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Screening of an FDA-approved compound library identifies four small-molecule inhibitors of Middle East respiratory syndrome coronavirus replication in cell culture

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Abstract

Coronaviruses can cause respiratory and enteric disease in a wide variety of human and animal hosts. The 2003 outbreak of severe acute respiratory syndrome (SARS) first demonstrated the potentially lethal consequences of zoonotic coronavirus infections in humans. In 2012, a similar previously unknown coronavirus emerged, Middle East respiratory syndrome coronavirus (MERS-CoV), thus far causing over 550 laboratory-confirmed infections, with an unexplained steep rise in the number of cases being recorded over recent months. The human MERS fatality rate of ~30% is alarmingly high, even though many deaths were associated with underlying medical conditions. Registered therapeutics for the treatment of coronavirus infections are not available. Moreover, the pace of drug development and registration for human use is generally incompatible with strategies to combat emerging infectious diseases. Therefore, we have screened a library of 348 FDA-approved drugs for anti-MERS-CoV activity in cell culture. If such compounds would prove sufficiently potent, their efficacy might be directly assessed in MERS patients. We identified four compounds (chloroquine, chlorpromazine, loperamide, and lopinavir) inhibiting MERS-CoV replication in the low-micromolar range (EC_{50} values 3-8 µM). Moreover, these compounds also inhibit the replication of SARS-coronavirus and human coronavirus 229E. Although their protective activity (alone or in combination) remains to be assessed in animal models, our findings may offer a starting point for treatment of patients infected with zoonotic coronaviruses like MERS-CoV. Although they may not necessarily reduce viral replication to very low levels, a moderate viral load reduction may create a window to mount a protective immune response.

Introduction

In June 2012, a previously unknown coronavirus was isolated from a patient who died from acute pneumonia and renal failure in Saudi Arabia (1, 2). Since then the virus, now known as the Middle East respiratory syndrome coronavirus (MERS-CoV; (3)), was contracted by hundreds of others in geographically distinct locations in the Middle East and evidence for limited human-to-human transmission accumulated (4). Travel-related MERS-CoV infections were reported from a variety of countries in Europe, Africa, Asia and the U.S.A., causing small local infection clusters in several cases (http://www.who.int/csr/disease/coronavirus_infections/en/). About 200 laboratory-confirmed human MERS cases were registered during the first two years of this outbreak, but recently, for reasons that are poorly understood thus far, this number has almost tripled within just two months' time (April-May 2014; (5)). This sharp increase in reported infections has enhanced concerns that we might be confronted with a repeat of the 2003 severe acute respiratory syndrome (SARS) episode, concerns aggravated by the fact that the animal reservoir for MERS-CoV remains to be identified with certainty (6-9). Furthermore, at about 30%, the current human case fatality rate is alarmingly high, even though many deaths were associated with underlying medical conditions. MERS-CoV infection in humans can cause clinical symptoms resembling SARS, such as high fever and acute pneumonia, although the two viruses were reported to use different entry receptors, dipeptidyl peptidase 4 (DPP4; (10)) and angiotensin-converting enzyme 2 (ACE2; (11)), respectively.

Coronaviruses are currently divided across four genera (alpha-, beta-, gamma-, and deltacoronaviruses; (12)). MERS-CoV was identified as a member of lineage C of the genus *Betacoronavirus* (2), which also includes coronaviruses of bat (13, 14) and hedgehog origin (6). Following the 2003 SARS epidemic, studies into the complex genome, proteome, and replication cycle of coronaviruses were intensified. Coronaviruses are enveloped viruses with a positive-sense RNA genome of unprecedented length (25 to 32 kb; (12, 15, 16)). The crystal structures of a substantial number of viral nonstructural and structural proteins were solved, and targeted drug design was performed for some of those (reviewed in (17)). Unfortunately, thus far none of these efforts resulted in antiviral drugs that were advanced beyond the preclinical phase (18). The 2003 SARS-CoV epidemic was controlled within a few months after its onset and since then the virus has not re-emerged, although close relatives continue to circulate in bat species (14). Consequently, the interest in anti-coronavirus drug development has been limited, until the emergence of MERS-CoV. Despite the modest size of this CoV outbreak thus far, the lack of effective methods to prevent or treat coronavirus infections in humans is a serious concern for the control of MERS-CoV or the next zoonotic coronavirus.

Antiviral research in the post-SARS era resulted in the identification of several compounds that may target coronavirus replication directly or modulate the immune response to coronavirus infection. For example, entry inhibitors targeting the coronavirus spike protein were developed (reviewed in (19)). In addition, several of the replicative enzymes (including both proteases and the helicase) were targeted with small-molecule inhibitors, some of which can inhibit coronavirus infection in cell culture at low-micromolar concentrations ((20-26) and reviewed in (26) and (27)). Broad spectrum antiviral agents, like the nucleoside analogue

ribavirin and interferon (IFN), were tested for their ability to inhibit SARS-CoV infection and were – to a limited extent - used for the treatment of SARS patients during the outbreak (reviewed by (28) and (29)). In the case of ribavirin, mixed results were reported from studies in different cell lines, animal models, and patients. Also the merits of treating SARS patients with immunomodulatory corticosteroids have remained a matter of debate (reviewed in (28-30)). For MERS-CoV, partial ribavirin sensitivity was observed in cell culture and in a macaque animal model, but only when using very high doses of the compound in combination with interferon-α2b (31, 32). However, in a small-scale clinical trial, this combination therapy did not benefit critically ill MERS patients (33). Nevertheless, the anti-coronavirus effects of type I IFN treatment deserve further evaluation, in particular since MERS-CoV seems to be considerably more sensitive than SARS-CoV (34, 35). Treatment with type I IFNs inhibits SARS-CoV and MERS-CoV replication in cell culture (31, 34-41) and, for example, protected macaques against SARS-CoV (36) or MERS-CoV infection (32). Based on experiments in cell culture, mycophenolic acid was recently reported to inhibit MERS-CoV infection (41, 42), and we and others showed that low-micromolar concentrations of cyclosporin A inhibit coronavirus replication (34, 43-45).

We recently described (34) a high-throughput assay for antiviral compound screening that is based on the pronounced cytopathic effect (CPE) caused by MERS-CoV infection in Vero and Huh7 cells. This assay was now further exploited to screen a library of 348 FDA-approved drugs for their potential to inhibit MERS-CoV replication. Chloroquine, chlorpromazine, loperamide, and lopinavir were found to inhibit MERS-CoV replication *in vitro* at low-micromolar concentrations. In addition, these molecules appear to be broad-spectrum coronavirus inhibitors, as they blocked the replication of human coronavirus 229E and SARS-

CoV with comparable efficacy. Since these compounds have already been approved for clinical use in humans, their anti-MERS-CoV activity merits further investigation, in particular in a small-animal model for MERS-CoV infection, of which a first example has recently been described (46).

Materials and Methods

Cell culture and virus infection - Vero, Vero E6, and Huh7 cells were cultured as described previously (34, 47). Infection of Vero and Huh7 cells with MERS-CoV (strain EMC/2012; (1)) at high or low multiplicity of infection (MOI) and SARS-CoV infection of Vero E6 cells (strain Frankfurt-1; (48)) were done as described before (34). Infection with GFP-expressing recombinant HCoV-229E (HCoV-229E-GFP; (49)) was performed in DMEM containing 8% FCS, 2 mM L-Glutamine (PAA), non-essential amino acids (PAA), and antibiotics. HCoV-229E-GFP was used to infect monolayers of Huh7 cells at an MOI of 5 as described previously (43). MERS-CoV and SARS-CoV titrations by plaque assay were performed essentially as described before (50). For titrations after high-MOI MERS-CoV infections (MOI of 1), cells were washed twice with PBS and the virus titer at 1 h post infection (p.i.) was determined to correct for the remainder of the inoculum. All work with live MERS-CoV and SARS-CoV was performed inside biosafety cabinets in biosafety level 3 facilities at Leiden University Medical Center or Erasmus Medical Center Rotterdam.

146 *Screening of an FDA-approved compound library* - A library of 348 FDA-approved drugs was
147 purchased from Selleck Chemicals (Houston, TX, USA). Compounds were stored as 10-mM stock
148 solutions in DMSO at 4°C until use. Compound stocks were diluted to a concentration of 200 or
149 60 µM in Iscove's Modified Dulbecco's Medium (Life Technologies) containing 1% FCS (PAA) and
150 antibiotics. For MERS-CoV studies, Vero cells were seeded in 96-well plates at a density of $2x10^4$
151 cells per well. After overnight incubation of the cells at 37°C, each well was given 50 µl of
152 compound dilution, which was mixed with 100 µl of EMEM medium containing 2% FCS
153 (EMEM/2%FCS) and 50 µl of MERS-CoV inoculum in EMEM/2% FCS. The MOI used was 0.005
154 and final compound concentrations tested were 15 or 50 µM. As solvent control, a subset of
155 wells was given 0.5% DMSO instead of compound dilution. At 3 days post infection (d p.i.),
156 differences in cell viability caused by virus-induced CPE and/or compound-specific side effects
157 were analyzed using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation
158 (monotetrazolium salt; MTS) Assay (Promega), as described previously (34). Cytotoxic effects of
159 compound treatment were monitored in parallel plates containing mock-infected cells, which
160 were given regular medium instead of virus inoculum.
161
162 *Compound validation* - For validation experiments, we separately re-ordered chlorpromazine
163 (CPZ; S2456; SelleckChem), lopinavir (LPV; ABT-378; SelleckChem), and loperamide (LPM;
164 S2480; SelleckChem), which were dissolved in DMSO, and chloroquine (CQ; C6628; Sigma)
165 which was dissolved in PBS. For all compounds 20-mM stock solutions were stored at -20°C as
166 aliquots for single use. To verify the antiviral effect of CQ, CPZ, LPM, and LPV on MERS-CoV
167 replication, the assay above described was repeated in 96-well plates using Huh7 cells (10^4 cells

168 seeded per well on the day before infection), and cell viability was assayed at 2 d p.i. Likewise,
169 compounds were tested for their inhibitory effect on SARS-CoV infection at 3 d p.i. (10^4 Vero E6
170 cells seeded per well, MOI 0.005). For HCoV-229E-GFP infections, 10^4 Huh7 cells were seeded
171 per well, incubated overnight, and infected at an MOI of 5. Medium containing 0 to 50 µM of a
172 compound was given 1 h before the start of infection (t=-1), and the compound remained
173 present during infection. HCoV-229E-GFP-infected Huh7 cells were fixed at 24 h p.i. and GFP
174 expression was quantified by fluorometry, as described previously (43).
175
176 *Statistical analysis* - The half-maximal effective concentration (EC_{50}) and the compound-specific
177 toxicity (50% cytotoxic concentration; CC_{50}) were calculated with GraphPad Prism 5 software
178 using the non-linear regression model. The relative efficacy of a compound in specifically
179 inhibiting viral replication (as opposed to inducing cytopathic side-effects) was defined as the
180 selectivity index (SI; calculated as CC_{50}/EC_{50}). Statistical analyses were performed using the
181 results of at least two independent experiments.
182
183
184 ## Results
185
186 **Screening for FDA-approved compounds with anti-MERS-CoV activity.** A primary library screen
187 was performed using a set of 348 FDA-approved drugs which were evaluated for their ability to
188 inhibit the replication of MERS-CoV in Vero cells (for a complete list of compounds tested, see

189 Supplemental Table S1) according to a recently published method that employs a colorimetric
190 cell viability assay to quantify virus-induced CPE (34).

191 The primary screen resulted in the identification of 11 hits that showed at least 50%
192 inhibition of virus-induced CPE in the absence of cytotoxicity (which was defined as >75%
193 viability in compound-treated mock-infected cultures). Next, these drugs, as well as the earlier
194 reported coronavirus inhibitor chloroquine (51-55), were tested over a broader concentration
195 range (2 to 62.5 μM; Supplemental Fig. 1). In this screen, compounds were considered as
196 confirmed hits when they inhibited MERS-CoV-induced CPE by >60% at non-toxic
197 concentrations (defined as >75% remaining viability in compound-treated mock-infected
198 cultures). Following this second round of testing, Cilnidipine, Fluoxetine HCl, Ivermectin,
199 Manidipine, Oxybutynin, Pyrimethamine, Rifabutinin, and Rifapentine were not further retained
200 (Supplemental Fig. 1).

201

202 **Low-micromolar concentrations of chloroquine, chlorpromazine, loperamide, and lopinavir**
203 **inhibit MERS-CoV replication.** Four compounds were selected for further validation.
204 Chloroquine (CQ) was found to inhibit MERS-CoV replication in a dose-dependent manner with
205 an EC_{50} of 3.0 μM (SI 19.4; Fig. 1A and Table 1). Interestingly, also another reported inhibitor of
206 clathrin-mediated endocytosis (56), chlorpromazine (CPZ), was found to inhibit MERS-CoV-
207 induced CPE (EC_{50} 4.9 μM; SI 4.3) with a 12-μM dose achieving complete inhibition (Fig. 1B and
208 Table 1). Loperamide (LPM), an antidiarrheal agent, inhibited MERS-CoV-induced CPE with an
209 EC_{50} of 4.8 μM (Fig. 1C and Table 1), but proved relatively toxic in Huh7 cells. An SI of 3.2 was
210 calculated and a maximum of 82% inhibition was observed at 8 μM, a concentration that was

not cytotoxic. The fourth hit was the human immunodeficiency virus-1 (HIV-1) protease inhibitor lopinavir (LPV), which was previously shown to inhibit SARS-CoV main protease activity and SARS-CoV replication *in vitro* (24). LPV inhibited MERS-CoV-induced CPE with an EC_{50} of 8.0 µM (SI 3.1; Fig. 1D and Table 1) and a maximal protective effect (89% inhibition) was observed at a dose of 12 µM. Two other MERS-CoV isolates (MERS-HCoV/KSA/UK/Eng-2/2012 and MERS-HCoV/Qatar/UK/Eng-1/2012) (57) were found to be equally sensitive to CQ, CPZ, LPM, while being somewhat less sensitive to treatment with LPV (data not shown).

CQ, CPZ, LPV, and LPM also inhibit replication of SARS-CoV and HCoV-229E. To investigate whether the MERS-CoV inhibitors identified above are potential broad-spectrum coronavirus inhibitors, we assessed their activity against two other coronaviruses: the alphacoronavirus HCoV-229E and the lineage B betacoronavirus SARS-CoV (MERS-CoV belongs to lineage C). All four compounds inhibited SARS-CoV-induced CPE in a dose-dependent manner (Fig. 2 and Table 1). For CQ, an EC_{50} value of 4.1 µM was observed (Fig. 2A), which is in line with earlier reports (51, 52). This compound did not affect the metabolism of Vero E6 cells or induce alterations in cell morphology at concentrations of up to 128 µM (CC_{50} of >128 µM; SI >31). LPM and CPZ blocked SARS-CoV CPE with comparable EC_{50} values (4.8 versus 4.9 µM; Fig. 2B-C). LPV completely blocked SARS-CoV induced CPE at 12 µM, with an EC_{50} of 8.0 µM (Fig. 2D).

Anti-HCoV-229E activity was assessed employing a GFP-expressing recombinant virus, as described previously (43, 49). All four compounds inhibited HCoV-229E-GFP replication at concentrations comparable to those needed to inhibit MERS-CoV and SARS-CoV replication (Fig. 3 and Table 1). The CQ EC_{50} value of 3.3 µM (SI of >15) for HCoV-229E-GFP was in the same

range as the previously reported concentration (10 µM) needed to significantly reduce HCoV-229E production in the human cell line L132 (53). Furthermore, CPZ, LPM, and LPV inhibited HCoV-229E-GFP replication with EC_{50} values of 2.5 µM (SI 9.4), 4.2 µM (SI 6.0) and 6.6 µM (SI 5.7), respectively.

Time-of-addition experiments suggest that CQ, CPZ, and LPM inhibit an early step in the replicative cycle whereas LPV inhibits a post-entry step. Both CQ and CPZ are known inhibitors of clathrin-mediated endocytosis and may thus inhibit MERS-CoV infection at a very early stage. To investigate this, both compounds were added to cells 1 h before (t=-1) or after (t=+1) infection (MOI of 1). Viral titers were determined at 24 h p.i. by plaque assay (Fig. 4). Virus production was not affected by CQ treatment when the compound was added at 1 h p.i. However, when added prior to infection, 16- and 32-µM concentrations of CQ induced a ~1-log and 2-log reduction in virus production, respectively (Fig. 4A). Comparable results were obtained upon CQ treatment of MERS-CoV-infected Huh7 cells (Fig. 4B). The results were less unambiguous for CPZ: addition 1 h prior to infection led to a ~2-log reduction of virus progeny titers, however, when added at 1 h p.i. a modest effect (0.5 to 1 log reduction) was observed (Fig. 4C-D), suggesting that the compound may also affect MERS-CoV infection at a post-entry stage. Treatment with 16 µM LPM in Vero cells reduced virus production by ~2 log when added prior to infection, while a 1-log reduction was observed when LPM was added at 1 h p.i. (Fig. 4E). Although this suggests a more pronounced effect early in MERS-CoV replication, this difference was not clearly observed when using Huh7 (compare Fig. 4E and 4F). Treatment with LPV from t=-1 or t=+1 h p.i. was equally effective in inhibiting MERS-CoV progeny production (2

to 3 log reduction), suggesting that LPV blocks a post-entry step in the MERS-CoV replicative cycle (Fig. 4G-H).

Discussion

The ongoing MERS-CoV outbreak has made it painfully clear that our current options for treatment of life-threatening zoonotic coronavirus infections in humans are very limited. At present, no drug is available for the treatment of any of the human or zoonotic coronaviruses (reviewed in (58)), despite the extensive research efforts triggered by the 2003 SARS outbreak (reviewed in (26, 27)). The brevity of that epidemic is a major reason why, thus far, none of the prototypic coronavirus inhibitors was advanced beyond the (early) preclinical stage. Like SARS-CoV a decade ago and MERS-CoV at present, future emerging coronaviruses will likely continue to pose a threat to global public health. Therefore, the search for broad-spectrum inhibitors that may reduce the impact of coronavirus infections in humans remains a challenging research priority. Given the time-consuming nature of antiviral drug development and registration, existing therapeutics for other conditions may constitute the only immediate treatment option in the case of emerging infectious diseases. For most of these drugs, ample experience is available with dosing in man and their safety and ADME profile is well known.

At the time of this study, a MERS-CoV infection model in (small) animals was not available. For initial antiviral testing, we therefore used our cell culture-based screening assay (34) to search for compounds that may inhibit MERS-CoV infection. We identified four FDA-approved compounds (chloroquine, chlorpromazine, loperamide and lopinavir) that inhibit the

277 *in vitro* replication of MERS-CoV at low-micromolar concentrations (Fig. 1 and Table 1). While
278 for some of these molecules the SI was limited (<10), for each of them we established at least
279 one concentration at which MERS-CoV replication was inhibited by more than 80% without a
280 detectable reduction of cell viability. The same four drugs were also found to inhibit, with
281 comparable potency, the *in vitro* replication of two other coronaviruses, i.e. HCoV-229E and
282 SARS-CoV (Fig. 2 and 3 and Table 1).

283 CQ inhibited MERS-CoV replication with an EC_{50} value of 3.0 µM (Fig. 1A) and blocked
284 infection at an early step (Fig. 5A). CQ has a tendency to accumulate in lysosomes where it
285 sequesters protons and increases the pH. In addition, it interacts with many different proteins
286 and cellular processes, resulting in the modulation of autophagy and the immune response (for
287 a review see (59)). CQ has also been reported to inhibit the replication of multiple flaviviruses,
288 influenza viruses, HIV (reviewed in (60)), Ebola virus (61), Nipah-Hendra virus (62), as well as
289 several coronaviruses, including SARS-CoV, in cell culture (51-55, 63, 64). Early reports showed
290 that high doses of CQ inhibit an early step of the replication of the coronavirus mouse hepatitis
291 virus (MHV). However, in SARS-CoV-infected BALB/c mice, systemically administered CQ did not
292 result in a significant viral load reduction in the lungs. Intranasal administration of CQ
293 (50mg/kg) resulted in a minor reduction of viral titers in the lung (65). When pregnant mice
294 were treated with CQ (at 15 mg/kg) their newborn offspring was protected against a lethal
295 challenge with HCoV-OC43 (54). Likely, the accumulation of CQ in the milk glands, resulting in
296 high drug concentrations in maternal milk, was a major factor in reaching a sufficiently high
297 plasma concentration of the drug in blood. CQ was also shown to inhibit the *in vitro* replication
298 (EC_{50} 2 µM) of the feline coronavirus infectious peritonitis virus (FIPV) (55). Treatment of

299 naturally infected cats with CQ resulted in a clinical improvement, which was however not
300 attributed to a direct antiviral effect and likely due to the immunomodulatory properties of CQ.
301 These results highlight that, e.g. drug delivery route, virus strain used, and drug dosage might
302 influence the outcome in animal models. In BALB/c mice steady-state plasma concentrations of
303 8 µM were observed following repeated administration of CQ at 90 mg/kg (61), which is above
304 the EC_{50} of CQ for inhibition of MERS-CoV-induced CPE in this study. Plasma levels of 9 µM were
305 observed in humans following CQ treatment with 8 mg/kg/day for three consecutive days (66).
306 The second FDA-approved drug found to block MERS-CoV infection was CPZ, the first
307 antipsychotic drug developed for treatment of schizophrenia (67). CPZ affects the assembly of
308 clathrin-coated pits at the plasma membrane (56) and has been reported to inhibit the
309 replication of alphaviruses (68), hepatitis C virus (69), and the coronaviruses SARS-CoV (70),
310 infectious bronchitis virus (71) and MHV-2 (72). Our time-of-addition studies, however, suggest
311 that CPZ inhibits MERS-CoV replication at both an early and a post-entry stage, implying that an
312 effect on clathrin-mediated endocytosis is unlikely to be the sole antiviral mechanism (Fig. 4C-
313 D). Plasma concentrations of CPZ in patients treated for psychotic disorders range between 0.3
314 and 3 µM (73), which is somewhat below the observed EC_{50} values observed here (which range
315 between 2 and 9 µM).
316 The replication of MERS-CoV *in vitro* was also inhibited by LPM, an anti-diarrheal opioid-
317 receptor agonist that reduces intestinal motility (reviewed in (74)). LPM also inhibits the
318 replication of two other coronaviruses at low-micromolar concentrations (4 to 6 µM). Upon oral
319 or intravenous administration, the molecule rapidly concentrates in the small intestine. Less
320 than 1% of orally taken LPM is absorbed from the gut lumen and its tendency to concentrate at

the site of action is the probable basis for its anti-diarrheal effect (75). This same property would very much limit systemic use for the treatment of respiratory coronavirus infections, although administration in the form of an aerosol might be explored. In the veterinary field, it would be interesting to test whether the compound has the potential to inhibit enteric coronaviruses such as the porcine transmissible gastroenteritis coronavirus.

Finally, the HIV-1 protease inhibitor (PI) LPV was shown to inhibit MERS-CoV replication with EC_{50} values of about 8 µM, which is in the range of the LPV plasma concentrations (8-24 µM) that have been observed in AIDS patients (76). LPV was previously shown to block the SARS-CoV main protease (M^{pro}) (24). This is somehow unexpected since the retro- and coronavirus proteases belong to different protease families (the aspartic and chymotrypsin-like protease families, respectively). Since MERS-CoV and SARS-CoV are relatively closely related, LPV may also target the M^{pro} of MERS-CoV. However, several anti-HIV PI's are also known to influence intracellular pathways leading to side effects in patients undergoing highly active anti-retroviral therapy, including lipodystrophy and insulin resistance (77). The exact cellular targets of these PI's have not yet been identified and most likely multiple pathways are involved. It remains to be investigated if the effect of LPV on these intracellular pathways is associated with the anti-CoV activity found here. Interestingly no selective anti-CoV activity was found for two other HIV PI's in the compound library (Atazanavir and Ritonavir - see supplemental data set S1). During the SARS outbreak, treatment with LPV, in combination with ritonavir, was explored with some success in non-randomized clinical trials (for reviews, see (78, 79)).

The efficacy of the most promising compounds identified in this study, CQ and LPV, should now be evaluated in (small-)animal models for MERS-CoV infection, which are still in

development. In a non-human primate model (macaques), only mild clinical signs developed, in contrast to the frequently severe clinical outcome in humans (80, 81). Unfortunately, Syrian hamsters (82), BALB/c mice (83), and ferrets (84) were found to resist MERS-CoV infection. A very recent study (46) reported that mice can be rendered susceptible to MERS-CoV infection by prior transduction with a recombinant adenovirus that expresses human DPP4, a documented receptor for MERS-CoV entry (10). Subsequent MERS-CoV infection resulted in severe pneumonia and high MERS-CoV titers in the lungs (46). Despite some practical and conceptual limitations, this model may provide a useful starting point for further evaluation of inhibitors of MERS-CoV infection.

In 2003, the ~10% mortality rate among SARS patients was one of the major reasons for the worldwide public unrest caused by the emergence of SARS-CoV. Clearly, and despite the recent sharp increase in number of registered cases (5), the course of the MERS-CoV outbreak has been quite different thus far. Although only 550-600 laboratory-confirmed cases have been registered in the two years that have passed since the first documented human infections, in particular the ~30% mortality rate within this group remains a grave concern. In this context, efficacious anti-coronavirus drugs, administered alone or in combination, can constitute an important first line of defense. It typically takes over 10 years to develop a newly discovered molecule and obtain approval for clinical use. To the best of our knowledge, there are currently no potent and selective coronavirus inhibitors in (early or advanced) preclinical development. Hence, drugs that have been registered for the treatment of other conditions and that also inhibit MERS-CoV replication might be used (off-label) in an attempt to save the life of MERS patients. A combination of two or more of such drugs may cause a modest reduction in viral

load, which might aid to control viral replication, slow down the course of infection and allow the immune system to mount a protective response. In an accompanying paper, CQ and CPZ were identified as inhibitors of the MERS-CoV as well (Dyall *et al.* 2014). Follow-up studies will include in-depth mechanism of action studies, including resistance development of MERS-CoV against the compounds identified. Furthermore, the efficacy of combinations of two or more of these drugs will be explored, also in combination with interferon. In particular CQ and LPV may constitute valuable candidates for further testing in animal models or direct off-label use, since the concentrations needed to inhibit viral replication in cell culture are in the range of the concentrations that can be achieved in human plasma.

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Figure legends

Figure 1. Low-micromolar amounts of chloroquine, chlorpromazine, loperamide, and lopinavir inhibit MERS-CoV-induced cytopathology.

Huh7 cells in 96-well plates were infected with MERS-CoV isolate EMC/2012 (MOI 0.005) in the presence of A) 0-32 µM CQ, B) 0-16 µM CPZ, C) 0-8 µM LPM, or D) 0-20 µM LPV. Cells were incubated for 2 days and cell viability was monitored using an MTS assay. In addition, the potential toxicity of compound treatment only was monitored in parallel mock-infected Huh7 cell cultures. Graphs show the results (average and SD) of a representative experiment that was performed in quadruplo. All experiments were repeated at least twice. For each compound, the calculated EC_{50}, CC_{50}, and SI values are given.

Figure 2. Low-micromolar amounts of chloroquine, chlorpromazine, loperamide, and lopinavir inhibit SARS-CoV-induced cytopathology.

Vero E6 cells in 96-well plates were infected with SARS-CoV isolate Frankfurt-1 (MOI 0.005) in the presence of A) 0-32 µM CQ, B) 0-16 µM CPZ, C) 0-32 µM LPM, or D) 0-32 µM LPV, given at t=+1 h p.i. Cells were incubated for 3 days and viability was monitored using an MTS assay. In parallel, potential compound cytotoxicity was monitored in mock-infected Vero E6 cells. Graphs show the results (average and SD) of a representative experiment that was performed in quadruplicate. All experiments were repeated at least twice. For each compound, the EC_{50}, CC_{50}, and SI values are given.

Figure 3. HCoV-229E-GFP replication is inhibited by low-micromolar amounts of chloroquine, chlorpromazine, loperamide, and lopinavir .

Huh7 cells in 96-well plates were infected with HCoV-229E-GFP (MOI 5) in the presence of 0-50 µM A) CQ, B) CPZ, C) LPM, or D) LPV. Compounds were given at t=-1 and remained present during infection. Cells were fixed at 24 h p.i. and GFP reporter gene expression was measured and normalized to the signal in control cells (100 %; black bars), which were treated with the solvent used for the various compounds. The effect of compound treatment on the viability of mock-infected Huh7 cells, compared with solvent-treated control cells, was determined by using an MTS assay (grey lines). Graphs show the results (average and SD) of a representative quadruplicate experiment. All experiments were repeated at least twice; n.d., not detected.

Figure 4. Chloroquine, chlorpromazine, loperamide, and lopinavir affect various stages of the MERS-CoV replication cycle.

Vero (A, C, E, G) and Huh7 cells (B, D, F, H) were infected with MERS-CoV isolate EMC/2012 (MOI 1). At t=-1 or t=+1, the indicated concentrations of CQ (A, B), CPZ (C, D), LPM (E, F), and LPV (G, H) were given and virus titers in the culture supernatant (n=4, average and SD are shown) were determined at 24 h p.i. using plaque assays; n.d., not detected.

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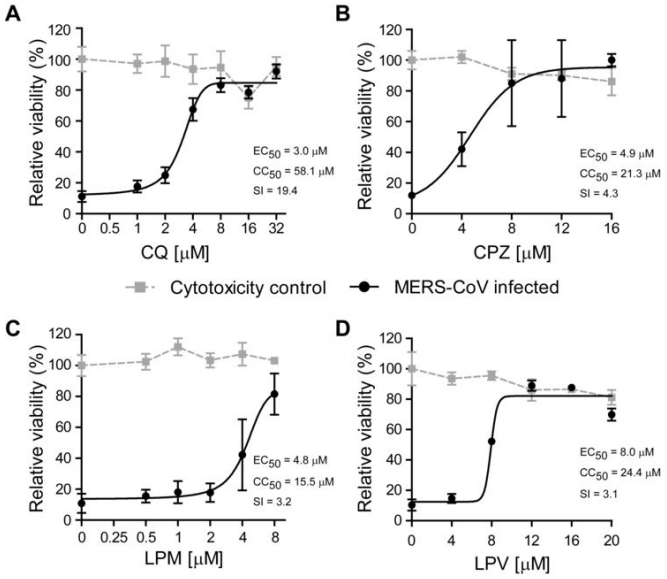

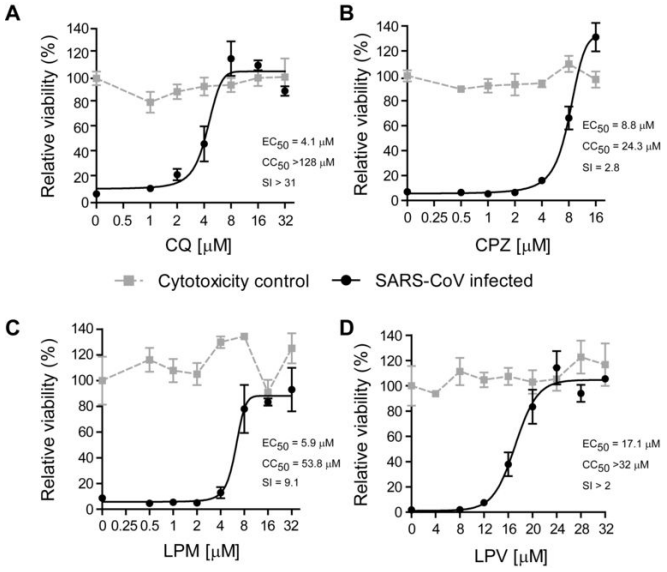

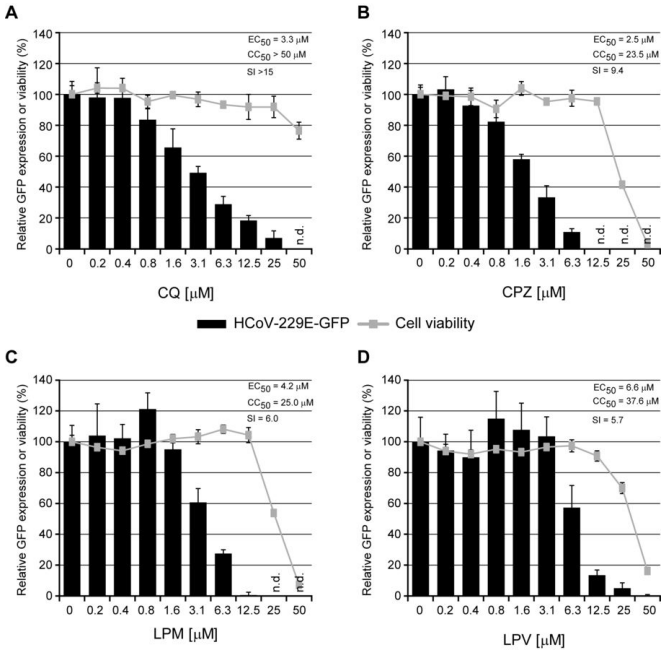

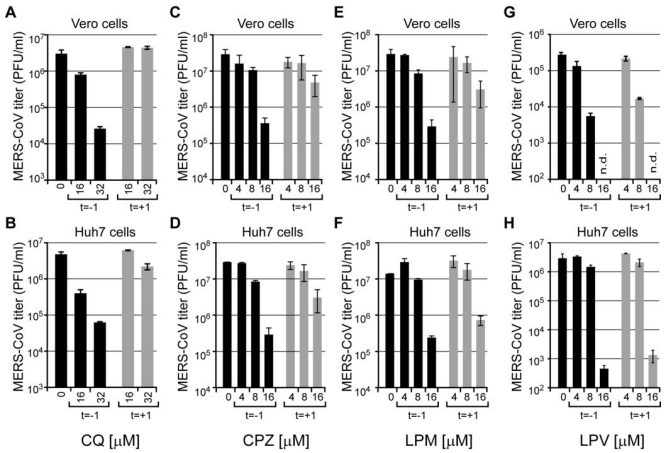

Table 1. Antiviral activity of chloroquine, chlorpromazine, loperamide and lopinavir against MERS-CoV, SARS-CoV and HCoV-229E-GFP

	MERS-CoV			SARS-CoV			HCoV-229E-GFP		
Compound	EC_{50}[a] (µM)	CC_{50}[a] (µM)	SI	EC_{50} (µM)	CC_{50} (µM)	SI	EC_{50} (µM)	CC_{50} (µM)	SI
Chloroquine	3.0 (± 1.1)	58.1 (± 1.1)	19.4	4.1 (± 1.0)	>128	>31	3.3 (± 1.2)	>50	>15
Chlorpromazine	4.9 (± 1.2)	21.3 (± 1.0)	4.3	8.8 (± 1.0)	24.3 (± 1.1)	2.8	2.5 (± 1.0)	23.5 (± 1.0)	9.4
Loperamide	4.8 (± 1.5)	15.5 (± 1.0)	3.2	5.9 (± 1.1)	53.8 (± 1.7)	9.1	4.0 (± 1.1)	25.9 (± 1.0)	6.0
Lopinavir	8.0 (± 1.5)	24.4 (± 1.0)	3.1	17.1 (± 1.0)	>32	>2	6.6 (± 1.1)	37.6 (± 1.3)	5.7

[a] EC_{50} and CC_{50} values are means (± SD) from a representative experiment (n=4) that was repeated at least twice. Antiviral activity was determined in Huh7 cells (for MERS-CoV and HCoV-229E-GFP) or VeroE6 cells (for SARS-CoV). See text for more details.