Fig. 6d), but none have yet been approved.

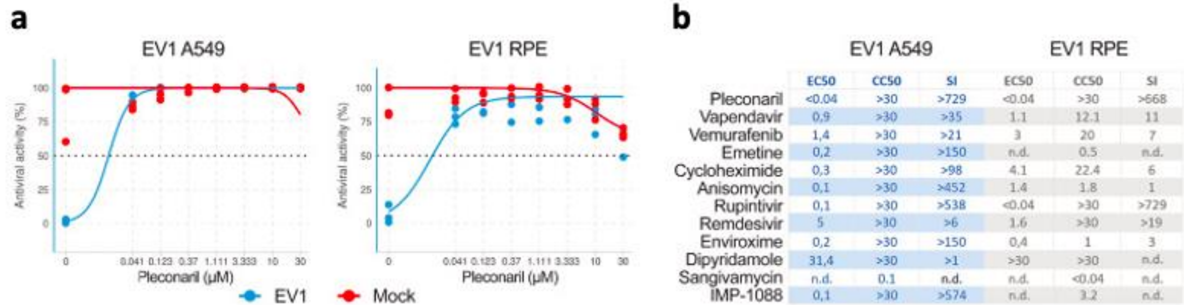


Virus	Drug 1	Drug 2	Drug 3	Drug 4
CVB1	MDL-860	Oxogluacine	Pleconaril	
CVB1	Disoxaril	Guanidine HCl	Oxogluacine	
CVB1	Disoxaril	Guanidine HCl	Oxogluacine	PTU-23
CVB3	Disoxaril	Guanidine HCl	Oxogluacine	
CVB3	MDL-860	Oxogluacine	Pleconaril	

Figure 6. *Literature review of anti-enteroviral drugs. (a) Effect of antiviral agents on enterovirus replication. (b) Overview of which viruses the drugs have been tested against, and what state of testing they have reached. (c) Two-drug combinations tested against EV1, PV, and HRV-B14 (31). (d) Table of published 3- and 4-drug combinations and which enteroviruses they have been tested against.*

1.2 Previous work at supervisor's lab

In the initial experiments, EV1 infected A549 and RPE cells were treated with the twelve drugs presented in Figure 6 separately at seven different concentrations. The efficacy and toxicity of the compounds were assessed by measuring cell viability using the CTG assay. The results were utilized to determine the drugs' EC50, CC50, and SI values. EC50 represents the concentration of a drug that induce 50% of its maximum biological effect (32), while CC50 is defined as the concentration that causes cytotoxicity in 50% of the cells compared to untreated cells (33). The ratio of these two parameters gives the selectivity index (SI), which is used to evaluate the safety and therapeutic window of a drug. A higher SI indicates a safer drug, as it is effective at lower concentrations than those needed to produce toxic effects (34). As presented in Figure 7b, several of the drugs demonstrated a SI >3, and had an inhibitory effect on the viral infections without being toxic to the host cells (31).



	EV1 A549			EV1 RPE		
	EC50	CC50	SI	EC50	CC50	SI
Pleconaril	<0.04	>30	>729	<0.04	>30	>668
Vapendavir	0,9	>30	>35	1.1	12.1	11
Vemurafenib	1,4	>30	>21	3	20	7
Emetine	0,2	>30	>150	n.d.	0.5	n.d.
Cycloheximide	0,3	>30	>98	4.1	22.4	6
Anisomycin	0,1	>30	>452	1.4	1.8	1
Rupintivir	0,1	>30	>538	<0.04	>30	>729
Remdesivir	5	>30	>6	1.6	>30	>19
Enviroxime	0,2	>30	>150	0,4	1	3
Dipyridamole	31,4	>30	>1	>30	>30	n.d.
Sangivamycin	n.d.	0.1	n.d.	n.d.	<0.04	n.d.
IMP-1088	0,1	>30	>574	n.d.	3.2	n.d.

Figure 7. *Antiviral activity of a selection of drugs targeted towards EV1. (a) Antiviral activity of pleconaril at different concentrations in EV1 infected A549 and RPE cells. CTG assay was used to determine cell viability. (b) Table shows the EC50, CC50 and SI (=CC50/EC50) of the drugs tested on EV1 infected A549 and RPE cells (31).*

Further, experiments were performed to make a complete version of Figure 6c with all pairs of the twelve drugs, and to calculate Bliss synergy scores for the combinations using SynergyFinder. The drug pairs were tested on EV1 infected A549 cells, and cell viability was measured. The synergy score is used to evaluate the effect of combining multiple drugs, categorizing them in synergistic, additive, or antagonistic effects (35). 25 of the combinations were classified as synergistic with a synergy score >5 (Fig. 8b), and successfully increased cell viability. As pleconaril had shown to be highly efficient, pleconaril-containing triple combinations with varying concentrations were tested against EV1 infected cells. High synergy was found for the combinations consisting of 1) pleconaril, rupintrivir and remdesivir and 2) pleconaril, rupintrivir and vapendavir. These combinations were tested against EV1 infected A549 cells, and further testing against other enteroviruses will be performed in this thesis.

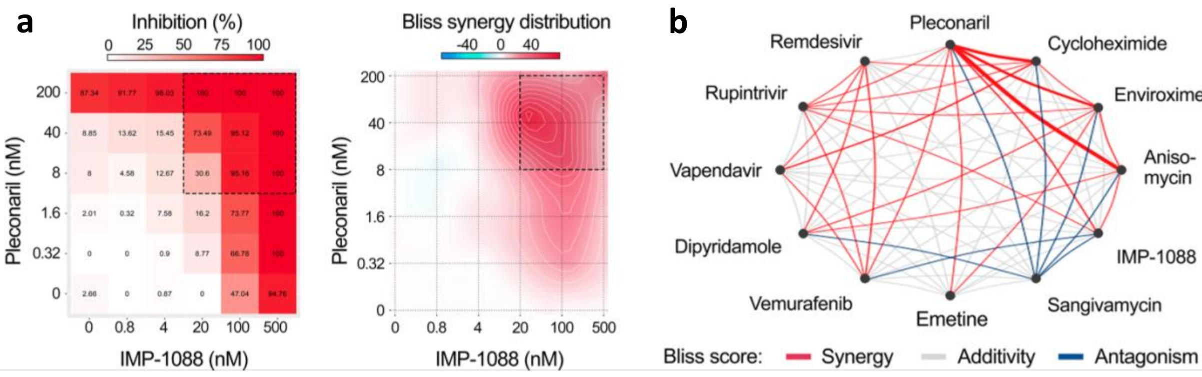


Figure 8. *Double combinations of antiviral drugs. (a) Effect of pleconaril combined with IMP-1088, in different concentrations, on EV1 in A549 cells. Viability was measured with CTG assay, and figures show inhibition of virus replication and calculated Bliss synergy scores. (b) Diagram showing the antiviral activity of 65 double combinations of the 12 drugs. Bliss scores were calculated, and blue lines represent antagonistic combinations (Bliss score <5), grey lines are additive combination (Bliss score 0-5), and red lines*

correspond to synergistic combinations (Bliss score >5). Thicker red lines represent a higher synergy score (31).

The drugvirus.info 2.0 database has also been created by Prof. Kainov and his team as an aid to make information about antivirals more easily accessible for further research and drug repurposing. It consists of a BSA and BCC database, with the BSA database providing info on what virus the different drugs inhibit. The BCC database contains BSA combinations against viruses and the stage of their development (36).

2 Aim and objectives

The first part of the thesis involves testing of two triple drug combinations against enteroviruses. Combination one includes remdesivir, pleconaril and rupintrivir, while the second consists of vapendavir, pleconaril and rupintrivir. The aim is to determine the most efficient concentrations that, when combined, produce the highest synergistic activity without inducing toxic effects. Another goal is to evaluate how the enteroviruses potentially mutate and develop resistance when exposed to the drugs over time, with the hope of finding combinations that delay the emergence of resistant enteroviral strains. This research will be conducted in collaboration with Amsterdam UMC, where sequencing will be performed. Additionally, the two combinations will be tested on organoids; however, this part will be done by Carlemi Calitz, PhD, at Amsterdam UMC, and dr. Qiuwei Abdullah Pan at Erasmus MC in Rotterdam. Their results will be briefly presented in this thesis.

The second objective is to update the drugvirus.info database with triple combinations of antivirals. Prior to the start of this project, drugvirus.info consisted of single and double combinations. Maintaining an updated database is important to provide researchers with easily accessible information regarding the drugs of interest and their combinations. This can contribute to time savings in future research by eliminating the need to search for appropriate combinations and concentrations, allowing scientists to focus on other parts of the research.

3 Materials and methods

3.1 Cell cultures

HE and RPE cells were supplied by Pål Magnus Holien from the Department for Medical Microbiology at St. Olavs Hospital. HE cells were grown in BME medium with 5% FBS and 1% Pen-Strep, while DMEM-F12 supplemented with 10% FBS and 1% Pen-Strep was used as cell growth media for the RPE cells. A549 cells, stored at -80°C from previous experiments, were thawed and cultured in DMEM high glucose with the addition of 10% FBS and 1% Pen-Strep. Incubation was done at 37°C with 5% CO_2 until ~80% confluence.

3.2 Viruses

A total of 15 viruses were used in the experiment (Table A1). Among these, 13 were isolated and provided by Pål Magnus Holien from St. Olavs Hospital. EV1 was the ATCC Farouk strain, and EV6 was previously isolated in the laboratory. The virus stocks were stored at -80°C. Virus growth media used for amplification in A549 cells consisted of DMEM high glucose supplemented with 0.2% BSA (bovine serum albumin) and 1% Pen-Strep. For HE cells, BME medium containing 2% FBS and 1% Pen-Strep was used, while DMEM-F12 with 0.2% BSA and 1% Pen-Strep was used for virus amplification in RPE cells. Incubation was done at 37°C with 5% CO_2.

3.3 Drugs

Remdesivir, vapendavir, pleconaril and rupintrivir were made as 10 mM stocks in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Germany) or milli-Q water, and stored at -20°C. Supplier and other details of the drugs are presented in Table B1. An overview of structure, mechanism of action, and drug-type is presented in Table S1.

3.4 Virus infectivity

HE, RPE, and A549 cells where infected with the 15 viruses. 2 mL A549 cells were added to 16 wells in a 24-well plate. After 24 h incubation until ~80% confluence, the medium was removed and replaced with 1 mL virus growth media. Subsequently, 2.5 µL of each of the 15 virus isolates were added to their respective wells, and the cells were further incubated for 72 h. No virus was added to the last well, which served as a mock.

For the two other cell lines, 500 µL cells from culture were seeded in 24-well plates, one plate for RPE and one for HE, and incubated for 24 h. The medium was then removed and replaced with 1 mL virus growth media, followed by the addition of 2.5 µL of the virus isolates. After 72 h incubation, CTG assay was performed to determine cell viability and the viruses' infectivity. All the media was removed from the wells and replaced with 100 µL CTG. 33 µL was transferred in triplicates to 96-well plates and analyzed using FLUOstar omega.

3.5 Drug combination test

A549 cells were infected with mock, EV6, EV11, and CVB5, separately, with the presence or absence of the drugs in the two combinations. This had previously been done for EV1 by Prof. Denis Kainov. 5-fold dilutions of the drugs were prepared in virus growth medium to create five concentrations in addition to 0 µM. The concentrations were based on the drug's EC_{50} determined from previous experiments performed with EV1 (see section 1.2).

For the first combination, the starting concentration of remdesivir was 10 µM, and for pleconaril and rupintrivir it was 0.1 µM, which was then diluted 5-fold five times. 33 µL of remdesivir concentrations was added to their separate 96-well plates with A549 cells, while 33 µL of the serial dilutions of pleconaril and rupintrivir were added to all the plates. Pleconaril concentrations decreased vertically, and rupintrivir decreased horizontally. 2 µL virus (moi 0.1) from stock was added to each well. After 48 h incubation, CTG assay was performed.

For the second combination consisting of vapendavir, rupintrivir and pleconaril, the same method was followed, and cells were infected with the same viruses. Vapendavir started at 0.5 µM, rupintrivir at 0.3 µM and pleconaril at 0.1 µM, and 5-fold dilutions were made from these to five concentrations, plus 0 µM.

3.6 Drug-resistance test

A549 cells were exposed to EV1 and antivirals from the two sets of combinations through twelve passages. Solutions consisting of the three drugs alone and in double and triple combinations were made to a concentration of 2 µM remdesivir, 0.02 µM pleconaril and 0.02 µM rupintrivir in virus growth medium. The solutions were made fresh between each passage. The second combination consisting of vapendavir, pleconaril and rupintrivir was made to 0.1

μM of each drug. The drug solutions were frozen at -20°C and reused for the following passages. 100 μL of each antiviral combination was added in triplicates to 96-well plates with a monolayer of approximately 4 x 10^4 A549 cells that had been prepared 24 h earlier. Included was a triplicate of wells containing only cells, and three wells with cells exposed to EV1 without adding drug compounds. For the first passage, 2 μL EV1 was added from stock, while for the following, 5 μL supernatant from the previous passage was added. The plates were incubated at 37°C with 5% CO_2, and CTG assay was performed after 48 h to evaluate cell viability. The supernatant from each passage was frozen at -20°C.

For cells exposed to the first combination consisting of remdesivir, pleconaril and rupintrivir, RNA was isolated from the frozen supernatants from passage eight by using the RNeasy® Plus Mini Kit (QIAGEN) and sent to Amsterdam University Medical Centers for sequencing.

3.7 Cell toxicity test

To assess whether the drugs had toxic effects on the A549 cells, 100 μL of the drug combinations from Section 3.6 were added to plates seeded with A549 cells, in triplicate, without the addition of virus. A triplicate of wells containing cells without adding drugs served as controls. Following a 48-h incubation at 37°C with 5% CO_2, cell viability was measured using the CTG assay. This was repeated three times to ensure consistent results.

3.8 Organoids

The two triple-drug combinations were tested on different organoids, with concentrations based on our findings from Section 3.5. However, the experiments were performed externally in Amsterdam and Rotterdam. The triple combination consisting of remdesivir, pleconaril and rupintrivir was tested on human intestinal enteroids infected with EV-A71 at Amsterdam UMC by Carlemi Calitz, PhD. The concentrations used were 1 μM remdesivir, 0.1 μM pleconaril and 0.1 μM rupintrivir. The second combination, consisting of vapendavir, pleconaril and rupintrivir, all at a concentration of 0.1 μM, was tested on EV1-infected human air-way organoids (HAOs) by dr. Qiuwei Abdullah Pan at Erasmus MC in Rotterdam.

3.9 Update of drugvirus.info database

To identify previously tested drug combinations involving more than two drugs against enteroviruses and other human viruses, PubMed was utilized as the primary database. The search terms "Antiviral triple combination enterovirus", "Synergistic antiviral triple combination", and "Antiviral triple combination" were employed. Additionally, drugs.com was used to find combinations with three or more drugs that are already approved for treatment of human viral diseases. The results were compiled into an Excel file and sorted alphabetically within each virus type. Duplicate combinations were combined and all PMIDs were saved for reference.

4 Results

4.1 Determination of cells and viruses used in the experiment

A549, RPE and HE cells were infected with the 15 viruses to assess their susceptibility to enteroviral infections. CTG assay was used to measure cell viability 72 h post-infection. Cell viability (%) was calculated relative to mock-infected cells, with viability exceeding 80% indicating a healthy cell culture. Values below 80% indicated that the specific viruses successfully infected the host cells. In A549 cells, viability dropped below 80% when infected with EV1 (10%), EV6 (18%), EV11 (51%), EV-A71 (65%), CVA6 (79%) and CVB5 (33%). RPE cells exhibited viability below 80% when infected with EV1 (38%), EV6 (80%) and CVB5 (76%). Similarly, HE cells showed viability below 80% when infected with EV1 (10%), EV6 (12%) and EV-A71 (63%) (see Fig. 9).

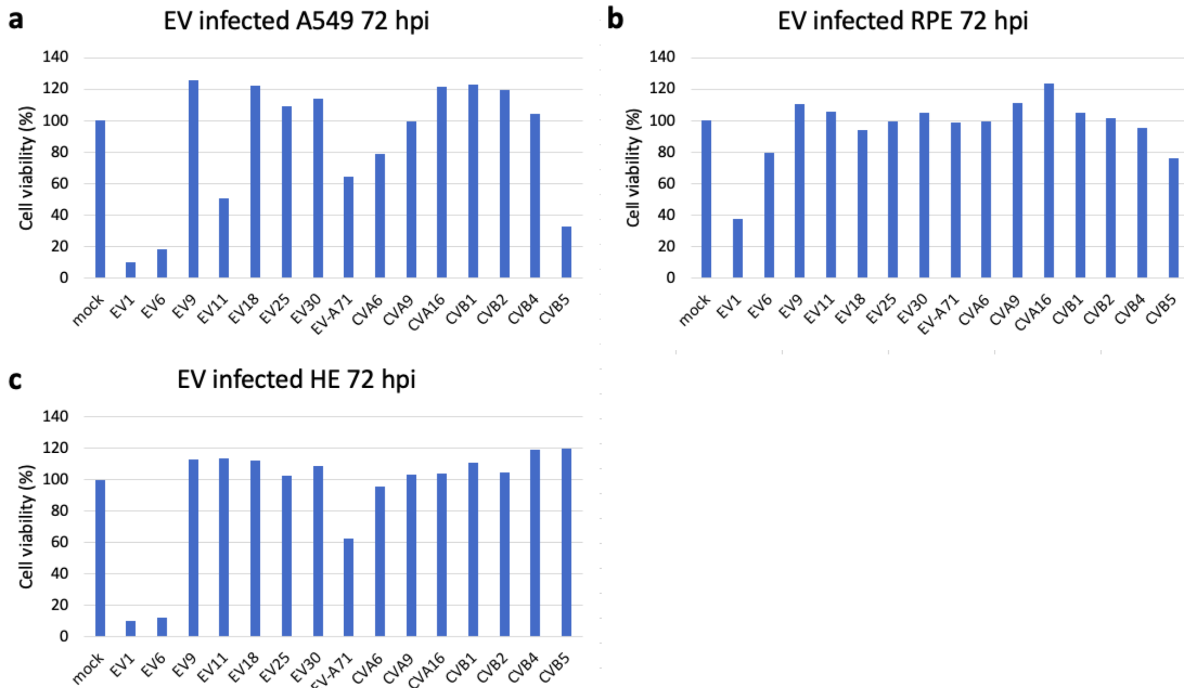


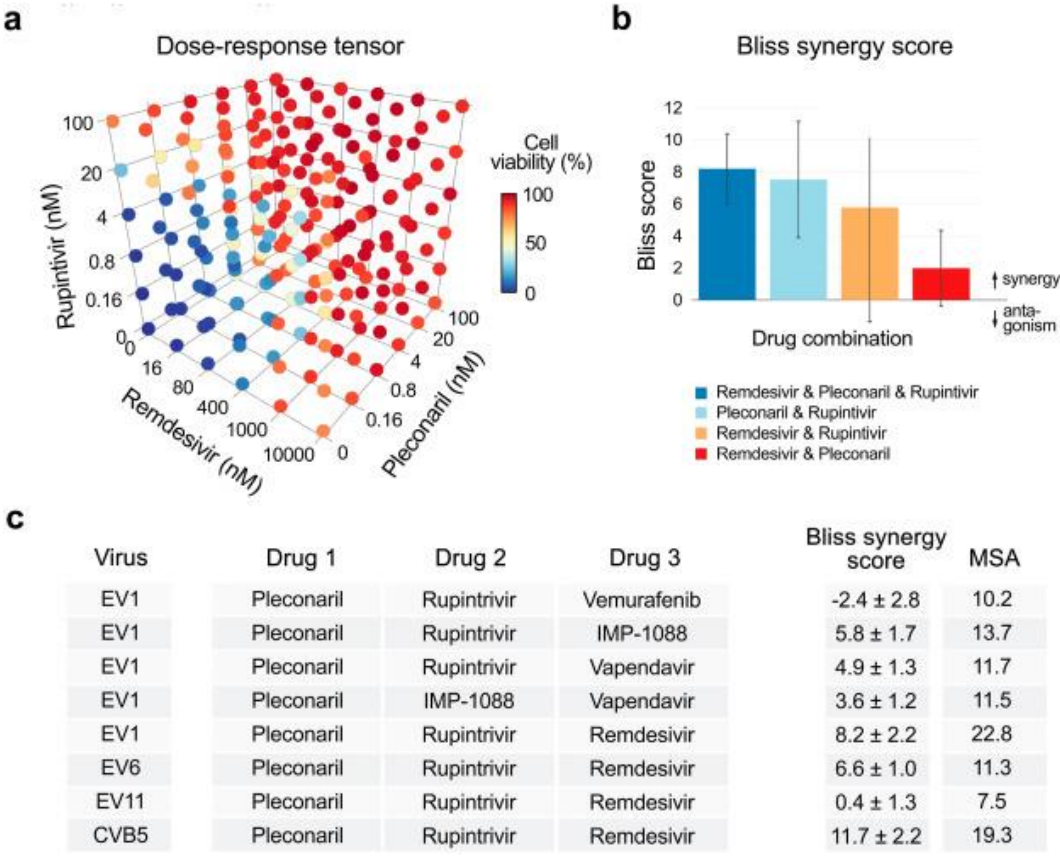
Figure 9. *Susceptibility of A549, RPE, and HE cells to enteroviral infection. Figure (a) shows viability of A549 cells 72 h post infection with the 15 viruses and mock. The same was done for RPE cells (b), and HE cells (c). Cell viability was measured with the CTG assay.*

4.2 Drug combination test on A549 cells

A549 cells infected with EV6, EV11, CVB5, and mock were treated with various combinations of remdesivir or vapendavir, pleconaril, and rupintrivir at different concentrations to determine the optimal concentrations of these drugs for maximum efficacy without inducing toxicity in the host cells. The same test had been done previously on EV1-infected A549 cells (Table S3 and S5).

The calculated cell viability for all combinations of remdesivir, pleconaril, and rupintrivir concentrations used against infected A549 cells are presented in Table S2. Mock-infected cells exhibited viability >80% for most concentrations. Pleconaril at the highest concentrations (≥0.02 μM) inhibited viral replication, resulting in cell viability exceeding 80% across all three virus types, regardless of the concentrations of remdesivir and rupintrivir, including when pleconaril was used alone. Remdesivir and rupintrivir demonstrated similar effects to pleconaril only when combined with the other drugs. The calculated Bliss synergy score and MSA for this combination are presented in Figure 10c for EV1, EV6, EV11 and CVB5. Figures 10a-b display the dose-response results and Bliss synergy scores from previous testing of this combination against EV1-infected A549 cells.

For the second combination comprising vapendavir, pleconaril and rupintrivir, the measured cell viability (%) is presented in Table S4. Mock-infected cells showed no drug-induced toxicity, with >80% viability for all combinations of the different concentrations. The most efficient combinations for infected cells varied depending on the type of virus. For EV6-infected cells, vapendavir contributed to maintaining cell viability >80% in most cases, even at low pleconaril and rupintrivir concentrations. EV11-infected cells required higher rupintrivir concentrations in combination with pleconaril and vapendavir. CVB5-infected cells benefited from higher pleconaril concentrations. The Bliss synergy score and MSA for this combination previously tested against EV1 is presented in Figure 10c.



Virus	Drug 1	Drug 2	Drug 3	Bliss synergy score	MSA
EV1	Pleconaril	Rupintrivir	Vemurafenib	-2.4 ± 2.8	10.2
EV1	Pleconaril	Rupintrivir	IMP-1088	5.8 ± 1.7	13.7
EV1	Pleconaril	Rupintrivir	Vapendavir	4.9 ± 1.3	11.7
EV1	Pleconaril	IMP-1088	Vapendavir	3.6 ± 1.2	11.5
EV1	Pleconaril	Rupintrivir	Remdesivir	8.2 ± 2.2	22.8
EV6	Pleconaril	Rupintrivir	Remdesivir	6.6 ± 1.0	11.3
EV11	Pleconaril	Rupintrivir	Remdesivir	0.4 ± 1.3	7.5
CVB5	Pleconaril	Rupintrivir	Remdesivir	11.7 ± 2.2	19.3

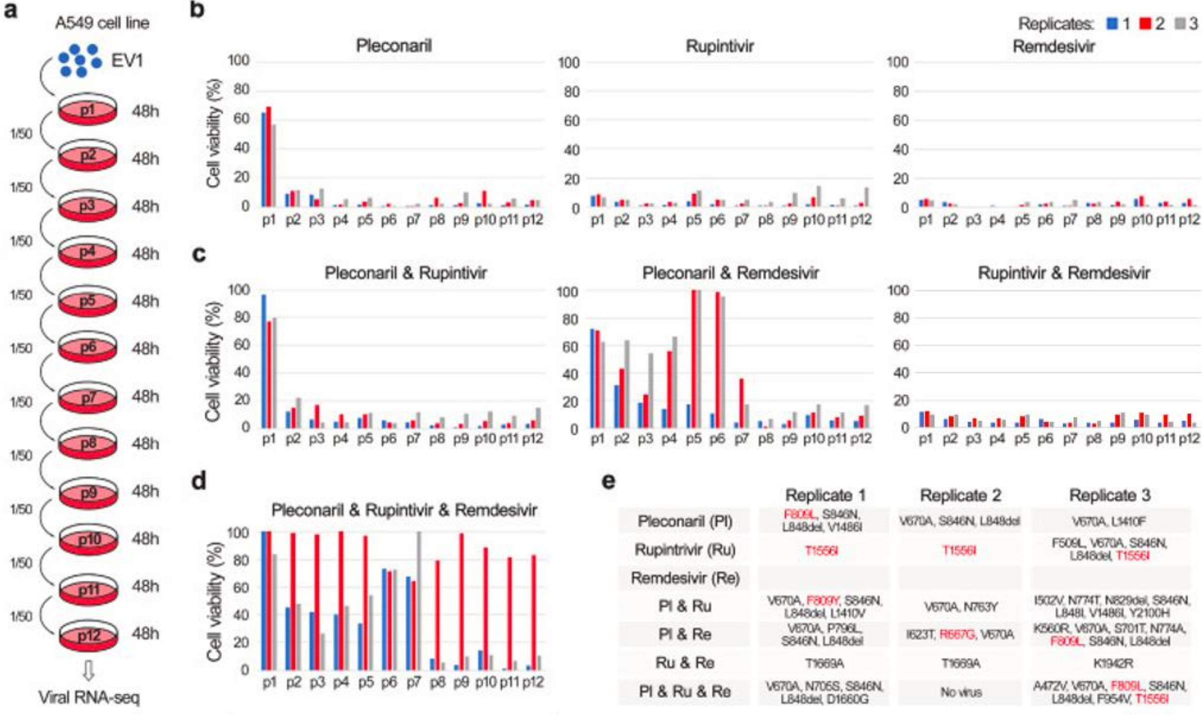
Figure 10. *Effect of triple drug combinations tested on A549 cells infected with enteroviruses. (a) EV1, or mock, infected A549 cells were treated with increasing concentrations of pleconaril, rupintrivir and remdesivir, and combinations of these. Cell viability was measured 48 h later with the CTG assay and is presented in the figure in a color gradient. (b) Results from experiment (a) was used to calculate Bliss synergy scores for double- and triple combinations of pleconaril, rupintrivir and remdesivir. (c) Table of the synergy scores and most synergistic area (MSA) for pleconaril-containing triple combinations tested against EV1, EV6, EV11, and CVB5 (31).*

4.3 Drug-resistance test

During the twelve passages of the remdesivir (2 µM), pleconaril (0.02 µM), and rupintrivir (0.02 µM) combinations, the cell viability was quickly decreased to a level indicating cell death (Fig. 11b-c). Pleconaril initially provided some protection in the first passage, both in single and double treatments, but its effectiveness was reduced significantly in subsequent passages. The triple combination, however, maintained a higher cell viability until passage eight (Fig. 11d).

Sequencing of RNA from viral proteins isolated from passage eight revealed several mutations potentially causing drug resistance (Fig. 11e). The T1556I mutations were found in all replicates where rupintrivir was used as monotherapy. F809L and R667G mutations were

identified in several replicates where pleconaril was used. No viral RNA was detected in replicate two of the triple combination.



	Replicate 1	Replicate 2	Replicate 3
Pleconaril (Pl)	F809L, S846N, L848del, V1486I	V670A, S846N, L848del	V670A, L1410F
Rupintrivir (Ru)	T1556I	T1556I	F509L, V670A, S846N, L848del, T1556I
Remdesivir (Re)			
Pl & Ru	V670A, F809Y, S846N, L848del, L1410V	V670A, N763Y	I502V, N774T, N829del, S846N, L848I, V1486I, Y2100H
Pl & Re	V670A, P796L, S846N, L848del	I623T, R667G, V670A	K560R, V670A, S701T, N774A, F809L, S846N, L848del
Ru & Re	T1669A	T1669A	K1942R
Pl & Ru & Re	V670A, N706S, S846N, L848del, D1660G	No virus	A472V, V670A, F809L, S846N, L848del, F954V, T1556I

Figure 11. *Development of resistance against pleconaril, remdesivir and rupintrivir in EV1. (a) Illustration of the course of the experiment. (b) Viability of A549 cells exposed to EV1 passaged 12 times in the presence of 0.02 μM pleconaril, 0.02 μM rupintrivir, or 2 μM remdesivir. (c-d) Cell viability of EV1 infected A549 cells with double/triple combinations of the drugs through 12 passages. (e) Mutations found in EV1 RNA after 8 passages in the presence of single drugs and in double- or triple combinations (31).*

For the second combination consisting of vapendavir (0.1 μM), pleconaril (0.1 μM) and rupintrivir (0.1 μM), combinations containing rupintrivir significantly enhanced cell viability across the passages compared to vapendavir and pleconaril, and the combination of these (Fig. 12). This was observed for monotherapy with rupintrivir and double- and triple combinations, and by passage 12, cell viability remained above 80%. It is worth noting that for the combination of vapendavir and rupintrivir, the second replicate exhibited viability below 30% starting from the fourth passages and throughout the subsequent passages.

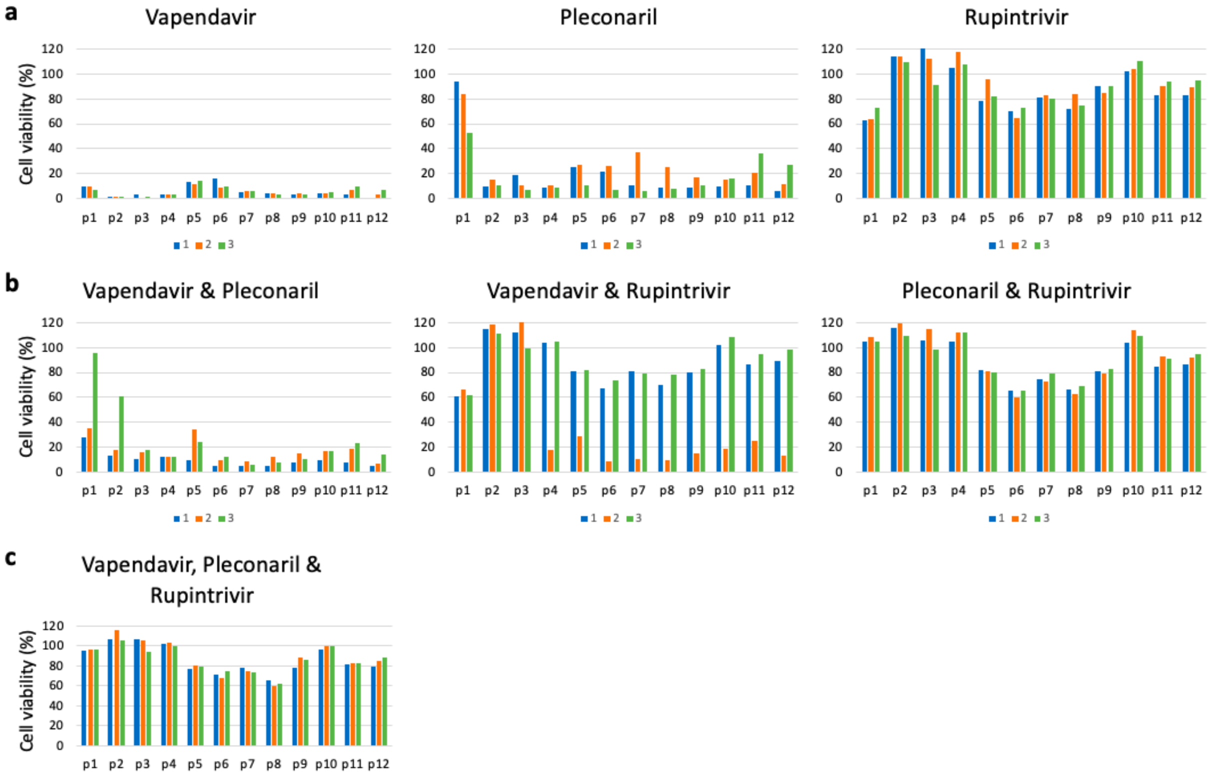


Figure 12. *EV1 resistance to vapendavir, pleconaril and rupintrivir. (a) Viability of A549 when infected with EV1 passaged 12 times in presence of vapendavir, pleconaril or rupintrivir. (b-c) Cell viability of EV1 infected A549 cells with the presence of double and triple combinations of vapendavir, pleconaril, and rupintrivir.*

4.4 Cytotoxic effects of the drugs

The results obtained from exposing the A549 cells to 2 µM remdesivir, 0.02 µM pleconaril and 0.02 µM rupintrivir, and the combinations of these, for 48 h revealed minimal to no toxicity (Fig. 13). This was similarly observed for the combination of 0.1 µM vapendavir, 0.1 µM rupintrivir and 0.1 µM pleconaril. While both triple combinations affected the cells slightly more than single- and double combinations, cell viability remained well above 80%, indicating a healthy cell culture. The reported values represent the mean percentage cell viability from three measurements, with mock treatment considered as 100%.

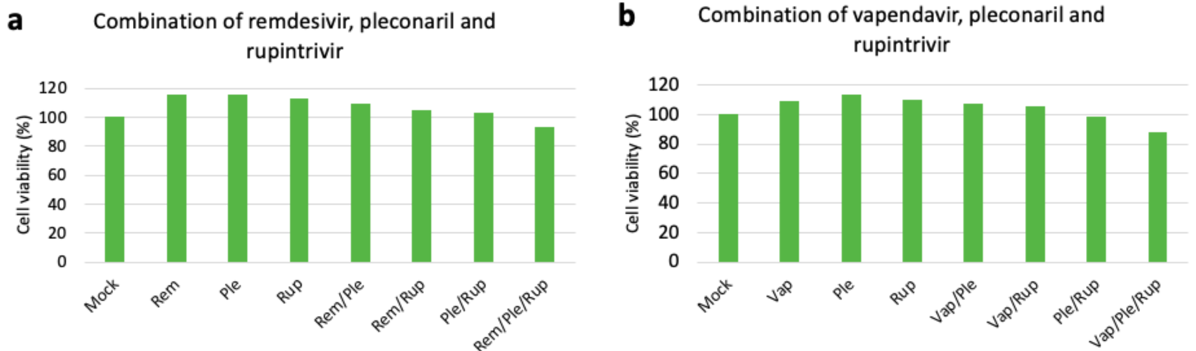


Figure 13. *Cytotoxic effects of the drug combinations on A549 cells measured by the CTG assay. (a) Percentage of viable A549 cells after 48 h incubation in the presence or absence of remdesivir, pleconaril and rupintrivir. (b) Percentage of viable A549 cells after 48 h incubation in the presence or absence of vapendavir, pleconaril and rupintrivir.*

4.5 Organoid test

Carlemi Calitz at Amsterdam UMC conducted tests on the combination comprising 0.1 µM pleconaril, 0.1 µM rupintrivir and 1 µM remdesivir on human intestinal organoids infected with EV-A71 to assess efficacy and toxicity. As illustrated in Figure 14c, the triple combination notably decreased the amount of viral RNA present in the organoids compared to the single- and double combinations. Additionally, Figure 14b demonstrates that the viability of the organoids exposed to the drugs for 72 h remained above 80% (31).

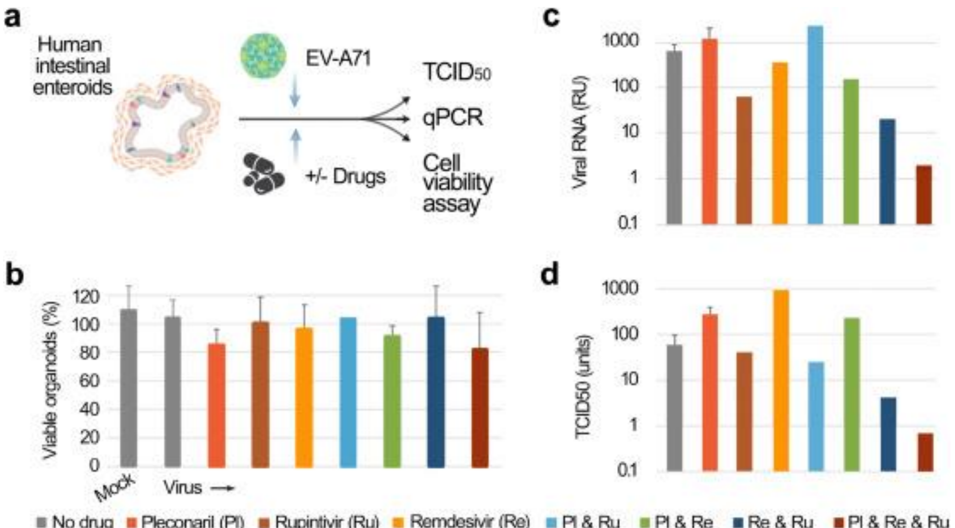


Figure 14. *Combinations of 1 µM remdesivir, 0.1 µM pleconaril and 0.1 µM rupintrivir tested on human intestinal organoids infected with EV-A71 or mock. (a) Illustration of the course of the experiment. (b) Percentage viable organoids 72 h after infection with EV-A71 in the presence of the drugs and combinations of these, including mock and infected organoids without antiviral treatment. Cell viability was measured with the CTG assay. (c) EV-A71 genes analyzed by RT-qPCR to determine viral copy number. (d) TCID50 assay of the infected enteroids treated with the drugs (31).*

As for the second combination consisting of 0.1 μM vapendavir, 0.1 μM pleconaril, and 0.1 μM rupintrivir, all compounds containing rupintrivir, whether as monotherapy or in double- and triple combinations, significantly inhibited replication of EV1 without inducing toxicity in the HAOs (Fig. 15).

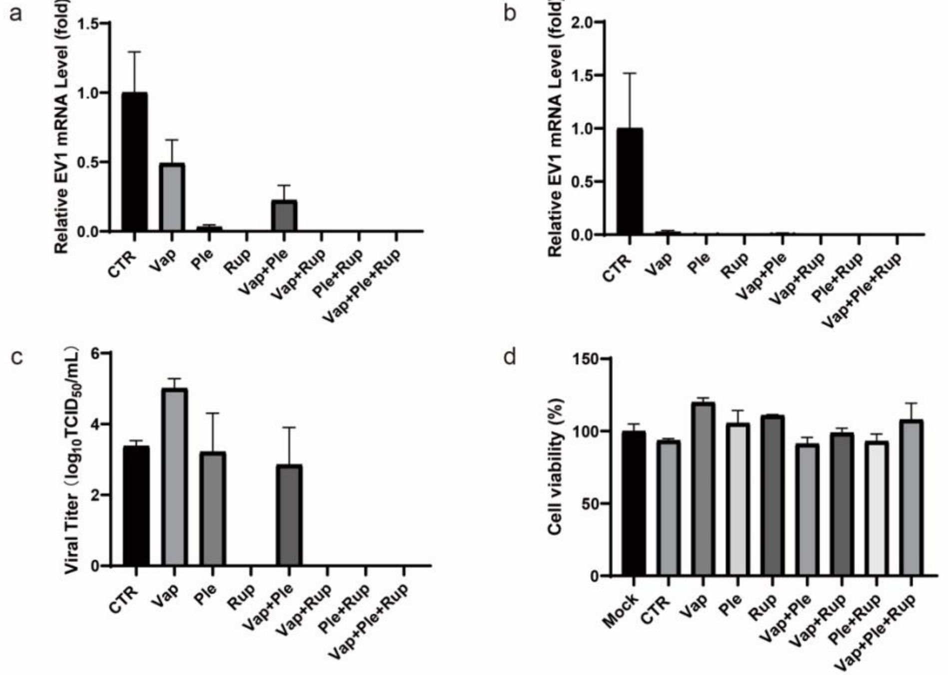


Figure 15. *Combinations of 0.1 μM vapendavir,0.1 μM pleconaril and 0.1 μM rupintrivir tested on EV1 or mock infected HAOs. (a) Relative EV1 mRNA level in the HAOs determined by RT-qPCR. (b) Relative EV1 mRNA level in culture supernatant. (c) Viral titer in supernatant determined by TCID50 assay. (d) Viability of the HAOs when infected with EV1 or mock was determined by using the Alamar Blue assay.*

4.6 Update of drugvirus.info database

A total of 68 antiviral combinations containing more than two drugs targeting enteroviruses and other human viruses were identified through searches on PubMed and drugs.com. Among these, 14 combinations were already approved and in use, while five combinations were aimed at enteroviruses and 49 targeted other human viruses, undergoing either clinical or pre-clinical testing. The Excel file containing the results was forwarded to Prof. Denis Kainov for updating the database at a later stage. Included is also our combination of remdesivir, pleconaril, and rupintrivir, bringing the total count of combinations to 69. A PDF-version of the Excel file has been included as a supplement (Table S6).

5 Discussion

Treating viral infections with single drugs often requires higher concentrations to effectively combat the virus. However, this approach can result in increased toxicity, which, in some cases, may be more harmful than the infection itself. Another challenge is the development of resistant strains, which further complicates treatment efforts. A strategy that has shown to be promising is to combine two or more drugs with different targets, either cellular or viral. Double combinations have proven to be more efficient than single therapies, but the rapid development of resistance remains a concern. Consequently, researchers have turned focus to triple combinations, with some already approved for use against certain viruses, primarily HIV-1 and hepatitis C.

The compilation of published triple combinations that are already approved or are under pre-clinical or clinical testing, enhances accessibility to this information once integrated into the drugvirus.info database. This addition expands the database from solely encompassing single drugs and double combinations to now including triple combinations as well. Currently, 69 unique triple combinations have been identified, but this number is expected to increase by the time the database has been updated. Notably, the triple combination used in the experiments of this thesis, consisting of vapendavir, pleconaril and rupintrivir, has not yet been added to the Excel file. However, findings on this combination are expected to be published as soon as experiments are completed, increasing the total count to 70.

Both triple combinations tested in this thesis demonstrated the ability to promote cell survival by inhibiting viral replication without inducing cellular toxicity. In comparison to single and double treatments, both triple combinations maintained a higher cell viability. Moreover, the observed synergistic effects obtained when combining the drugs enabled the use of lower concentrations. This reduction in required concentration contributed to a decrease in drug toxicity. Additionally, the other objective of combining drugs was to prevent or delay the emergence of resistant strains. Through passaging of EV1 propagated in A549 cells with the presence or absence of the two triple combinations, it was observed that both combinations, to varying extent, successfully reduced viral replication, and thereby increased cell viability. They also delayed the development of resistant strains compared to single and double therapies.

For the first combination consisting of remdesivir, pleconaril and rupintrivir, mono- and double therapies of these had minimal to no effect on viral replication throughout the passages, as indicated by cell viability measurements below 20%. When comparing the cell viability from the first passage to when the different concentrations of this combination was previously tested against EV1 infected A549 cells (Table S3), the results are mostly comparable. However, viability measured from cells exposed to 2 μM remdesivir was at 86%, which do not correlate with the <10% measured cell viability from passage 1. A possible explanation could be a mistake in the preparation of the drug solution, or different condition during the experiment.

Of the double combinations, the combination of pleconaril and remdesivir appeared to exert the best effect on the cells but remained below 80% viability. Although the combination of pleconaril and rupintrivir inhibited viral replication at a significant degree in the first passage, cell viability dropped below 20% already in the second passage, suggesting quick occurrence of mutations rendering the EV1 resistant to the drugs. This was also indicated by the sequencing results, were several mutations were identified. One of them was the F809Y in replicate 1 of the pleconaril and rupintrivir combination, which affects the binding site for pleconaril in the VP1 protein. When the drug is not able to bind to its target, its effects are lost, providing no protection against the virus. Additionally, F809L and R667G mutations were found in RNA from viruses exposed to pleconaril alone or in combination, having the same effect as the F809Y mutation. For rupintrivir-exposed viruses, the T1556I mutation was detected in all replicates exposed to rupintrivir alone and in one replicate for the triple combination. This mutation, located in the rupintrivir-binding pocket of the viral 3C-protease, can explain the loss of rupintrivir's effectiveness throughout the passages.

The triple combination of remdesivir, pleconaril, and rupintrivir provided a prolonged maintenance of a higher number of viable cells compared to both single therapies and double combinations. However, by passage eight, a significant reduction in viable cells was observed, which indicated the emergence of resistant strains. Notably, this emergence of resistance occurred later than for the single and double therapies, and thereby provides a successful delay in the development of resistance against the triple combination. Although the combination kept a more stable cell viability for longer, it was still measured below 80%. An increase in the concentration of one, or more, drugs could potentially give a better effect. Several mutations were identified through sequencing of RNA from the triple-drug replicates, including the F809L and T1556I mutations previously mentioned. The second replicate for the triple combination

yielded deviating results, which can be explained by the absence of viral RNA from isolation, possibly due to pipetting errors in the early passages. Moreover, various other mutations were detected across all samples, as depicted in Figure 11e. Consequently, it cannot be concluded that the mutations highlighted in the text solely contribute to the resistance, nor can it be determined whether a combination of multiple mutations is required for resistance development. Nonetheless, this information is valuable for comparison in future experiments.

In the second combination comprising vapendavir, pleconaril, and rupintrivir, the wells containing rupintrivir, whether as single treatment or in combination, exhibited a more stable cell viability. It is worth noting that the concentration of rupintrivir in this combination is higher than in the first combination, 0.1 μM compared to 0.02 μM, suggesting a potential impact. Contrary, for the single and double combinations without rupintrivir, the development of resistant strains appeared to occur as early as the second passage, as seen from the significantly lower cell viability compared to rupintrivir-containing treatments. This observation supports the concept that resistant strains tend to emerge more rapidly in single and double regimens compared to treatment with triple combinations. In addition, since vapendavir and pleconaril share the same mechanism of action, their failure to collectively inhibit viral replication, compared to other double and triple combinations, underscores the advantage of combining drugs targeting different stages of the viral replication cycle.

Testing of the drugs on human intestinal organoids confirmed that the triple combination of remdesivir, pleconaril and rupintrivir has higher efficacy than the single and double therapies as the viral replication was clearly inhibited. When exposed to the triple combination, viable organs were measured to >80%, indicating an absence of toxic effects. These findings support the promising potential of this combination, and further research should be done to assess its in vivo effects. The results from testing the second combination consisting of vapendavir, pleconaril and rupintrivir on human lung organoids are also very promising. Both drug resistance testing and organoid experiments demonstrated that rupintrivir-containing combinations were the most effective in inhibiting viral replication. Since both triple combinations provided promising results, they are both very relevant for further testing. However, the organoid tests do not take account for the development of resistant strains, which the remdesivir-pleconaril-rupintrivir combination demonstrated. Because of this, the vapendavir-containing combination seems to be the best option going forward. It could also be considered to increase the concentrations of drugs from the first combination, as this had great

results for rupintrivir, or test a different combination of the four drugs, e.g. remdesivir, vapendavir, and rupintrivir. In this case, we would avoid using two drugs with the same MoA, which was the case for vapendavir and pleconaril in the second combination.

The cell line employed in these experiments was A549 cells, which was identified as susceptible to infection by EV1, EV6, EV11, EV-A71, and CVB5. Additionally, RPE and HE cells were tested and found to be susceptible for EV1 and CVB5, and EV1, EV6 and EV-A71, respectively. This information will help future research by identifying which cell types are suitable for studying different enteroviruses, and thereby saving time and resources.

The triple combinations investigated have demonstrated significant efficacy in inhibiting the replication of EV1, EV6, EV11, and CVB5. This indicates broad-spectrum effects of the drugs which potentially also extending to other enteroviruses that have not been examined in this study. To further evaluate the broad-spectrum potential of these combinations, they should be tested against several other enteroviruses, particularly those of great clinical relevance, such as EV-D70, EV-A71 and HRVs.

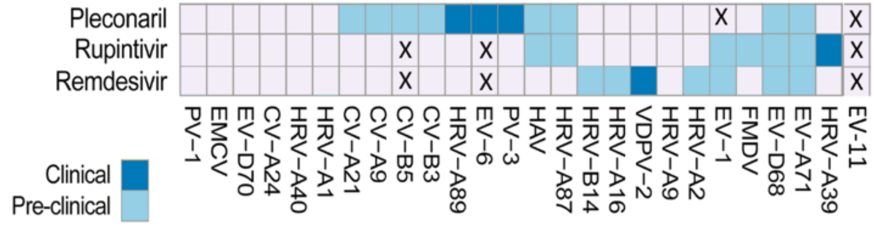


Figure 16. *Potential coverage of picornaviruses by triple BCC*

The results presented here suggest promising clinical applicability, as evidenced by the absence of cytotoxic effects on host cells and the delayed emergence of resistant strains, notably in the combination comprising vapendavir, pleconaril, and rupintrivir. The prospect of an approved treatment option for enteroviruses in the future holds significant promise, offering the potential to mitigate severe diseases, reduce risk of transmission between individuals, and reduce the emergence of strains resistant to the treatments. As seen previously for viruses such as HIV and HCV, approvement of BCCs significantly improve the treatment and prognosis of patients.

In a future perspective, the clinical relevance of the BCCs should be further evaluated, and identification of potentially safe-for-man combinations with the best therapeutic potential and minimal side effects will be performed using data from clinical studies on BSAs. In addition, an economic evaluation considering the costs of treatment from the perspective of health care system and society should be done. These analyses can use relevant subgroup characteristics to estimate subgroup-specific transition probabilities and outcomes, such as hospitalization rates, case-fatality ratios, quality adjusted life expectancy, in countries with accessible data. Evaluations should be statistically analyzed, including probabilistic sensitivity and variable importance analysis, as well as bi-variate and threshold analyses on drug price and relative efficacy.

6 Conclusion

In vitro testing has provided promising results for both triple combinations against enteroviruses as the viral replication was inhibited or reduced for the viruses tested in A549 cells without inducing toxicity. Combining drugs with different targets has shown potential in reducing required concentrations. Particularly, the combination containing vapendavir, rupintrivir, and pleconaril exhibited positive results as it notably delayed development of resistant EV1 strains compared to the remdesivir-containing combination. In addition, both combinations showed beneficial effects on EV1-infected organoids without toxicity. Considering results from drug resistance testing and organoid studies, the vapendavir, pleconaril and rupintrivir combination appears as the most promising, as it effectively inhibited viral replication through all twelve passages and reduced viral RNA in human air-way organoids. However, further in vitro testing is necessary to assess their effects and potential toxicity.

In a future perspective, it will be important to expand testing to evaluate the broad-spectrum potential of these combinations against a wider range of enteroviruses. Additionally, economic evaluations and statistical analyses will provide valuable insights into the cost-effectiveness and therapeutic potential of these combinations. Overall, the development and approval of triple combination therapies is a promising approach to improve treatment outcomes for viral infections, potentially reducing disease severity, transmission risk, and the emergence of drug-resistant strains.

The prepared update of the drugvirus.info database with 69 combinations containing three or more drugs, will enhance efficiency in drug combination research by providing readily available information on previously tested combinations. By the time the database update is conducted, an increase in the number of combinations is expected.

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Appendix A - Viruses used in the experiments

Table A1. The table presents the viruses used, their species, and full name.

Abbreviation	Species	Virus
EV1	Enterovirus B	Echovirus 1
EV6	Enterovirus B	Echovirus 6
EV9	Enterovirus B	Echovirus 9
EV11	Enterovirus B	Echovirus 11
EV18	Enterovirus B	Echovirus 18
EV25	Enterovirus B	Enterovirus 25
EV30	Enterovirus B	Enterovirus 30
EV-A71	Enterovirus A	Enterovirus A71
CVA6	Enterovirus A	Coxsackievirus A6
CVA9	Enterovirus A	Coxsackievirus A9
CVA16	Enterovirus A	Coxsackievirus A16
CVB1	Enterovirus B	Coxsackievirus B1
CVB2	Enterovirus B	Coxsackievirus B2
CVB4	Enterovirus B	Coxsackievirus B4
CVB5	Enterovirus B	Coxsackievirus B5

Appendix B - Antiviral drugs used in the experiments

Table B1. Supplier, and other details, of the drugs utilized in the experiments (37).

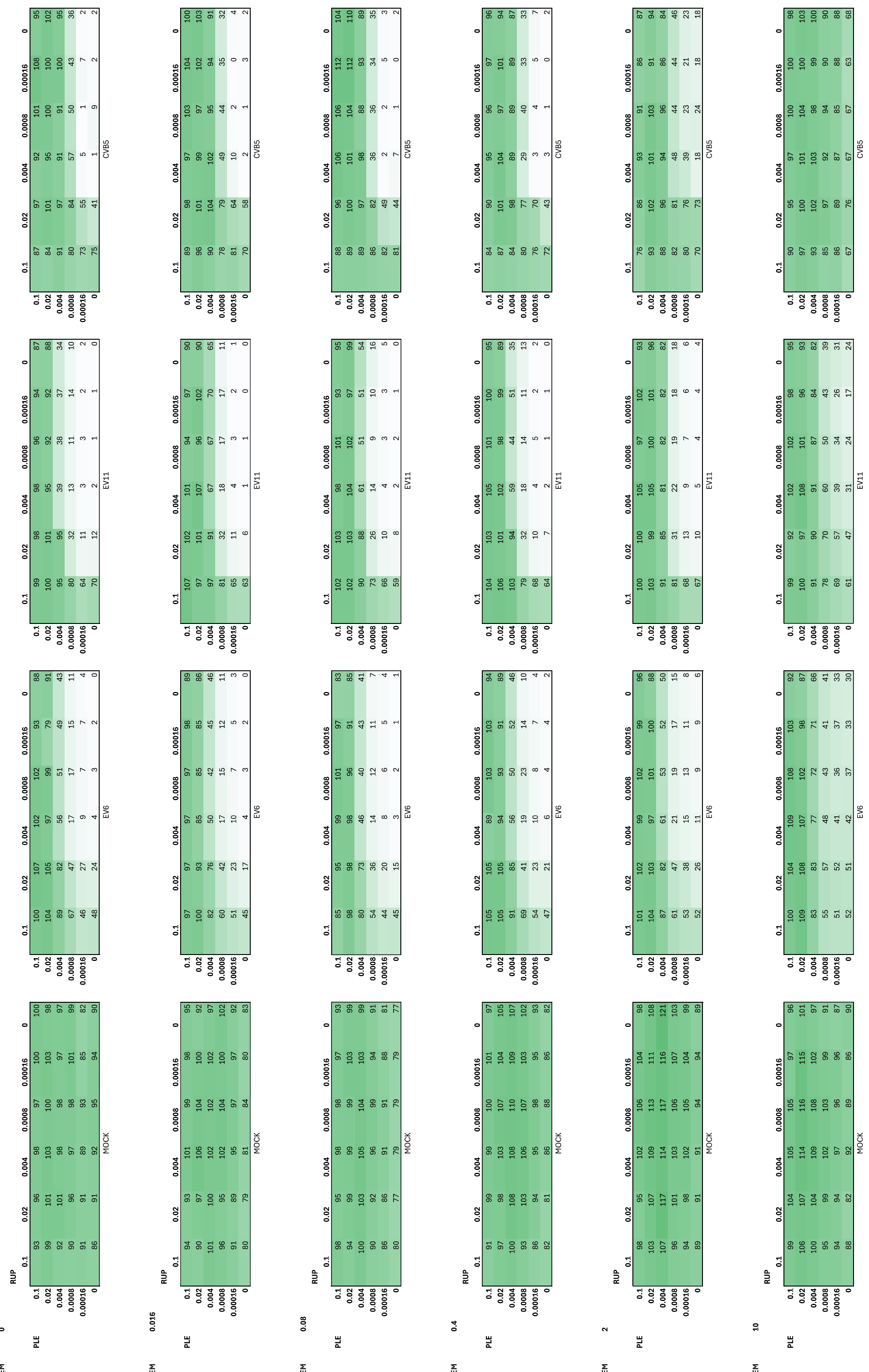
Drug	CAS	Supplier	Purity (%)	Cat. #	Formula	MW
Pleconaril	153168-05-9	Cayman Chemical	≥98	CAYM28461	$C_{19}H_{20}FNO_3$	323.3
Remdesivir	1809249-37-3	Cayman Chemical	>98	30354	$C_{27}H_{35}N_6O_8P$	602.6
Rupintrivir	223537-30-2	Sigma-Aldrich	≥98	PZ0315-5MG	$C_{27}H_{32}N_4O_3$	481.5
Vapendavir diphosphate	1198151-75-5	MedChemExpress	98.8	HY-106254A	$C_{21}H_{32}N_4O_{11}P_2$	578.5

Supplementary

Table S1. The table presents the four drugs used in the experiment, their structure taken from PubChem, type of antiviral, target and mechanism of action, and their drug bank ID.

Drug	Structure	Type	Target	MoA	Drug bank ID
Remdesivir		Small molecule	Viral RNA polymerase	Inhibitor of replication	DB14761
Vapendavir/ BTA798		Small molecule	VP1	Inhibitor of viral entry	DB05181
Pleconaril		Small molecule	VP1	Inhibitor of viral entry	DB05105
Rupintrivir		Small molecule	3C protease	Inhibitor of polyprotein processing	DB05102

Table S2. Measured cell viability of A549 cells infected with mock, EV6, EV11, and CVB5 and treated with different concentrations of remdesivir, pleconaril and rupintrivir.



REM 0

PLE / RUP	MOCK 0.1	MOCK 0.02	MOCK 0.004	MOCK 0.0008	MOCK 0.00016	MOCK 0	EV6 0.1	EV6 0.02	EV6 0.004	EV6 0.0008	EV6 0.00016	EV6 0	EV11 0.1	EV11 0.02	EV11 0.004	EV11 0.0008	EV11 0.00016	EV11 0	CVB5 0.1	CVB5 0.02	CVB5 0.004	CVB5 0.0008	CVB5 0.00016	CVB5 0
0.1	93	96	98	97	100	100	100	107	102	102	93	88	99	98	98	96	94	87	87	97	92	101	108	95
0.02	99	101	103	100	103	98	104	105	97	99	79	91	100	101	95	92	92	88	84	101	95	100	100	102
0.004	92	101	98	98	97	97	89	82	56	51	49	43	95	95	39	38	37	34	91	97	91	91	100	95
0.0008	90	96	97	98	101	99	67	47	17	17	15	11	80	32	13	11	14	10	80	84	57	50	43	36
0.00016	91	91	89	93	85	82	46	27	9	7	7	4	64	11	3	3	2	2	73	55	5	1	7	2
0	86	91	92	95	94	90	48	24	4	3	2	0	70	12	2	1	1	0	75	41	1	9	2	2

REM 0.016

PLE / RUP	MOCK 0.1	MOCK 0.02	MOCK 0.004	MOCK 0.0008	MOCK 0.00016	MOCK 0	EV6 0.1	EV6 0.02	EV6 0.004	EV6 0.0008	EV6 0.00016	EV6 0	EV11 0.1	EV11 0.02	EV11 0.004	EV11 0.0008	EV11 0.00016	EV11 0	CVB5 0.1	CVB5 0.02	CVB5 0.004	CVB5 0.0008	CVB5 0.00016	CVB5 0
0.1	94	93	101	99	98	95	97	97	97	97	98	89	107	102	101	94	97	90	89	98	97	103	104	100
0.02	90	97	106	104	100	92	100	93	85	85	85	86	97	101	107	96	102	90	96	101	99	97	102	103
0.004	101	100	102	102	102	97	82	76	50	42	45	46	97	91	67	67	70	65	90	104	102	95	94	91
0.0008	96	95	102	104	100	102	60	42	17	15	12	11	81	32	18	17	17	11	78	79	49	44	35	32
0.00016	91	89	95	97	97	92	51	23	10	7	5	3	65	11	4	3	2	1	81	64	10	2	0	4
0	80	79	81	84	80	83	45	17	4	3	2	0	63	6	1	1	0	0	70	58	2	1	3	2

REM 0.08

PLE / RUP	MOCK 0.1	MOCK 0.02	MOCK 0.004	MOCK 0.0008	MOCK 0.00016	MOCK 0	EV6 0.1	EV6 0.02	EV6 0.004	EV6 0.0008	EV6 0.00016	EV6 0	EV11 0.1	EV11 0.02	EV11 0.004	EV11 0.0008	EV11 0.00016	EV11 0	CVB5 0.1	CVB5 0.02	CVB5 0.004	CVB5 0.0008	CVB5 0.00016	CVB5 0
0.1	98	95	98	98	97	93	85	95	99	101	97	83	102	103	98	101	93	95	88	96	106	106	112	104
0.02	94	99	99	99	103	99	98	98	98	96	91	85	102	103	104	102	97	99	89	100	101	104	112	110
0.004	100	103	105	104	103	99	80	73	46	40	43	41	90	88	61	51	51	54	89	97	98	88	93	89
0.0008	90	92	96	99	94	91	54	36	14	12	11	7	73	26	14	9	10	16	86	82	36	36	34	35
0.00016	86	86	91	91	88	81	44	20	8	6	5	4	66	10	4	3	3	5	82	49	2	2	5	3
0	80	77	79	79	79	77	45	15	3	2	1	1	59	8	2	2	1	0	81	44	7	1	0	2

REM 0.4

PLE / RUP	MOCK 0.1	MOCK 0.02	MOCK 0.004	MOCK 0.0008	MOCK 0.00016	MOCK 0	EV6 0.1	EV6 0.02	EV6 0.004	EV6 0.0008	EV6 0.00016	EV6 0	EV11 0.1	EV11 0.02	EV11 0.004	EV11 0.0008	EV11 0.00016	EV11 0	CVB5 0.1	CVB5 0.02	CVB5 0.004	CVB5 0.0008	CVB5 0.00016	CVB5 0
0.1	91	99	99	100	101	97	105	105	89	103	103	94	104	103	105	101	100	95	84	90	95	96	97	96
0.02	97	98	103	107	104	105	105	105	94	93	91	89	106	101	102	98	99	89	87	101	104	97	101	94
0.004	100	108	108	110	109	107	91	85	56	50	52	46	103	94	59	44	51	35	84	98	89	89	89	87
0.0008	93	103	106	107	103	102	69	41	19	23	14	10	79	32	18	14	11	13	80	77	29	40	33	33
0.00016	86	94	95	98	95	93	54	23	10	8	7	4	68	10	4	5	2	2	76	70	3	4	5	7
0	82	81	86	88	86	82	47	21	6	4	4	2	64	7	2	1	1	0	72	43	3	1	0	2

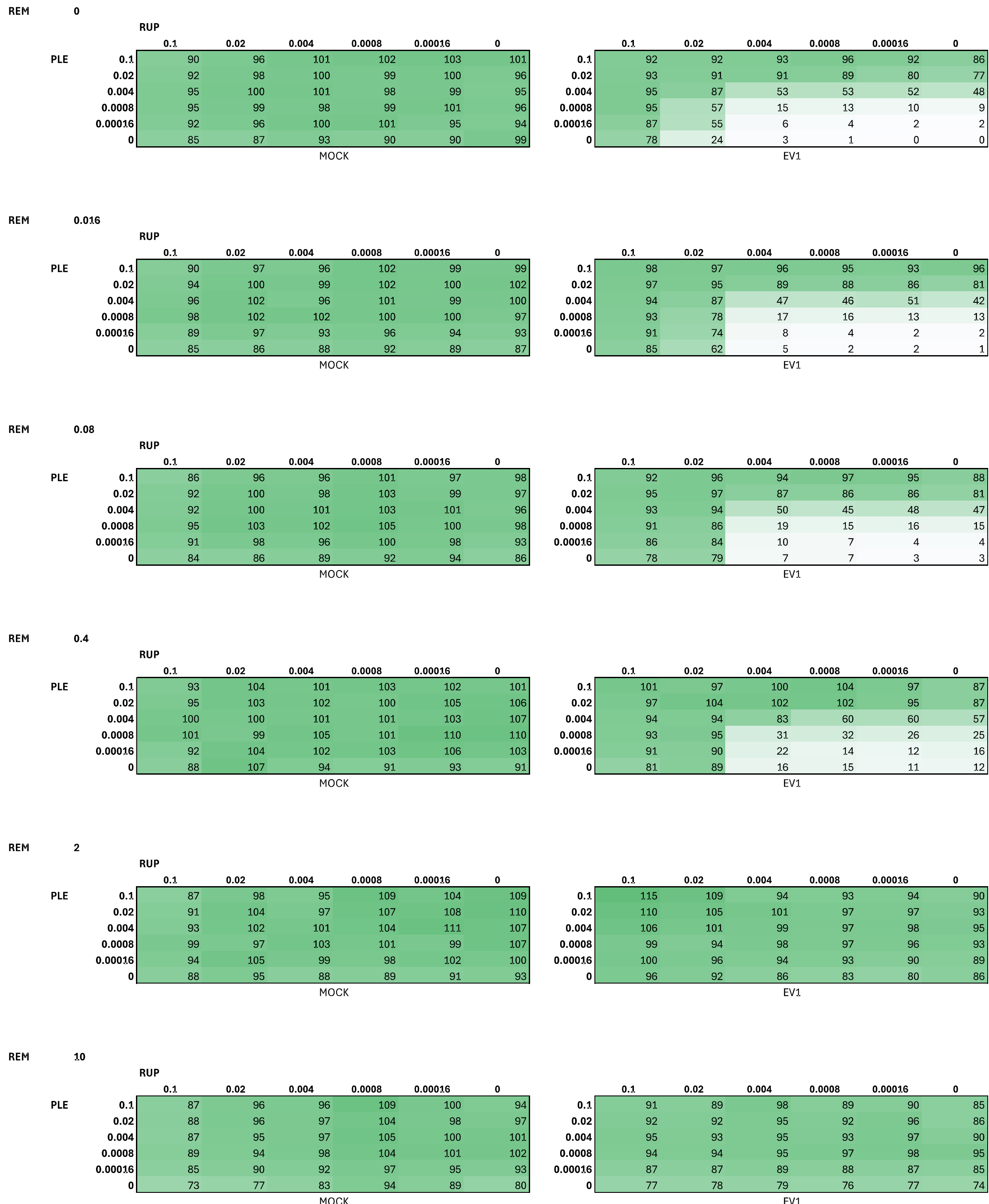
REM 2

PLE / RUP	MOCK 0.1	MOCK 0.02	MOCK 0.004	MOCK 0.0008	MOCK 0.00016	MOCK 0	EV6 0.1	EV6 0.02	EV6 0.004	EV6 0.0008	EV6 0.00016	EV6 0	EV11 0.1	EV11 0.02	EV11 0.004	EV11 0.0008	EV11 0.00016	EV11 0	CVB5 0.1	CVB5 0.02	CVB5 0.004	CVB5 0.0008	CVB5 0.00016	CVB5 0
0.1	98	95	102	106	104	98	101	102	99	102	99	96	100	100	105	97	102	93	76	86	93	91	86	87
0.02	103	107	109	113	111	108	104	103	97	101	100	88	103	99	105	100	101	96	93	102	101	103	91	94
0.004	107	117	114	117	116	121	87	82	61	53	52	50	91	85	81	82	82	82	88	96	94	96	86	84
0.0008	96	101	103	106	107	103	61	47	21	19	17	15	81	31	22	19	18	18	82	81	48	44	44	46
0.00016	94	98	102	105	104	99	53	38	15	13	11	8	68	13	9	7	6	6	80	76	39	23	21	23
0	89	91	91	94	94	89	52	26	11	9	9	6	67	10	5	4	4	4	70	73	18	24	18	18

REM 10

PLE / RUP	MOCK 0.1	MOCK 0.02	MOCK 0.004	MOCK 0.0008	MOCK 0.00016	MOCK 0	EV6 0.1	EV6 0.02	EV6 0.004	EV6 0.0008	EV6 0.00016	EV6 0	EV11 0.1	EV11 0.02	EV11 0.004	EV11 0.0008	EV11 0.00016	EV11 0	CVB5 0.1	CVB5 0.02	CVB5 0.004	CVB5 0.0008	CVB5 0.00016	CVB5 0
0.1	99	104	105	105	97	96	100	104	109	108	103	92	99	92	102	102	98	95	90	95	97	100	100	98
0.02	106	107	114	116	115	101	109	108	107	102	98	87	100	97	108	101	96	93	97	100	101	104	100	103
0.004	100	104	109	108	102	97	83	83	77	72	71	66	91	90	91	87	84	82	93	102	103	98	99	100
0.0008	95	99	102	103	99	91	55	57	48	43	41	41	78	70	60	50	43	39	85	97	92	94	90	90
0.00016	94	94	97	96	96	87	51	52	41	36	37	33	69	57	39	34	26	31	86	89	87	85	88	88
0	88	82	92	89	86	90	52	51	42	37	33	30	61	47	31	24	17	24	67	76	67	67	63	68

Table S3. Measured cell viability of A549 cells infected with mock, and EV1 and treated with different concentrations of remdesivir, pleconaril and rupintrivir. Experiment conducted by Prof. Denis Kainov.



REM 0

MOCK

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	90	96	101	102	103	101
0.02	92	98	100	99	100	96
0.004	95	100	101	98	99	95
0.0008	95	99	98	99	101	96
0.00016	92	96	100	101	95	94
0	85	87	93	90	90	99

EV1

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	92	92	93	96	92	86
0.02	93	91	91	89	80	77
0.004	95	87	53	53	52	48
0.0008	95	57	15	13	10	9
0.00016	87	55	6	4	2	2
0	78	24	3	1	0	0

REM 0.016

MOCK

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	90	97	96	102	99	99
0.02	94	100	99	102	100	102
0.004	96	102	96	101	99	100
0.0008	98	102	102	100	100	97
0.00016	89	97	93	96	94	93
0	85	86	88	92	89	87

EV1

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	98	97	96	95	93	96
0.02	97	95	89	88	86	81
0.004	94	87	47	46	51	42
0.0008	93	78	17	16	13	13
0.00016	91	74	8	4	2	2
0	85	62	5	2	2	1

REM 0.08

MOCK

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	86	96	96	101	97	98
0.02	92	100	98	103	99	97
0.004	92	100	101	103	101	96
0.0008	95	103	102	105	100	98
0.00016	91	98	96	100	98	93
0	84	86	89	92	94	86

EV1

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	92	96	94	97	95	88
0.02	95	97	87	86	86	81
0.004	93	94	50	45	48	47
0.0008	91	86	19	15	16	15
0.00016	86	84	10	7	4	4
0	78	79	7	7	3	3

REM 0.4

MOCK

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	93	104	101	103	102	101
0.02	95	103	102	100	105	106
0.004	100	100	101	101	103	107
0.0008	101	99	105	101	110	110
0.00016	92	104	102	103	106	103
0	88	107	94	91	93	91

EV1

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	101	97	100	104	97	87
0.02	97	104	102	102	95	87
0.004	94	94	83	60	60	57
0.0008	93	95	31	32	26	25
0.00016	91	90	22	14	12	16
0	81	89	16	15	11	12

REM 2

MOCK

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	87	98	95	109	104	109
0.02	91	104	97	107	108	110
0.004	93	102	101	104	111	107
0.0008	99	97	103	101	99	107
0.00016	94	105	99	98	102	100
0	88	95	88	89	91	93

EV1

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	115	109	94	93	94	90
0.02	110	105	101	97	97	93
0.004	106	101	99	97	98	95
0.0008	99	94	98	97	96	93
0.00016	100	96	94	93	90	89
0	96	92	86	83	80	86

REM 10

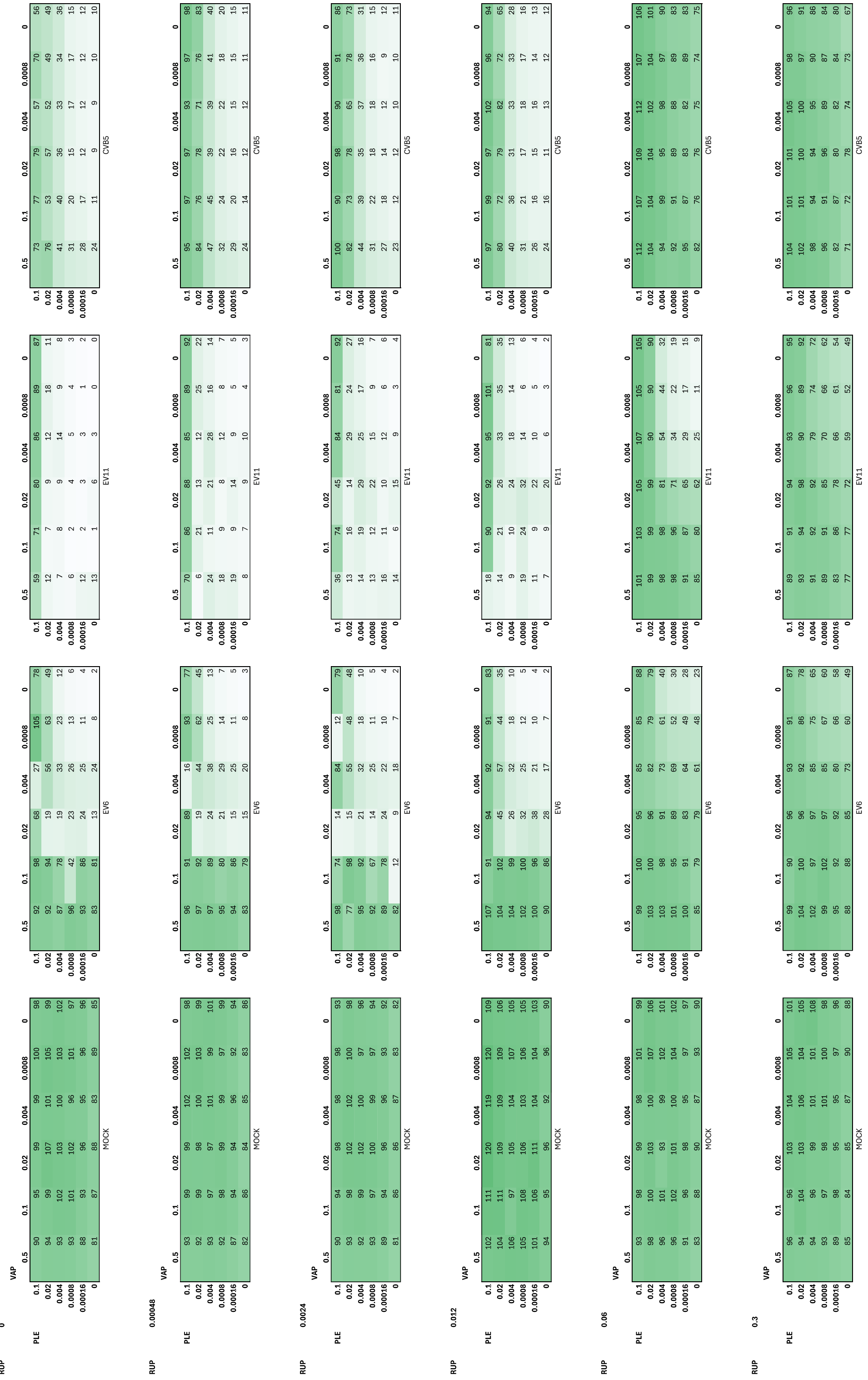
MOCK

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	87	96	96	109	100	94
0.02	88	96	97	104	98	97
0.004	87	95	97	105	100	101
0.0008	89	94	98	104	101	102
0.00016	85	90	92	97	95	93
0	73	77	83	94	89	80

EV1

PLE \ RUP	0.1	0.02	0.004	0.0008	0.00016	0
0.1	91	89	98	89	90	85
0.02	92	92	95	92	96	86
0.004	95	93	95	93	97	90
0.0008	94	94	95	97	98	95
0.00016	87	87	89	88	87	85
0	77	78	79	76	77	74

Table S4. Measured cell viability of A549 cells infected with mock, EV6, EV11, and CVB5 and treated with different concentrations of vapendavir, pleconaril and rupintrivir.



RUP 0

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	90	95	99	99	100	98
0.02	94	99	107	101	105	99
0.004	93	102	103	100	103	102
0.0008	93	101	102	96	101	97
0.00016	88	93	96	95	96	96
0	81	87	88	83	89	85

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	92	98	68	27	105	78
0.02	92	94	19	56	63	49
0.004	87	78	19	33	23	12
0.0008	96	42	23	26	13	6
0.00016	93	86	24	25	11	4
0	83	81	13	24	8	2

EV6

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	59	71	80	86	89	87
0.02	12	7	9	12	18	11
0.004	7	8	9	14	9	8
0.0008	6	2	4	5	4	3
0.00016	12	2	3	3	1	2
0	13	1	6	3	0	0

EV11

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	73	77	79	57	70	56
0.02	76	53	57	52	49	49
0.004	41	40	36	33	34	36
0.0008	31	20	15	17	17	15
0.00016	28	17	12	12	12	12
0	24	11	9	9	10	10

CVB5

RUP 0.00048

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	93	99	99	102	102	98
0.02	92	99	98	100	103	99
0.004	93	97	97	101	99	101
0.0008	92	98	99	99	97	99
0.00016	87	94	94	96	92	94
0	82	86	84	85	83	86

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	96	91	89	16	93	77
0.02	97	92	19	44	62	45
0.004	97	89	24	38	25	13
0.0008	95	80	21	29	14	7
0.00016	94	86	15	25	11	5
0	83	79	15	20	8	3

EV6

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	70	86	88	85	89	92
0.02	6	21	13	12	25	22
0.004	24	11	21	28	16	14
0.0008	18	9	8	12	8	7
0.00016	19	9	14	9	5	5
0	8	7	9	10	4	3

EV11

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	95	97	97	93	97	98
0.02	84	76	78	71	76	83
0.004	47	45	39	39	41	40
0.0008	32	24	22	22	18	20
0.00016	29	20	16	15	15	15
0	24	14	12	12	11	11

CVB5

RUP 0.0024

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	90	94	98	98	98	93
0.02	93	98	102	102	100	98
0.004	92	99	102	100	97	96
0.0008	93	97	100	99	97	94
0.00016	89	94	96	96	93	92
0	81	86	86	87	83	82

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	98	74	14	84	12	79
0.02	77	98	15	55	48	48
0.004	95	92	21	32	18	10
0.0008	92	67	14	25	11	5
0.00016	89	78	24	22	10	4
0	82	12	9	18	7	2

EV6

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	36	74	45	84	81	92
0.02	13	16	14	29	24	27
0.004	14	19	29	25	17	16
0.0008	13	12	22	15	9	7
0.00016	16	11	10	12	6	6
0	14	6	15	9	3	4

EV11

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	100	90	98	90	91	86
0.02	82	73	78	65	78	73
0.004	44	39	35	37	36	31
0.0008	31	22	18	18	16	15
0.00016	27	18	14	12	9	12
0	23	12	12	10	10	11

CVB5

RUP 0.012

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	102	111	120	119	120	109
0.02	104	111	109	109	109	106
0.004	106	97	105	104	107	105
0.0008	105	108	106	103	106	105
0.00016	101	106	111	104	104	103
0	94	95	96	92	96	90

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	107	91	94	92	91	83
0.02	104	102	45	57	44	35
0.004	104	99	26	32	18	10
0.0008	102	100	32	25	12	5
0.00016	100	96	38	21	10	4
0	90	86	28	17	7	2

EV6

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	18	90	92	95	101	81
0.02	14	21	26	33	35	35
0.004	9	10	24	18	14	13
0.0008	19	24	32	14	6	6
0.00016	11	9	22	10	5	4
0	7	9	20	6	3	2

EV11

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	97	99	97	102	96	94
0.02	80	72	79	82	72	65
0.004	40	36	31	33	33	28
0.0008	31	21	17	18	17	16
0.00016	26	16	15	16	14	13
0	24	16	11	13	12	12

CVB5

RUP 0.06

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	93	98	99	98	101	99
0.02	98	100	103	100	107	106
0.004	96	101	93	99	102	101
0.0008	96	102	101	100	104	102
0.00016	91	96	98	95	97	97
0	83	88	90	87	93	90

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	99	100	95	85	85	88
0.02	103	100	96	82	79	79
0.004	103	98	91	73	61	40
0.0008	101	95	89	69	52	30
0.00016	100	91	83	64	49	28
0	85	79	79	61	48	23

EV6

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	101	103	105	107	105	105
0.02	99	99	99	90	90	90
0.004	98	98	81	54	44	32
0.0008	98	96	71	34	22	19
0.00016	91	87	65	29	17	15
0	85	80	62	25	11	9

EV11

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	112	107	109	112	107	106
0.02	104	104	104	102	104	101
0.004	94	99	95	98	97	90
0.0008	92	91	89	88	89	83
0.00016	95	87	83	82	89	83
0	82	76	76	75	74	75

CVB5

RUP 0.3

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	96	96	103	104	105	101
0.02	94	104	103	106	104	105
0.004	94	96	99	101	101	108
0.0008	93	97	98	101	100	98
0.00016	89	98	95	95	97	96
0	85	84	85	87	90	88

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	99	90	96	93	91	87
0.02	104	100	96	92	86	78
0.004	102	97	97	85	75	65
0.0008	99	102	97	85	67	60
0.00016	95	92	92	80	66	58
0	88	88	85	73	60	49

EV6

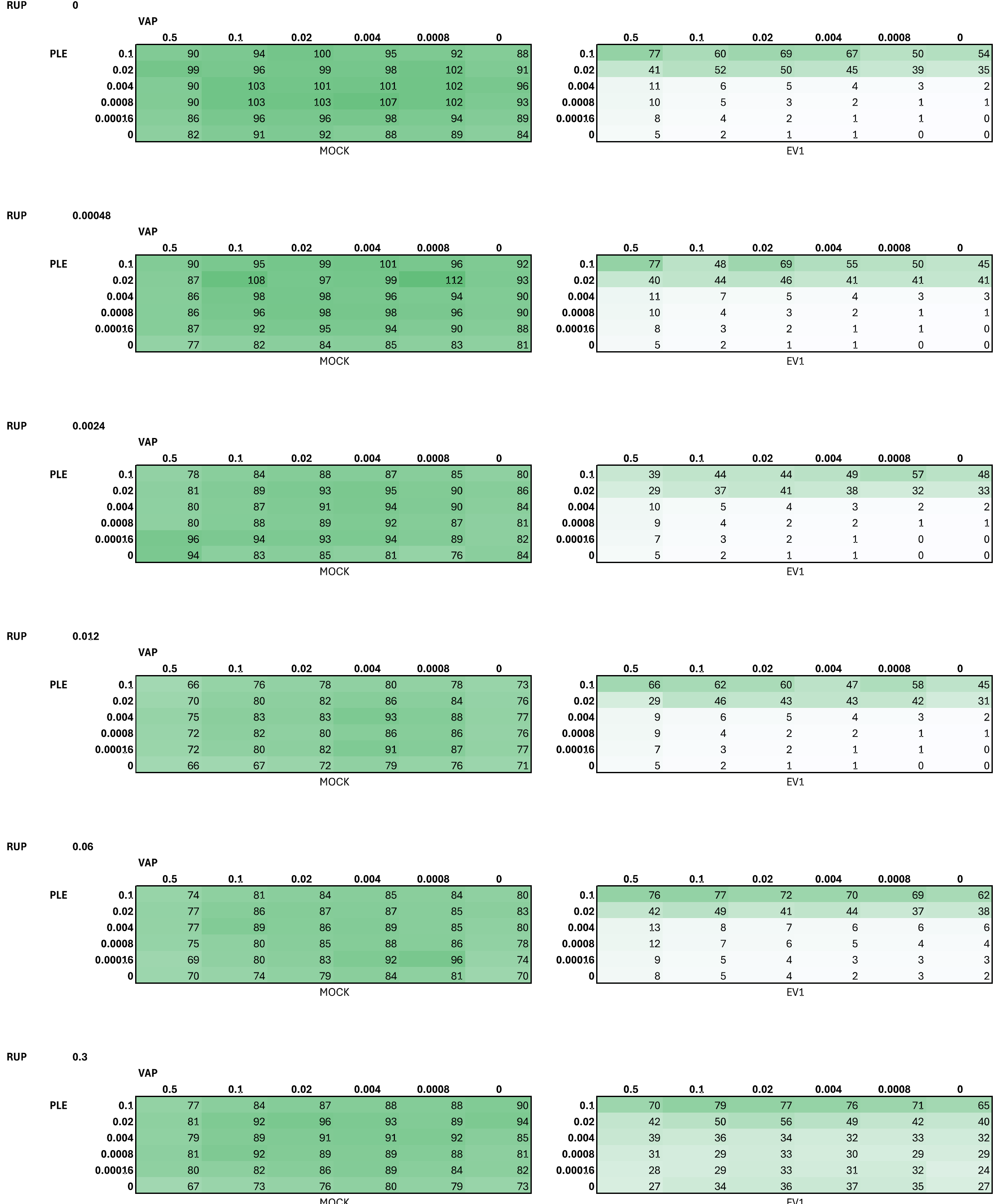
PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	89	91	94	93	96	95
0.02	93	94	98	90	89	92
0.004	91	92	92	79	74	72
0.0008	89	91	85	70	66	62
0.00016	83	86	78	66	61	54
0	77	77	72	59	52	49

EV11

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	104	101	101	105	98	96
0.02	102	101	100	100	97	91
0.004	98	94	94	95	90	86
0.0008	96	91	96	89	87	84
0.00016	82	87	80	82	84	80
0	71	72	78	74	73	67

CVB5

Table S5. Measured cell viability of A549 cells infected with mock, and EV1 and treated with different concentrations of vapendavir, pleconaril and rupintrivir. Experiment conducted by Prof. Denis Kainov.



RUP 0

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	90	94	100	95	92	88
0.02	99	96	99	98	102	91
0.004	90	103	101	101	102	96
0.0008	90	103	103	107	102	93
0.00016	86	96	96	98	94	89
0	82	91	92	88	89	84

EV1

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	77	60	69	67	50	54
0.02	41	52	50	45	39	35
0.004	11	6	5	4	3	2
0.0008	10	5	3	2	1	1
0.00016	8	4	2	1	1	0
0	5	2	1	1	0	0

RUP 0.00048

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	90	95	99	101	96	92
0.02	87	108	97	99	112	93
0.004	86	98	98	96	94	90
0.0008	86	96	98	98	96	90
0.00016	87	92	95	94	90	88
0	77	82	84	85	83	81

EV1

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	77	48	69	55	50	45
0.02	40	44	46	41	41	41
0.004	11	7	5	4	3	3
0.0008	10	4	3	2	1	1
0.00016	8	3	2	1	1	0
0	5	2	1	1	0	0

RUP 0.0024

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	78	84	88	87	85	80
0.02	81	89	93	95	90	86
0.004	80	87	91	94	90	84
0.0008	80	88	89	92	87	81
0.00016	96	94	93	94	89	82
0	94	83	85	81	76	84

EV1

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	39	44	44	49	57	48
0.02	29	37	41	38	32	33
0.004	10	5	4	3	2	2
0.0008	9	4	2	2	1	1
0.00016	7	3	2	1	0	0
0	5	2	1	1	0	0

RUP 0.012

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	66	76	78	80	78	73
0.02	70	80	82	86	84	76
0.004	75	83	83	93	88	77
0.0008	72	82	80	86	86	76
0.00016	72	80	82	91	87	77
0	66	67	72	79	76	71

EV1

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	66	62	60	47	58	45
0.02	29	46	43	43	42	31
0.004	9	6	5	4	3	2
0.0008	9	4	2	2	1	1
0.00016	7	3	2	1	1	0
0	5	2	1	1	0	0

RUP 0.06

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	74	81	84	85	84	80
0.02	77	86	87	87	85	83
0.004	77	89	86	89	85	80
0.0008	75	80	85	88	86	78
0.00016	69	80	83	92	96	74
0	70	74	79	84	81	70

EV1

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	76	77	72	70	69	62
0.02	42	49	41	44	37	38
0.004	13	8	7	6	6	6
0.0008	12	7	6	5	4	4
0.00016	9	5	4	3	3	3
0	8	5	4	2	3	2

RUP 0.3

MOCK

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	77	84	87	88	88	90
0.02	81	92	96	93	89	94
0.004	79	89	91	91	92	85
0.0008	81	92	89	89	88	81
0.00016	80	82	86	89	84	82
0	67	73	76	80	79	73

EV1

PLE \ VAP	0.5	0.1	0.02	0.004	0.0008	0
0.1	70	79	77	76	71	65
0.02	42	50	56	49	42	40
0.004	39	36	34	32	33	32
0.0008	31	29	33	30	29	29
0.00016	28	29	33	31	32	24
0	27	34	36	37	35	27

Table S6. 3- and 4-drug expansion of the drugvirus.info database, separated in enteroviruses and other human viruses.

ENTEROVIRUSES

Source	Virus	Drug name	Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Status	PMID			
pubmed	CVB1		Pleconaril	MDL-860	Oxogluacine			Pre-clinical	27815331	26196747		
	CVB1		Disoxaril	Guanidine HCl	Oxogluacine			Pre-clinical	27442375			
	CVB1		Disoxaril	Guanidine HCl	Oxogluacine	PTU-23		Pre-clinical	19900482			
	CVB3		Disoxaril	Guanidine HCl	Oxogluacine			Pre-clinical	27552486			
	CVB3		Pleconaril	MDL-860	Oxogluacine			Pre-clinical	33090201			
	EV1		Pleconaril	Remdesivir	Rupintrivir			Pre-clinical	38417531			

OTHER HUMAN VIRUSES

Source	Virus	Drug name	Drug 1	Drug 2	Drug 3	Drug 4	Drug 5	Status	PMID			
drugs.com	HIV-1	*Atripla*	Efavirenz	Emtricitabine	Tenofovir			Discontinued				
	HIV-1	*Biktarvy*	Bictegravir	Emtricitabine	Tenofovir			Approved				
	HIV-1	*Complera*	Emtricitabine	Rilpivirine	Tenofovir			Approved				
	HIV-1	*Delstrigo*	Doravirine	Lamivudine	Tenofovir disoproxil fumarate			Approved				
	HIV-1	*Genvoya*	Cobicistat	Elvitegravir	Emtricitabine	Tenofovir		Approved				
	HIV-1	*Odefsey*	Emtricitabine	Rilpivirine	Tenofovir			Approved				
	HIV-1	*Stribild*	Cobicistat	Elvitegravir	Emtricitabine	Tenofovir		Approved				
	HIV-1	*Symfi (Lo)*	Efavirenz	Lamivudine	Tenofovir			Approved				
	HIV-1	*Symtuza*	Cobicistat	Darunavir	Emtricitabine	Tenofovir		Approved				
	HIV-1	*Triumeq*	Abacavir	Dolutegravir	Lamivudine			Approved				
	HIV-1	*Trizivir*	Abacavir	Lamivudine	Zidovudine			Approved				
	HCV	*Viekira*	Dasabuvir	Ombitasvir	Paritaprevir	Ritonavir	+/- Ribavirin	Approved				
	HCV	*Vosevi*	Sofosbuvir	Velparasvir	Voxilaprevir			Approved				
	HCV	*Technivie*	Ombitasvir	Paritaprevir	Ritonavir			Approved				
pubmed	Adeno		Cidofovir	sCAR-Fc	siRNAs			Pre-clinical	26026665			
	Avian flu		MI-701	Oseltamivir carboxylate	Ribavirin			Pre-clinical	26022200			
	Ebola		IFN-b	Lamivudine	Zidovudine			Pre-clinical	26752302			
	Flavivirus		2'-CMC	6-azaU	IFNa2a			Pre-clinical	24663025			
	Flu-A		Amantadine	Oseltamivir	Ribavirin			Clinical	20179772	21968371	19620324	22220216
	Flu-A		Amantadine	Oseltamivir	Ribavirin	Zanamivir		Clinical	31014561			
	Flu-A		AMT	OSCN^-	Oseltamivir			Pre-clinical	35663985			
	Flu-A		anti-RBS	anti-Stem	Favipiravir			Pre-clinical	32382088			
	Flu-A		Celecoxib	Mesalazine	Zanamivir			Pre-clinical	18523003			

Flu-A	Ellagic acid	Isoprinosine	Oseltamivir		Pre-clinical	29241072			
Flu-A	Fibrates	Neuraminidase inh.	Statins		Pre-clinical	21944947			
HBV	Adefovir dipivoxil	Entecavir	Lamivudine		Clinical	22246827			
HBV	Amantadine	Biphenyl dimethyl dicarboxylate	Ursodeoxycholic acid		Pre-clinical	15918519			
HBV	Amdoxovir	Clevudine	Emtricitabine		Pre-clinical	12760857			
HCV	2'-C-MeC	IFN-a2b	Ribavirin		Pre-clinical	18610555			
HCV	2'-C-MeC	Tegobuvir	VX-950		Pre-clinical	26036224			
HCV	2'-F-C-MeC	IFN-a2b	Ribavirin		Pre-clinical	18610555			
HCV	Amantadine	IFN-alpha	Ribavirin		Clinical	15699763	12010649		
HCV	Asunaprevir	Beclabuvir	Daclatasvir		Clinical	26083155	26014906		
HCV	Asunaprevir	PEG-IFN	Ribavirin		Clinical	24373080			
HCV	Boceprevir	PEG-IFN	Ribavirin		Clinical	21205141	24373078	23886001	
HCV	Daclatasvir	Direct-acting antiviral	NS5A-syn		Pre-clinical	26711745			
HCV	Daclatasvir	PEG-IFN	Ribavirin		Clinical	24373074	24373080	26014906	
HCV	Danoprevir	PEG-IFN	Ribavirin		Clinical	24373080			
HCV	Dasabuvir	Ombitasvir	Paritaprevir/r	+/- Ribavirin	Clinical	26083155			
HCV	Didanosine	Interferon-a	Ribavirin		Pre-clinical	12700449			
HCV	Fluvastatin	PEG-IFN	Ribavirin	Telaprevir	Clinical	24732752			
HCV	Gelcaprevir	Pibrentasvir	Sofosbuvir	+/- Ribavirin	Clinical	30605721			
HCV	HCV protease	Interferon-a	Ribavirin		Pre-clinical	21817191			
HCV	PEG-IFN	Ribavirin	Thymalfasin		Clinical	15641210			
HCV	PEG-IFN	Ribavirin	Simeprevir		Clinical	24882512			
HCV	PEG-IFN	Ribavirin	Telaprevir		Clinical	21205141	22951253	24373078	23886001
HCV	PEG-IFN	Ribavirin	Sofosbuvir		Clinical	24289735			
HCV	Sofosbuvir	Velpatasvir	Voxilaprevir		Clinical	30605721	28753040		
HIV-1	Abacavir	Lamivudine	Tenoforvir		Pre-clinical	15918336			
HIV-1	Abacavir	Nelfinavir	Zidovudine		Clinical	12126455			
HIV-1	Carbosilane dendrimers	Maraviroc	Tenofovir		Pre-clinical	25867856			
HIV-1	Didanosine	Interferon-a	Ribavirin		Pre-clinical	12700449			
HIV-1	Efavirenz	Emtricitabine	Tenoforvir		Pre-clinical	19439089			
HIV-1	Indinavir	Lamivudine	Zidovudine		Pre-clinical	10722511			
HIV-1	Lamivudine	Nelfinavir	Zidovudine		Clinical	12126455			
HIV-1	PGDM1400	PGT121	VRC07-523LS		Clinical	35551291			
SARS-CoV-2	Azithromycin	Ciclesonide	Hydroxychloroquine		Clinical	33054978			
SARS-CoV-2	Brequinar	Camostat	Molnupiravir		Pre-clinical	36190406			
SARS-CoV-2	Hydroxychloroquine	Lopinavir	Ritonavir		Clinical	33974624			
SARS-CoV-2	IFN-b1b	Lopinavir	Ribavirin		Clinical	34111191	33041111		
SARS-CoV-2	IFN-b1b	Ribavirin	Ritonavir		Clinical	34111191	33041111		
SARS-CoV-2	mAbs	Nirmatrelvir	Remdesivir		Clinical	36976301			
SARS-CoV-2	mAbs	Remdesivir	Ritonavir		Clinical	36976301			

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