



Short communication

Alisporivir inhibits MERS- and SARS-coronavirus replication in cell culture, but not SARS-coronavirus infection in a mouse model



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ABSTRACT

Currently, there is no registered treatment for infections with emerging zoonotic coronaviruses like SARS- and MERS-coronavirus. We here report that in cultured cells low-micromolar concentrations of alisporivir, a non-immunosuppressive cyclosporin A-analog, inhibit the replication of four different coronaviruses, including MERS- and SARS-coronavirus. Ribavirin was found to further potentiate the antiviral effect of alisporivir in these cell culture-based infection models, but this combination treatment was unable to improve the outcome of SARS-CoV infection in a mouse model. Nevertheless, our data provide a basis to further explore the potential of Cyp inhibitors as host-directed, broad-spectrum inhibitors of coronavirus replication.

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1. Main text

The outbreak of the Severe Acute Respiratory Syndrome-coronavirus (SARS-CoV) in 2003 and the continuing circulation of the Middle East respiratory syndrome-coronavirus (MERS-CoV; since 2012) have highlighted the potentially lethal consequences of zoonotic coronavirus infections in humans. Approximately 8000 people were infected during the SARS-CoV epidemic (~10% mortality; <http://www.who.int/csr/sars/en/>), while the global MERS tally is now over 1800 laboratory-confirmed cases, with a mortality of about 35% (http://www.who.int/csr/disease/coronavirus_infections/en/). In mid-2015, an air travel-related outbreak in South Korea (186 confirmed cases, 36 deaths, and about 17,000 potentially exposed individuals quarantined) again illustrated the socioeconomic impact of this kind of emerging pathogen, also outside regions in which the virus is endemic (<http://www.who.int/csr/don/07-july-2015-mers-korea/en/>). The lack of effective methods to prevent or treat coronavirus infections in humans remains a serious public health concern, especially given increasing evidence that SARS-like coronaviruses continue to circulate in bats and may

have the potential to readily cross the species barrier and emerge as human pathogens (Ge et al., 2013; Menachery et al., 2015).

Data from cell culture infection models (Chan et al., 2013a, 2013b; de Wilde et al., 2013b; Falzarano et al., 2013a; Kindler et al., 2013; Zielecki et al., 2013) and experiments in rhesus macaques (Falzarano et al., 2013b) and marmosets (Chan et al., 2015) suggested that interferons (IFNs) are potent inhibitors of MERS-CoV replication. The outcome of the clinical use of interferons was variable, with some reports questioning the long-term survival benefits (Al-Tawfiq et al., 2014; Omrani et al., 2014; Shalhoub et al., 2015), whereas others suggested that further investigation is warranted (Khalid et al., 2016), possibly in combination with the use of lopinavir/ritonavir (Kim et al., 2015). Unfortunately, some of the negative outcomes are confounded by the late-stage treatment of critically-ill patients.

Since the development and registration of novel therapeutic compounds is generally time consuming, the repurposing of approved drugs offers one of the few shortcuts to establishing anti-coronavirus therapy. Several FDA-approved compounds have been reported to inhibit MERS-CoV and SARS-CoV replication in cell culture (de Wilde et al., 2014; Dyall et al., 2014; Hart et al., 2013), but their efficacy in animal models remains to be determined. Previously, the FDA-approved drug cyclosporin A (CsA) was shown to inhibit the replication of a variety of viruses (reviewed in Nagy et al., 2011), including coronaviruses (Carbajo-Lozoya et al., 2014; de Wilde et al., 2013b, 2011; Kim and Lee, 2014; Pfeifferle et al., 2011;

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Tanaka et al., 2012). CsA targets members of the cyclophilin (Cyp) family, which are peptidyl-proline isomerases (PPIases) that act as chaperones in protein folding and function (Davis et al., 2010). Since the immunosuppressive properties of CsA (Schreiber and Crabtree, 1992) are an undesirable side-effect in the context of antiviral therapy, numerous non-immunosuppressive Cyp inhibitors have been developed. Among these, alisporivir (ALV) showed increased sustained viral response during treatment of chronic hepatitis C virus (HCV) infection in phase III clinical trials, when treatment with ribavirin and pegylated interferon was combined with ALV (Buti et al., 2015; Flisiak et al., 2012; Pawlotsky et al., 2015; Zeuzem et al., 2015).

In this study, we have first investigated whether ALV inhibits MERS- and SARS-CoV replication in cell culture. We employed previously described cell-culture based screening assays relying on the rapid cytopathic effect (CPE) observed in coronavirus-infected cells (de Wilde et al., 2013b, 2014). First, the viability of MERS-CoV-infected Vero cells (MOI 0.005) treated with increasing ALV concentrations was assessed at 3 days post infection (p.i.). We found that virus-induced CPE was prevented by ALV in a dose-dependent manner (EC_{50} 3.6 μ M; Fig. 1A). A similar efficacy was observed in MERS-CoV-infected Huh7 cells (MOI 0.005) (EC_{50} 3.4 μ M; Fig. 1B). The toxicity of ALV treatment was assessed for both cell lines and CC_{50} values were found to be comparable and 7–13 times higher than the observed EC_{50} values (26.4 and 43.8 μ M, respectively; see Fig. 1A–B). ALV sensitivity was not restricted to the original MERS-CoV isolate (EMC/2012; Zaki et al., 2012) as similar EC_{50} values of 1.5 and 3.0 μ M were determined when using MERS-CoV strain N3/Jordan in Vero (MOI 0.05) and Huh7 cells (MOI 0.005), respectively (Fig. 1C–D). The potential of ALV to inhibit other betacoronaviruses was illustrated by the complete block of SARS-CoV replication in VeroE6 cells. The SARS-CoV field isolate Frankfurt-1 was inhibited with an EC_{50} of 8.3 μ M (CC_{50} in VeroE6 cells is >50 μ M; Fig. 1E; de Wilde et al., 2014) and for the mouse-adapted SARS-CoV strain MA-15 (MOI 0.05; kindly provided by prof. Luis Enjuanes, National Center of Biotechnology (CNB-CSIC), Madrid, Spain) an EC_{50} of 1.3 μ M was determined. The EC_{50} , CC_{50} , and selectivity index (SI) values are summarized in Table 1.

We next established that ALV treatment also reduced the yield of infectious MERS-CoV progeny. Vero and Huh7 cells were infected with MERS-CoV (MOI 0.01) and treated with increasing but non-cytotoxic ALV concentrations (for cell viability data, see Fig. 1; grey lines) from 1 h post infection (p.i.) onward. Progeny virus from MERS-CoV-infected Vero cells was harvested at 48 h p.i. and titrated by plaque assay, which revealed a ~2 log reduction when using 6.3 μ M ALV (Fig. 2A). This effect was more pronounced in Huh7 cells, as a comparable decrease in progeny titer was already observed when using 3.1 μ M ALV (Fig. 2B). Similar results were obtained using fully differentiated primary human airway epithelial (HAE) cells, of which the non-ciliated cells were shown to be the primary target for MERS-CoV infection (Raj et al., 2013). First, air-liquid interface cultures derived from two donors were cultured for 14 days on semi-permeable transwell membranes (van Wetering et al., 2007). Next, these HAE cell cultures were pre-treated for 16 h with 25 μ M ALV or 0.13% EtOH (vehicle control) and infected with MERS-CoV isolate EMC/2012 (MOI of 2; titer determined on Vero cells). After a 48-h ALV treatment, MERS-CoV progeny titers were reduced by 1 or 3 log, depending on the donor (Fig. 2C–D).

In a second laboratory, the ability of ALV to inhibit MERS-CoV replication was assessed independently in Vero, LLC-MK2, and Huh7 cells. Cells were inoculated with MERS-CoV (EMC/2012) at an MOI of 0.001 and medium containing between 0.625 and 20 μ M of ALV was added at 1 h p.i. Supernatant was collected on day 3 and levels of infectious virus progeny assessed by TCID₅₀ assay. In parallel, toxicity was assessed using a cell viability assay. On day 3,

a 4- to 5-log decrease in infectious progeny was observed at a dose of 10 μ M ALV and EC_{50} values of 3.9, 2.8, and 4.0 μ M were calculated for Vero, LLC-MK2, and Huh7 cells, respectively (Fig. S1 and Table 1).

SARS-CoV infection (MOI 0.01) in Vero cells was sensitive to 3.1 μ M ALV (Fig. 2E), resulting in a near-complete block when using 6.3 μ M ALV. A ~1.5 log reduction in virus progeny could be achieved in VeroE6 cells using the same dose (Fig. 2F), suggesting that ALV treatment in VeroE6 cells was twice less effective compared to Vero cells. For both SARS-CoV and MERS-CoV, differences in ALV sensitivity in various cell lines were observed. One explanation for such difference would be that ALV uptake differs per cell line. Interestingly, we also observed remarkable difference in e.g. SARS-CoV-induced cell death between VeroE6 and Vero cells, ranging from severe CPE in the former to minimal CPE in the latter after the same incubation period (unpublished observations). This suggests that these related cell lines can respond quite differently to virus infection.

To investigate the potential of ALV as a broad-spectrum coronavirus inhibitor, we assessed the effect of ALV treatment on the replication of two additional coronaviruses, murine hepatitis virus (MHV; strain A59) and human coronavirus 229E (HCoV-229E). Following infection with an MOI of 0.01, media were harvested at 16 h p.i. for MHV on 17C11 cells or 48 h p.i. for HCoV-229E on Huh7 cells (Fig. 2G and H). When using an ALV dose of 6.3 μ M, MHV progeny titers were decreased by 1 log, while a 25- μ M dose resulted in a ~2-log reduction without showing any signs of toxicity in uninfected cells (cell viability values >85% of untreated 17C11 control cells were observed for all ALV concentrations tested; data not shown). HCoV-229E production was reduced to a similar extent (~1.5 and ~2-log reduction at 6.3 and 12.5 μ M ALV, respectively).

As ribavirin has previously been reported to inhibit MERS-CoV replication (Falzarano et al., 2013a) and ALV and ribavirin have been used together during clinical trials for hepatitis C treatment (Pawlotsky et al., 2015), this combination was tested in LLC-MK2 cells. Combining ribavirin and ALV treatment primarily had an additive effect on antiviral activity, with the exception of the combination of 25 μ g/ml ribavirin and 5 μ M ALV, which had a synergy value of 1.51 (Fig. 3A) as calculated using MacSynergy II (Prichard and Shipman, 1990). Increasing concentrations of ribavirin gradually lowered the cell viability measured without affecting cellular morphology when assessed microscopically (Fig. 3B).

The above findings suggested that a combination approach might have a chance of success in animal models. Therefore, we assessed whether similar inhibitory effects could be observed in a mouse model. We employed the mouse-adapted MA15 strain of SARS-CoV (Roberts et al., 2007) and animals were treated daily with ALV (60 mg/kg) and/or ribavirin (50 mg/kg). Unfortunately, treatment with ALV alone did not enhance survival (data not shown) and combined therapy with ALV and ribavirin did not prevent weight loss (Fig. 4A) or enhance survival (Fig. 4B).

Since the ALV concentration required for SARS-CoV inhibition in cell culture was >100-fold higher than that required for inhibition of HCV replication (Coelmont et al., 2010; Paeshuysse et al., 2006; Puyang et al., 2010), the negative outcome of this animal experiment may not be too surprising. Nevertheless, the use of Cyp inhibitors remains a promising and innovative antiviral strategy. This class of drugs can potentially inhibit against broad range of pathogenic viruses, including hepatitis B virus (Phillips et al., 2015; EC_{50} of 4.1 μ M), HCV (Coelmont et al., 2010; Paeshuysse et al., 2006; Puyang et al., 2010; EC_{50} values between 0.02 and 0.23 μ M), and human immunodeficiency virus 1 (Ptak et al., 2008; EC_{50} values in the low-nanomolar range). We previously reported that also the replication of two arteriviruses, which are distantly related to coronaviruses, can be blocked by the ALV-related non-immunosuppressive CsA analog Debio-064 (de Wilde et al., 2013a).

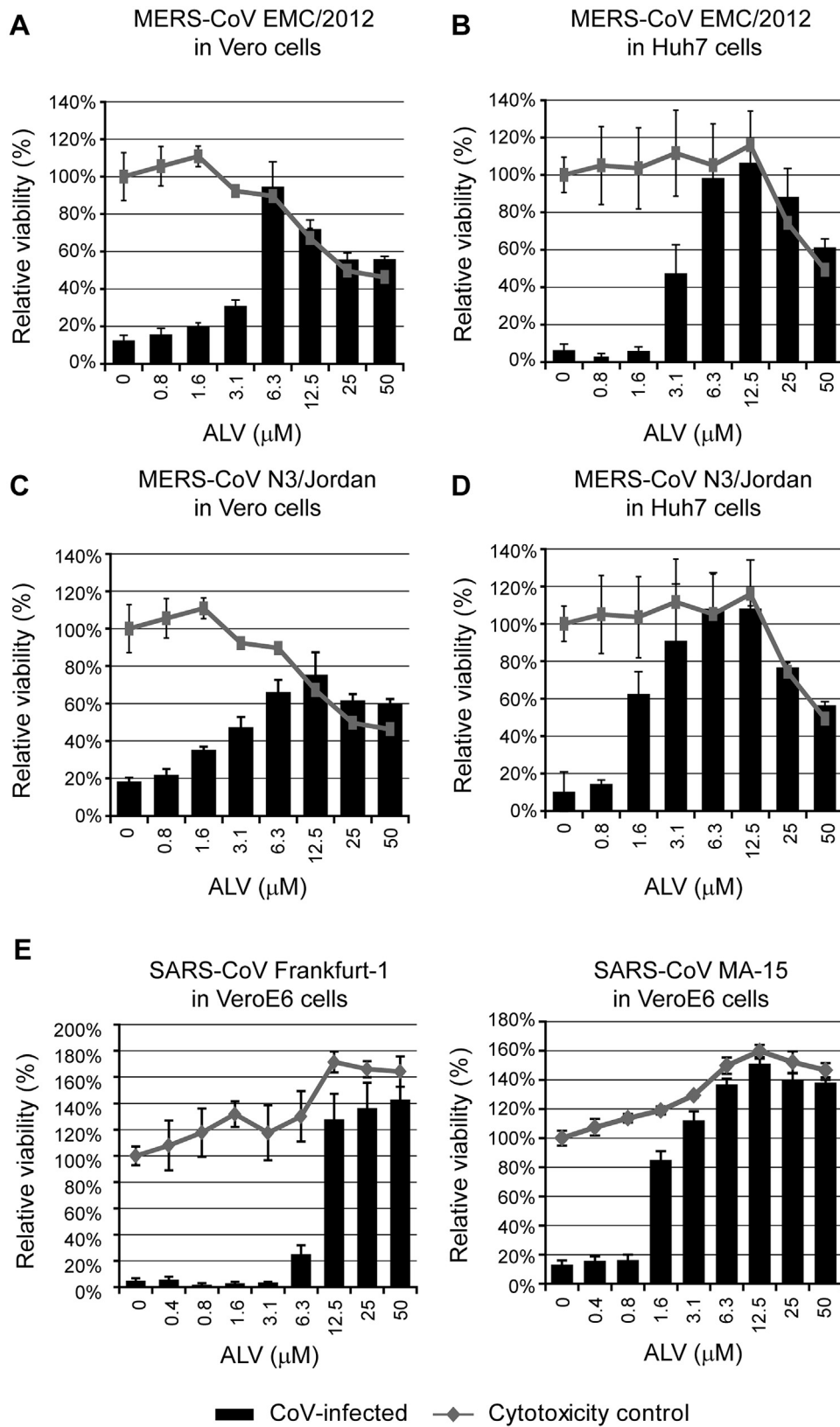


Fig 1. MERS-CoV- and SARS-CoV-induced cell death is strongly reduced by low-micromolar concentrations of alisporivir. (a,c) Vero or (b,d) Huh7 cells in 96-well plates were infected with (a,b) MERS-CoV EMC/2012 (MOI 0.005) or (c,d) MERS-CoV N3/Jordan (MOI 0.05) in Vero cells and MOI 0.005 in Huh7 cells in the presence of 0–50 μM ALV. (e,f) Vero E6 cells in 96-well plates were infected with (e) SARS-CoV isolate Frankfurt-1 (MOI 0.005) or (f) SARS-CoV strain MA-15 (MOI 0.05) in the presence of 0–50 μM ALV. Mock-infected cells that did not receive ALV or solvent were used as a reference for cell viability (their relative viability was set at 100%). Cells were incubated for 3 days with the exception for MERS-CoV EMC/2012 in Huh7 cells (2 days) and cell viability was monitored using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega). In addition, the (potential) toxicity of ALV treatment was monitored in parallel in mock-infected cell cultures. Graphs show the results (average and SD) of a representative experiment that was performed in quadruplicate. All virus-cell combinations were tested at least twice. Lines represent relative cell viability in the absence of infection (ALV toxicity control); bars represent relative cell viability after infection and ALV treatment.

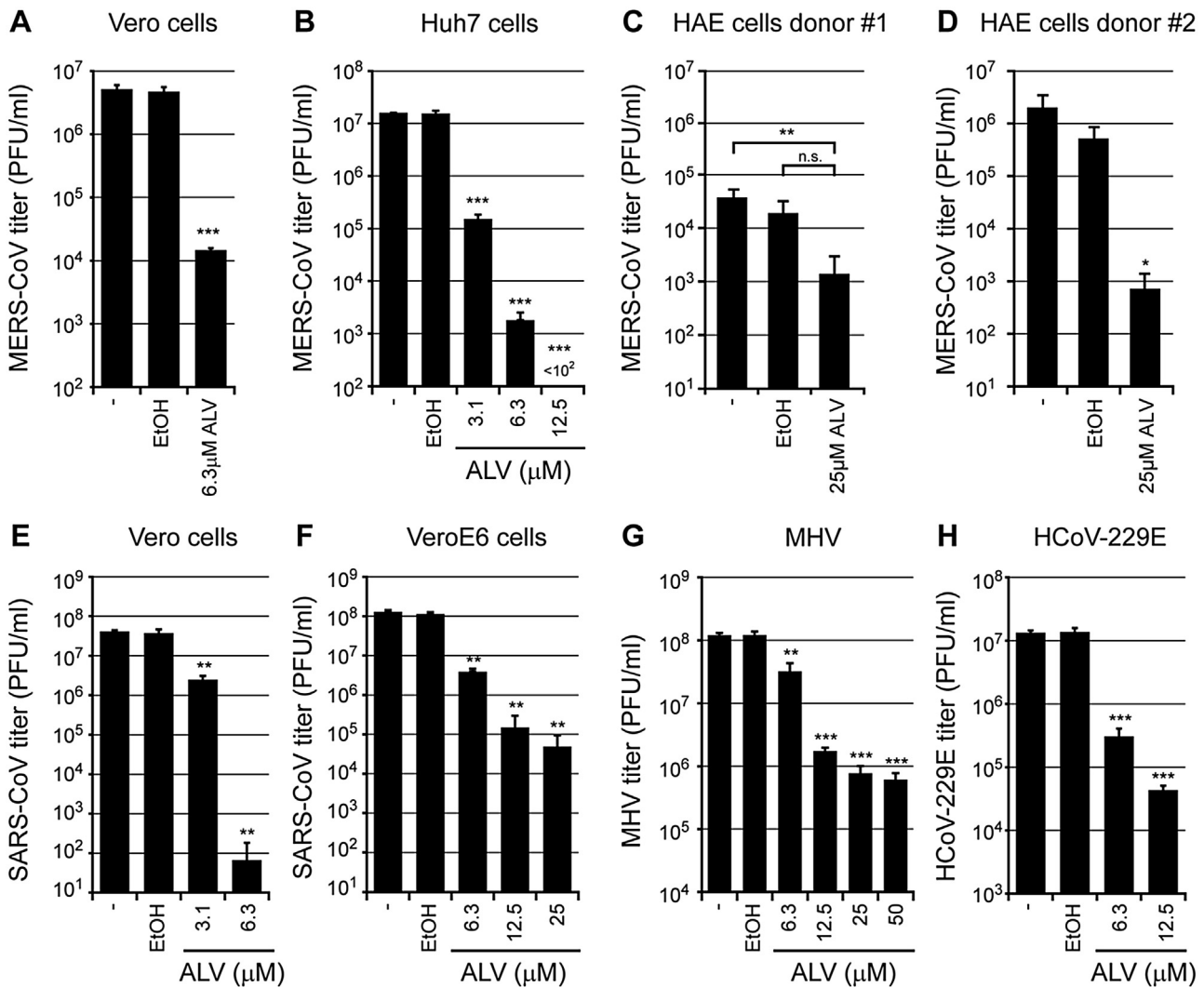


Fig. 2. Alisporivir inhibits the yield of MERS-CoV, SARS-CoV, MHV, and HCoV-229E from infected cells. (a, b) MERS-CoV EMC/2012-infected (MOI 0.01) (a) Vero and (b) Huh7 cells were treated with 3.1 or 6.3 μM ALV (Vero) or 3.1 to 12.5 μM ALV (Huh7) from 1 h p.i. onward. At 48 h p.i., virus titers in the culture medium were determined by plaque assays as described before (van den Worm et al., 2012). (c, d) HAE cells from two different donors were cultured on semi-permeable 12-well transwell membranes for 14 weeks. About 16 h prior to infection, 25 μM ALV, 0.13% EtOH, or medium was added to the basal side of the cell layer. Subsequently, cells were apically infected with MERS-CoV (MOI of 2; titer determined on Vero cells). After 48 h, virus release from the apical side of the cell layer was determined by harvesting the mucous fluid and subsequent plaque assay. (e, f) SARS-CoV-infected (e) Vero or (f) VeroE6 cells (MOI 0.01) were treated with various concentrations of ALV from 1 h p.i. onwards, and virus titers in the culture medium at 32 h p.i. were determined by plaque assay. (g) 17C1 cells infected with MHV-A59 (MOI 0.01) or (h) Huh7 cells infected with HCoV-229E (MOI 0.01) were treated with ALV from 1 h p.i. onwards, and infectious progeny titers were determined at 16 h p.i. and 48 h p.i., respectively. The graphs show the results of one representative experiment (mean ± SD, n = 3). For all experiments, control infections include cells that remained untreated (“-”) or are treated with an amount of EtOH equalling that present at the highest ALV concentration used. Two-sided Student’s *t* test (Graphpad Prism 7 software) was used to determine the significance of inhibition of virus replication between EtOH-treated and ALV-treated samples (* *p* < 0.05; ** *p* < 0.005; *** *p* < 0.001; n.s. not significant).

Our study reveals that ALV is a broad-spectrum coronavirus inhibitor in cell culture, as it inhibits the replication of both alpha- and betacoronaviruses (Figs. 1 and 2 and Carbajo-Lozoya et al., 2014). Further research is needed to elucidate the exact mechanism of action underlying ALV’s interference with coronavirus replication, as well as the involvement of CyPs in the coronavirus replication cycle. For HCV, ALV has been shown to disrupt functional interactions between cyclophilin A (CypA) and viral proteins and/or RNA (Coelmont et al., 2010; Garcia-Rivera et al., 2012; Nag et al., 2012). Interestingly, although ALV has a ~4 times higher affinity for CypA compared to CsA (unpublished data), the EC₅₀ values for both inhibitors are similar, leaving the possibility that ALV and other Cyp inhibitors target CoV replication independently of CypA.

In a previous study, a 4-log reduction in HCoV-NL63 virus progeny was reported upon treatment of infected CaCo-2 cells with 10 μM ALV (EC₅₀ 0.8 μM; Carbajo-Lozoya et al., 2014). The

observed variations in EC₅₀ values suggest that different coronaviruses may not be equally sensitive to the drug, although they may also reflect differences in e.g. experimental design, Cyp expression levels, and/or ALV uptake or turnover in different cell lines. The lack of ALV activity in the SARS-CoV animal model suggests that the drug itself may not be suited for the treatment of coronavirus infections. Nevertheless, Cyp inhibitors remain interesting leads for the development of host-directed anti-coronavirus therapy, as well as interesting tools to study the role of CyPs in coronavirus replication in more detail.

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Table 1
Inhibition of SARS-CoV and MERS-CoV infection by ALV treatment.

Virus	Strain	Cell line	EC ₅₀ ^a (μM)	CC ₅₀ ^a (μM)	SI ^b	Read-out
MERS-CoV	EMC/2012	Vero	3.6 ± 1.1	26.4 ± 1.0	7.3	CPE-based assay ^c
		Huh7	3.9 ± 1.7	>20	>5.1	Virus yield ^{d,e}
		Huh7	3.4 ± 1.0	43.8 ± 1.0	12.9	CPE-based assay
	N3/Jordan	LLC-MK2	2.8 ± 1.0	>20	>7.1	Virus yield
		Vero	4.0 ± 1.1	14.3 ± 1.8	3.6	Virus yield
		Huh7	3.0 ± 1.0	26.4 ± 1.0	8.8	CPE-based assay
SARS-CoV	Frankfurt-1	VeroE6	8.3 ± 1.0	43.8 ± 1.0	29.2	CPE-based assay
		VeroE6	1.5 ± 1.0	>50	>6.0	CPE-based assay
	MA-15	VeroE6	1.3 ± 0.05	>50	>38.5	CPE-based assay

Data are from two independent laboratories. EC₅₀ and CC₅₀ values were calculated as described previously (de Wilde et al., 2014; Falzarano et al., 2013a). The selectivity index (SI), the relative efficacy of a compound in specifically inhibiting virus replication, was calculated as CC₅₀/EC₅₀. Statistical analyses were performed using the results of at least two independent experiments.

^a EC₅₀ and CC₅₀ values are means (± SE) from a representative experiment (n = 4) that was repeated at least twice.

^b SI: Selectivity index, calculated as CC₅₀/EC₅₀.

^c Data is presented in Fig. 1.

^d Virus yield is determined by TCID₅₀ assay (data is presented in Fig. S1; Falzarano et al., 2013a).

^e Experiments performed to independently confirm the antiviral effect of ALV in a second laboratory.

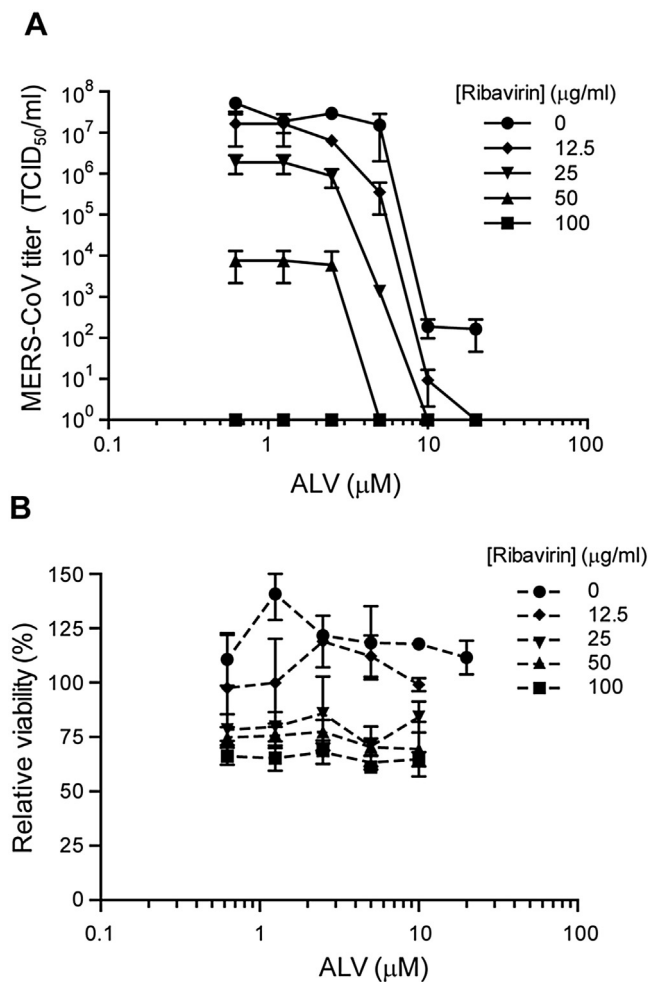


Fig. 3. Additive antiviral effects of combined Ribavirin and Alisporivir treatment in MERS-CoV-infected cell cultures. (a) LLC-MK2 cells infected with MERS-CoV at an MOI of 0.001 were treated with a combination of 0.625 to 20 μM ALV and 12.5 to 100 μg/ml ribavirin from 1 h p.i. onwards. Virus titers in the culture medium at 3 days p.i. were determined by TCID₅₀ as previously described (Falzarano et al., 2013a). (b) In parallel, control cells were treated with the same compound concentrations to determine cytotoxicity with a CellTiter 96 AQueous One solution cell proliferation assay.

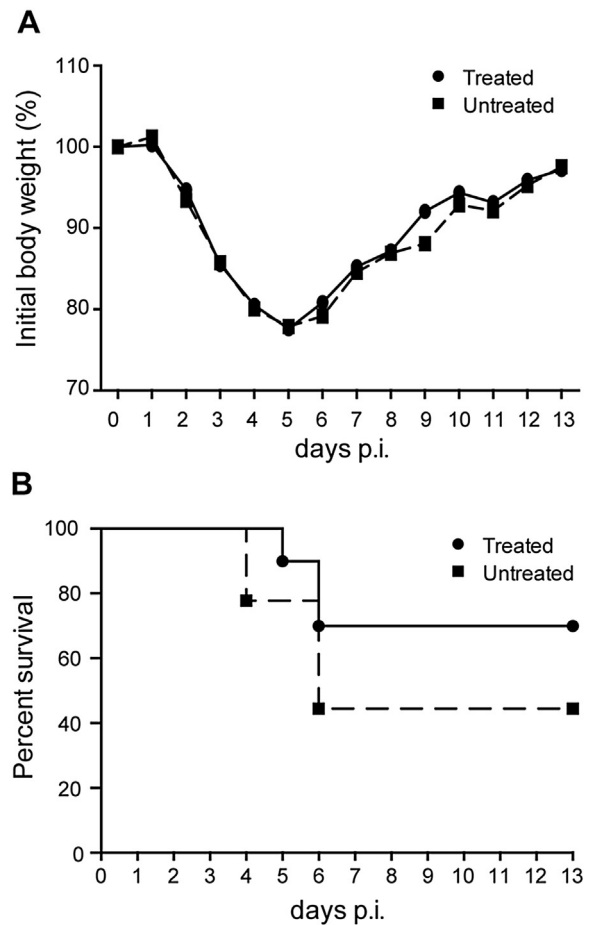


Fig. 4. Treatment with Ribavirin and alisporivir does not protect from SARS-CoV infection in a mouse model. (a,b) Combination therapy with alisporivir and ribavirin did not diminish morbidity (a) or mortality (b) in SARS-CoV MA15-infected mice. Six-week-old BALB/c mice were infected with 40,000 PFU of SARS-CoV-MA15, and then treated daily with 60 mg/kg alisporivir and 50 mg/kg ribavirin. Results of two experiments combined, n = 10 for both groups.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.virusres.2016.11.011>.

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