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Repurposing of clinically developed drugs for treatment of Middle East Respiratory Coronavirus Infection

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Running Title: Pharmaceuticals with activity against MERS-CoV

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ABSTRACT (209)

Outbreaks of emerging infections present the unique challenge of trying to select appropriate pharmacologic treatments in the clinic with little time available for drug testing and development. Typically clinicians are left with general supportive care and often untested convalescent plasma as available treatment options. Repurposing of approved pharmaceutical drugs for new indications presents an attractive alternative to clinicians, researchers, public health agencies, drug developers and funding agencies. Given development times and manufacturing requirements for new products, repurposing of existing drugs is likely the only solution for outbreaks due to emerging viruses. In the studies described here, a library of 290 compounds was screened for antiviral activity against Middle Eastern respiratory syndrome-coronavirus (MERS-CoV) and severe acute respiratory syndrome-coronavirus (SARS-CoV). Selection of compounds for inclusion in the library was dependent on current or previous FDA-approval or advanced clinical development. Some drugs were included that had a well-defined cellular pathway as target. In total, 27 compounds with activity against both MERS-CoV and SARS-CoV were identified. The compounds belong to thirteen different classes of pharmaceuticals including; inhibitors of estrogen receptors used for cancer treatment and inhibitors of dopamine receptor used as antipsychotics. The drugs identified in these screens provide new targets for *in vivo* studies as well as incorporation into ongoing clinical studies.

INTRODUCTION

Middle Eastern respiratory syndrome-coronavirus (MERS-CoV) is an emerging virus, and to date no antiviral or therapeutic has been approved for treating patients. Since September 2012, 206 patients, including 86 deaths, have been attributed to infection with MERS-CoV.

Currently, supportive care remains the only available treatment option. As the number of cases continues to rise and the geographic range of the virus increases, there is a growing urgency for candidate interventions.

Prior to 2002, coronaviruses were not considered to be significant human pathogens. Other human coronaviruses such as HCoV-229E and HCoV-OC43 resulted in only mild respiratory infections in healthy adults. This perception was shattered in 2002, when severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in the Guangdong Province, China. This virus rapidly spread to 29 different countries, resulting in 8273 confirmed cases, and 775 (9%) deaths (1). While the SARS-CoV predominantly impacted South-East Asia, with significant outbreaks throughout China, Hong Kong, Taiwan, Singapore and Vietnam, the virus was carried outside of the region. Importation of the virus into Canada resulted in 251 confirmed cases and 44 deaths (1). Implementation of infection control measures was able to bring the epidemic to an end in 2003.

In 2012, a novel coronavirus Middle Eastern respiratory syndrome coronavirus (MERS-CoV) was detected in a patient with severe respiratory disease in the kingdom of Saudi Arabia. To date, 206 laboratory-confirmed cases of MERS-CoV infection were reported, including 86 deaths, across nine countries (WHO Disease outbreak news, January 9, 2014; http://who.int/csr/don/20140109/en). Clinical features of MERS-CoV infection in humans range from asymptomatic to very severe pneumonia with the potential development of acute respiratory distress syndrome, septic shock and multi-organ failure resulting in death. Since the first case of MERS-CoV infection was reported in September 2012 and the virus was isolated, significant progress has been made toward understanding the epidemiology, ecology, and biology of the virus (2). Several assays for the detection of acute infection with MERS-CoV by

real-time RT-PCR have been developed and are now in widespread use (3). Over thirty whole or partial genome sequences from different MERS-CoV infected patients have been posted to Genbank and phylogenetic trees have been published by several groups (3). Dipeptidyl peptidase 4 (also known as CD26) has been identified as the functional cellular receptor for MERS-CoV (4, 5). Ecological studies have suggested that the virus is of animal origin and is most closely related to coronaviruses found in a number of species of bats with MERS-CoV viral sequences now found in camels in Saudi Arabia (6-9). Interestingly, a subset of MERS-CoV cases reported close contact with camels. Camels may constitute an intermediate animal host since camel serum samples collected in 2003 and 2013 had antibodies to MERS-CoV indicating that the MERS-CoV circulates in camels (10-12). The recent development of an animal model for MERS-CoV with adenovirus vectored human DPP4 in mice will now allow for further pathogenesis studies with various MERS-CoV strains (13).

The emergences of both SARS-CoV and MERS-CoV have demonstrated the importance of coronaviruses as potential emerging human pathogens and highlighted the necessity and value of effective communications within the international science community to facilitate rapid responses to emerging infectious diseases. In July 2013, the International Severe Acute Respiratory & Emerging Infection Consortium (ISARIC) compiled a list of drugs available to clinicians for treatment of MERS-CoV infection based on recent experience in treating SARS-CoV infection and pandemic influenza (14). The most promising and clinically available drugs were ribavirin and interferon, or a combination of the two since they demonstrated efficacy in an *in vivo* model for MERS-CoV infection (15, 16). This combination has failed to demonstrate benefit in the small number of severely ill MERS-CoV patients treated (17). Outside of ribavirin and IFN, the ISARIC recommendations had few alternatives for treating clinicians. It should be

92 noted that these recommendations are meant to be fluid and based on the best available
93 information at the time. As new data becomes available these recommendations may change.
94 Recently, we have shown mycophenolic acid (MPA) and IFN-β to be highly effective against
95 MERS-CoV infection *in vitro*. Interestingly, the activity of MPA was specific to MERS-CoV
96 with little activity observed against SARS-CoV infection, (18, 19).

97 In the work described here, we took the approach of screening a unique panel of both
98 approved drugs and drugs with a well-defined cellular pathway for *in vitro* efficacy against
99 MERS-CoV infection. This subset was identified previously as having antiviral activity against
100 a series of other viruses (P. J. Glass, personal communication). A subset of drugs was also
101 screened against SARS-CoV with the objective to identify drugs with broad activity against
102 coronaviruses in preparedness for potential future emerging coronaviruses. We utilized this
103 approach with the rationale that drugs that have been approved for use in humans would be more
104 readily accepted as potential therapeutic options for MERS-CoV infection if shown to have anti-
105 viral activity. The screening of approved drugs to identify therapeutics for drug repurposing is a
106 valid approach and several approved drugs have been identified with activity against many viral
107 diseases (20-22). Here we found that 66 of the screened drugs were effective at inhibiting either
108 MERS-CoV or SARS-CoV infection *in vitro* and 27 of these compounds were effective against
109 both MERS-CoV and SARS-CoV. These data demonstrate the efficiency of screening approved
110 or clinically developed drugs for identification of potential therapeutic options for emerging viral
111 diseases and also provide an expedited approach for supporting off-label use of approved
112 therapeutics.

113

MATERIALS and METHODS

Cell lines and virus.

Vero E6 cell line (ATCC# 1568, Manassas, VA) was maintained at the Integrated Research Facility (IRF, Frederick, MD) in Dulbecco's modified Eagle's medium (DMEM; Corning, Manassas, VA)) plus 10% fetal bovine serum (FBS). The Jordan strain of MERS-CoV (GenBank accession no. KC776174.1, MERS-CoV- Hu/Jordan-N3/2012 (23)), kindly provided by Drs. Kanta Subbarao (National Institutes of Health, Bethesda, MD) and Gabriel Defang (Naval Medical Research Unit-3, Cairo, EG) was amplified in Vero E6 cells at a multiplicity of infection (m.o.i.) of 0.01. On day 4 after infection, when the cytopathic effect (CPE) was visible, virus-containing supernatants were collected and clarified by centrifugation. MERS-CoV was titered on Vero E6 cells by plaque assay. All procedures using live MERS-CoV were performed at biosafety level 3 conditions at the IRF.

Vero E6 cell line (ATCC# 1568, Manassas, VA) at University of Maryland, Baltimore (UMB), was maintained in minimal essential medium (MEM; Corning, Manassas, VA) supplemented with 10% FBS (SAFC, Bioscience, Lenexa, KS), 1% penicillin/streptomycin (Gemini Bio-products, West Sacramento, CA) and 1% L-glutamine (Life Technologies, Grand Island, NY). Mouse adapted SARS-CoV (MA15) has been described previously (24). SARS-CoV was amplified in Vero E6 cells for 2 days, when the CPE was visible. SARS-CoV containing supernatants were collected and clarified by centrifugation. SARS-CoV was titered on Vero E6 cells by plaque assay. All procedures using live SARS-CoV were performed at biosafety level 3 conditions at UMB.

Reagents.

Chlorpromazine hydrochloride (CAS#69-09-0) was purchased from Sigma-Aldrich, St. Louis, MO. Imatinib mesylate (CAS# 220127-57-1), Gemcitabine hydrochloride (CAS#122111-03-9) and Toremifene citrate (CAS #89778-27-8) were purchased from Sequoia Research Products, Pangbourne, UK. Triflupromazine hydrochloride (CAS# 1098-60-8) was purchased from U.S. Pharmacopeia, Rockville, MD. Dasatinib (CAS# 302962-49-8) was purchased from Toronto Research Chemicals Inc., Toronto, CA. DMSO was used as solvent for the high throughput screening assay described below.

Drug library and compound plate preparation.

A library of approved drugs including some drugs with a well-defined cellular target was assembled, and has been previously described (25). A subset of 290 compounds was selected for screening against MERS-CoV and SARS-CoV based on the antiviral activity observed in screens against other RNA viruses (21). For the MERS-CoV and SARS-CoV screens, compounds were added to compound plates using an acoustic compound dispenser (Echo 555, Labcyte, Sunnyvale, CA). The compounds were shot in nl volumes directly on to 96-well plates from master stock solutions. Following addition of compound, 200 µl of DMEM media was added to plates and plates were frozen at -80 ^{0}C for a minimum of 24 h prior to shipment to the IRF and UMB investigators. Compound plates were thawed prior to the addition of compound to the infectivity assays described below at the IRF and UMB. For the MERS screen, compounds were plated in 200 µl of media at 4X the final concentrations such that the addition of 50 µL, to assay plates resulted in the appropriate final concentration (200 µl final assay volume). For the SARS screens, drugs were plated in 200 µl of media at 2X the final concentrations such that the addition of 50 µl resulted in the appropriate final concentration (100 µl final assay volume). All drug plates were blinded to those performing the infectivity assays.

Cell-based ELISA screen for MERS-CoV antiviral agents.

Vero E6 cells were seeded at 40,000 cells in 100 µl DMEM plus 10% FBS per well in black, opaque or clear bottom 96 well-plates. After 24 h, test drugs were transferred from compound plates and added to 3 cell plates in 50 µl using the 96-well liquidator (Rainin Instrument, Oakland, CA). DMSO concentration was kept at 0.05% or lower. Duplicate Vero E6 seeded plates were used for detecting inhibition of MERS-CoV, and one plate was used for determining cytotoxicity of compounds. For infection, duplicate plates pre-treated with drugs for 1 hour before the plates were transferred into the containment laboratory to add MERS-CoV strain -Hu/Jordan-N3/2012- at an m.o.i. of 0.1 in 50 µl of DMEM plus 10% FBS. After 48 h, plates were fixed with 10% neutral-buffered formalin and removed from biocontainment. MERS-CoV infection was detected with a rabbit polyclonal antibody to the HCoV-EMC/2012 Spike Protein (Sino Biological Inc., Beijing, CN, #40069-RP02) followed by staining with Alexa Fluor® 594 goat anti-rabbit IgG (H+L) antibody (Life Technologies, Grand Island, NY). Fluorescence was quantified on a plate reader (Infinite® M1000 Pro, Tecan US, Morrisville, NC) with excitation wavelength of 590 nm and emission wavelength at 617 nm. The drugs with >50% inhibition of Spike expression and <30% toxicity were then screened with SARS-CoV as described below in the methods.

To detect cellular toxicity of drugs in the MERS-CoV screen, one of the three plates that received the test drugs was used to evaluate cytotoxicity of drugs and was not infected with virus. At 48 h after drug addition, cell plates were analyzed using the CellTiter Glo luminescent cell viability assay kit according to the manufacturer's directions (Promega, Madison, WI), and luminescence was read on the Infinite® M1000 Pro plate reader.

SARS-CoV cytopathic effect (CPE) inhibition assay.

For the SARS-CoV screen, 174 of the 290 drugs were screened against SARS-CoV, including all the hits that blocked MERS-CoV (72 drugs). A different assay was used to screen for inhibition of SARS-CoV replication than was used for MERS-CoV replication due to different equipment for analysis at UMB and IRF/NIAID. For the SARS-CoV inhibitor screen at UMB, Duplicate Vero E6 cells were seeded into white opaque 96-well plates (Corning Costar) at $1x10^4$ cells per well and cultured overnight at 37°C. Cells were treated with the drugs for 2 h at 37°C and then mock infected or infected with SARS-CoV (MA15) at an m.o.i. of 1. Cells were cultured at 37°C for 48 hours and then analyzed for cell survival using the CellTiterGlo® luminescent cell viability assay (Promega, Madison, WI) according to the manufacturer's instructions and read on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). A third identical drug compound plate was used to assess drug toxicity in the absence of SARS-CoV using the same Cell-Titer Glo assay (Promega) as above with cells incubated in the presence of the drug for 48 hours before being assayed.

Data analysis.

For the MERS-CoV screen a minimum of four replicates were performed on two separate days. For the SARS-CoV screen a minimum of two replicates were performed on two separate days. Outlier data points were defined as a value > median+3σ and were excluded from calculations.

For MERS screening, raw phenotype measurements **T** from each treated well were converted to normalized fractional inhibition **I = 1-T/V** relative to the median **V** of vehicle-treated wells arranged around the plate. For SARS screening with a CPE endpoint, the calculation used to measure the antiviral activity of the compounds was the Percent Normal. The Percent Normal monitors the reduction in cytolysis of cells due to the presence of compound

treatment. Percent Normal = (**T**-**V**)/(**N**-**V**). **T** represents cells infected with SARS and treated with compound. **V** represents cells infected with SARS but vehicle treated. **N** represents the normal control where cells are neither infected nor treated with compound.

After normalization, average activity values were calculated between replicate measurements at the same treatment doses along with σ_1, the accompanying standard error estimates. Drug response curves were represented by a logistic sigmoidal function with a maximal effect level (A_{max}), the concentration at half-maximal activity of the compound (EC_{50}), and a Hill coefficient representing the sigmoidal transition. We used the fitted curve parameters to calculate the concentration (EC_{50}) at which the drug response reached an absolute inhibition of 50%, limited to the maximum tested concentration for inactive compounds.

Compounds were considered active if the antiviral activity observed was > 50 % **I** (or Percent Normal) with no or low corresponding cytoxicity (<30% **I**).

RESULTS

Overview of screening process. A primary screen of 290 compounds containing both approved drugs and developmental drugs with defined cellular target was performed with three-point dose response curves to identify compounds with activity against MERS-CoV using a cell-based ELISA assay (Fig. 1). The analysis of the raw screening data indicated that 72 compounds were active against MERS-CoV (>50% inhibition) with no or low cytotoxicity (< 30% toxicity). In the secondary screen, the 72 compounds were plated at eight doses for confirmation of antiviral activity against MERS-CoV as well as to determine EC_{50} values in the MERS-ELISA assay. The 72 compounds were also evaluated for their antiviral activity against SARS-CoV using a cytopathic effect (CPE) inhibition assay. An independent screen using a subset of 102

compounds against SARS-CoV infection identified 6 unique compounds with activity against SARS-CoV.

Overview of drugs active against SARS-CoV, MERS-CoV or both. Analysis of data from all screening activities resulted in a list of 66 compounds that were active against SARS-CoV, MERS-CoV, or both. In summary, we found six drugs that were active against SARS-CoV only, 33 drugs that were active against MERS-CoV only, and 27 drugs that were active against both SARS-CoV and MERS-CoV. These drugs were grouped based upon their recognized mechanism of action into thirteen different therapeutic classes that were active against SARS-CoV, MERS-CoV or both (Table 1.). The high hit rates of 21% (60 out of 290) for MERS-CoV inhibitors and 19% (33 out of 174) for SARS-CoV inhibitors can be explained by the fact that the library was enriched for compounds that have shown antiviral activity against other viruses (P. J. Glass et al. personal communication).

Pharmaceuticals that inhibited both coronaviruses included neurotransmitter inhibitors, estrogen receptor antagonists, kinase signaling inhibitors, inhibitors of lipid or sterol metabolism, protein processing inhibitors, and inhibitors of DNA synthesis/repair. Anti-parasitics or anti-bacterials were two classes of pharmaceuticals in which function was not obviously linked to coronaviruses, or viruses in general, but showed antiviral activity against SARS-CoV and MERS-CoV. We also found a cathepsin inhibitor, E-64-D, blocked both SARS-CoV and MERS-CoV, though this was not surprising since it is known that cathepsins are important for the fusion step during virus entry of coronaviruses (26).

Interestingly, classes of drugs were discovered that seem to inhibit only SARS-CoV or MERS-CoV, but not both. Though we only identified a small number of SARS-CoV only

inhibitors, they are primarily anti-inflammatories, which interfere with cell signaling associated with the immune response to virus infection. MERS-CoV was specifically blocked by inhibitors of ion transport, the cytoskeleton (specifically tubulin), and apoptosis.

Specific drugs. Twenty seven specific drugs inhibited both MERS-CoV and SARS-CoV infection (Table 2, Fig. S1-S2). We present a selection of drugs in Fig. 2-4 that are particularly interesting because they have similar structures, similar mechanisms of action or have been tested against other viruses. Data on antiviral activity and cytotoxicity for the remaining compounds that inhibit MERS-CoV and SARS-CoV are provided in Supplemental Material.

In total, 16 neurotransmitter antagonists were found to have activity against one or both of the coronaviruses (Table 1). Eleven of these antagonists were active against both MERS-CoV and SARS-CoV, two against only SARS-CoV and three against only MERS-CoV. Two of the neurotransmitter inhibitors that inhibit both MERS-CoV and SARS-CoV are chlorpromazine hydrochloride and triflupromazine hydrochloride (Table 2). Both of these drugs inhibit the dopamine receptor and have similar chemical structures (Fig. 2A) sharing the same core structure, with the only difference being the nature of the halide group: chlorpromazine hydrochloride has a single chlorine, while triflupromazine hydrochloride has three fluorine surrounding a carbon. Both chlorpromazine hydrochloride and triflupromazine hydrochloride strongly inhibit MERS-CoV and SARS-CoV, with micromolar EC_{50}s (range 5.76 μM to 12.9 μM) and low toxicity (Fig. 2B and 2C). No significant difference was observed between the effects of these drugs on MERS-CoV and SARS-CoV, for example triflupromazine hydrochloride inhibits both MERS-CoV and SARS-CoV with approximately the same EC_{50} (5.76 μM and 6.39 μM respectively, Fig. 2C). The similarity in the structure of chlorpromazine

hydrochloride and trifluprom azine hydrochloride would suggest that they inhibit MERS-CoV and SARS-CoV using the same mechanism of action. Chlorpromazine hydrochloride has been used to study virus entry by clathrin-mediated endocytosis of several viruses including West Nile virus (WNV) and influenza virus (27-31). SARS-CoV also utilizes the clathrin-mediated endocytosis pathway for entry (32) suggesting that this drug may act similarly on MERS-CoV and SARS-CoV and have potential as a broad spectrum coronavirus inhibitor.

We identified three inhibitors of the kinase signaling pathway, two (imatinib mesylate and dasatinib) that are active against both MERS-CoV and SARS-CoV, and one (nilotinib) that inhibits SARS-CoV only. Imatinib mesylate and dasatinib are known inhibitors of the Abelson murine leukemia viral oncogene homolog 1 (ABL1) pathway. The ABL1 pathway is a signaling pathway involved in cell differentiation, cell adhesion and the cellular stress response. Over-activation of the ABL1 pathway can lead to chronic myelogenous leukemia. Both imatinib mesylate and dasatinib were developed and approved as inhibitors of this pathway for treating human cancers including chronic myelogenous leukemia (33, 34). Both imatinib mesylate and dasatinib inhibit SARS-CoV and MERS-CoV with micromolar EC_{50}s (range 2.1 to 17.6 μM) and low toxicity (Fig. 3A and 3B). SARS-CoV does appear to be more sensitive to both ABL1 inhibitors; for example, the EC_{50} of dasatinib against SARS-CoV is 2.1 μM, whereas for MERS-CoV the EC_{50} is 5.4 μM (Fig. 3A). A third ABL1 inhibitor, nilotinib, was also used in this study. Nilotinib is able to inhibit SARS-CoV with a micromolar EC_{50} and low toxicity (data not shown), but does not significantly inhibit MERS-CoV, with the maximum inhibition of MERS-CoV being 39% at the highest dose tested (data not shown). However, the fact that nilotinib is able to inhibit SARS-CoV and partially inhibit MERS-CoV further points to the importance of the ABL1 pathway in coronavirus replication. Imatinib mesylate has been shown to block egress

295 of Ebola virus and of poxviruses and entry of coxsackie virus (20, 35, 36). These data suggest
296 that the ABL1 pathway may important for replication of many different virus families and,
297 therefore, inhibitors of this pathway have the potential to be broad-spectrum antivirals.

298 Gemcitabine hydrochloride is a deoxycytidine analog that inhibits DNA synthesis and
299 repair. Gemcitabine hydrochloride inhibits both MERS-CoV and SARS-CoV with micromolar
300 EC_{50}s (1.2 μM and 4.9 μM respectively) and low toxicity (Fig. 4A). Interestingly, we identified
301 four DNA synthesis inhibitors that were active against at least one coronavirus (Table 1),
302 suggesting that these drugs have potential as antivirals for coronaviruses. These data also
303 demonstrate the importance of screening large drug sets, rather than targeted screens of
304 suspected inhibitors, as it may not have been immediately obvious that a DNA synthesis
305 inhibitor would have any effect on the replication of an RNA virus.

306 Toremifene citrate is an estrogen receptor 1 antagonist that inhibits both MERS-CoV and
307 SARS-CoV with micromolar EC_{50}s (12.9 μM and 11.97 μM respectively) and low toxicity (Fig.
308 4B). Toremifene citrate has been tested against several filoviruses and was shown to block
309 filovirus entry (21, 37). In the screens described here, there were five estrogen receptor inhibitors
310 that blocked at least one coronavirus (Table 1) and two of these blocked both MERS-CoV and
311 SARS-CoV with micromolar EC_{50}s (Table 2) and low toxicity. While the antiviral mechanism is
312 unknown for MERS-CoV and SARS-CoV, these results suggest that estrogen receptor 1
313 inhibitors have the potential for broad-spectrum antiviral activity.

314

DISCUSSION

In order prevent the emergence of a novel virus from growing into a pandemic or established human pathogen it is critical that public health officials and clinicians can diagnose the infection, control its spread and treat those afflicted. First and foremost, we need more countermeasures that can be used for the early phase of an epidemic to provide an immediate treatment response while more appropriate therapies are being developed. Given the time and costs associated with licensure of novel therapeutics, one feasible and rapid response is through repurposing of existing clinically developed products. Repurposing of approved drugs has several advantages including known safety/tolerability profiles, availability, lower cost, and familiarity of clinicians in working with these drugs. Supplying the international community with robust sets of *in vitro* and *in vivo* data on potential drugs for treatment of emerging viral diseases continues to be a high priority, as it will allow clinicians to make educated decisions on clinically available drugs for testing in intervention trials.

Here we report that screening of a library of 290 drugs either clinically developed or with a well-defined cellular pathway identified 27 compounds with activity against MERS-CoV and SARS-CoV, 33 compounds with activity against MERS-CoV, and six compounds with activity against SARS-CoV alone. Overall, we have demonstrated that libraries of approved compounds can be used to screen for inhibitors of viruses and have identified a number of potential antivirals with activity against coronaviruses.

The drugs identified here belong to 13 different classes of pharmaceutical drugs. For two of the classes, kinase signaling inhibitors and estrogen receptor antagonists, previous work with other viruses has given insight into how these drugs may affect viral infections. Three tyrosine kinase inhibitors, imatinib mesylate (Gleevec), nilotinib (Tasigna) and dasatinib, were developed

to treat human cancers and were later shown to have activity against several viruses including poxviruses and Ebola virus (20, 36). Mechanism of action studies revealed that Abl1 tyrosine kinase regulates budding or release of poxviruses and Ebola virus, demonstrating that the c-Abl1 kinase signaling pathways play an important role in the egress of these viruses. Here we show that kinase signaling may also be important for replication of two members of the *Coronaviridae* family. Imatinib mesylate and dasatinib inhibit MERS-CoV and SARS-CoV, while nilotinib inhibits only SARS-CoV. The step in viral replication that these kinases are involved will need to be investigated further. *In vivo* studies performed in the mouse model of vaccinia virus infection showed that imatinib mesylate was more effective than dasatinib in blocking dissemination of the virus and this was attributed to the immunosuppressive effect of dasatinib (36). Nevertheless, dasatinib may have value for treating coronaviral infections if a dosing regimen can be defined that minimizes immunotoxicity while still blocking viral replication. Imatinib mesylate (Gleevec) and nilotinib (Tasigna) are FDA-approved oral cancer medicines and are considered promising candidates for development into antivirals against poxviruses (38).

Estrogen receptor modulators represent another class of FDA-approved drugs that have potential as antivirals in the clinic. Toremifene citrate, which we have shown blocks both MERS-CoV and SARS-CoV, has previously been shown to inhibit filoviruses (21). Mechanism of action studies showed that the drug acts at a late step of virus entry and may inhibit trafficking of the virus to the late endosome or triggering of fusion for filoviruses (21, 37). Interestingly, the estrogen signaling pathway is not involved in the virus entry step, indicating that these drugs may have off-target effects or the estrogen signaling pathway plays an as-yet undiscovered role in filovirus biology. Toremifene citrate also showed activity in the mouse model of Ebola virus infection (21).

Our screen also identified antiviral actives in the pharmaceutical class of neurotransmitter receptor antagonists. These antagonists have been developed for psychiatric care as anti-psychotics, antiemetics, anticholinergics and antidepressants and predominantly act by blocking the dopamine receptor or H_1 receptor (antihistamine). Chlorpromazine was shown to inhibit clathrin-mediated endocytosis of several viruses by preventing the formation of clathrin-coated pits at the plasma membrane (27). This drug is currently approved by the FDA as an antipsychotic and for the treatment of nausea (39), and is occasionally used for short term as off-label treatment of severe migraine (40), making it a promising candidate for testing as a broad-spectrum antiviral. Astemizole, an antihistamine that was identified in our screen, is a strong antagonist of the H_1 receptor (Fig. S1 and S2). Interestingly, it has been reported that astemizole is a potent inhibitor of malaria and showed efficacy in two animal models of malaria with similar mechanism of action to chloroquine (41). Although astemizole was withdrawn from the U.S. market in 1999, it may be worthwhile to re-examine this drug or existing analogs for short term use in an acute infection. Previous work on chloroquine in coronavirus infections by Barnard et al. has found that while the drug inhibits viral replication *in vitro*, chloroquine did not show efficacy in reducing SARS-CoV virus titers in a nonlethal mouse model (42). Protection studies using a mouse adapted SARS-CoV will be performed to identify the *in vivo* efficacy of targeted drugs from our screen.

While development of drugs with broad activity against a virus family or even unrelated viruses is advantageous for several reasons such as immediate availability, lowering costs, recycling of products from the strategic national stockpile, drug classes that are more selective in their activity and affect either MERS-CoV or SARS-CoV should also be further investigated. Our screen identified 33 MERS-CoV actives (Table 1) and the two largest classes were

cytoskeleton inhibitors (8 drugs) and ion channel inhibitors (11 drugs). Drugs targeting the cytoskeleton specifically interfere with microtubule polymerization and are antimitotics developed for treatment of cancer. Some of them, such as nocodazole, have also been used in cell biology labs to synchronize the cell division cycle. Nocodazole's ability to depolymerize microtubules has been used to investigate entry pathway of WNV and results show that an intact microtubule network is necessary for trafficking of internalized WNV from early to late endosomes (27). This drug had high activity against MERS-CoV, but had no activity against SARS-CoV, suggesting that, in addition to the application as therapeutics, these drugs may also have value in further elucidating differences in the virus replication cycle of MERS-CoV and SARS-CoV.

Two of the 9 ion channel inhibitors, monensin and salinomycin sodium, with activity against MERS-CoV represent polyether ionophores that are currently well-recognized candidates for anticancer drugs (43, 44). Studies on the mechanism of anticancer activity have shown that these compounds affect cancer cells by increasing their sensitivity to chemotherapy and reversing multidrug resistance (monensin) in human carcinoma. Furthermore, ionophore antibiotics also inhibit chemoresistant cancer cells by increasing apoptosis, and salinomycin was specifically shown to be able to kill human cancer stem cells (45). Interestingly, these compounds affected MERS-CoV, but not SARS-CoV indicating that MERS-CoV is uniquely susceptible to ionophore activities. Monensin has also been reported to inhibit La Crosse virus and Uukuniemi virus infection by blocking the formation and egress of virus particles (46, 47). Further studies will reveal if these drugs act at similar step during MERS-CoV infection.

Overall, we identified several pharmaceutical classes of drugs that could be beneficial for treatment of coronaviral infections. Interestingly, imatinib mesylate, gemcitabine hydrochloride

and chlorpromazine hydrochloride were also identified in a similar but independent study described in the accompanying paper by A. H. de Wilde et al. These drugs appear to target host factors, rather than viral proteins specifically and treatment of viral infections in patients aimed at host factors could reconfigure overt manifestations of viral pathogenesis into a less virulent subclinical infection and lower adverse disease outcome (38). The targets identified in this paper provide new candidates for future research studies and clinical intervention protocols.

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L.M.J. was employed at Zalicus Inc. during the time the researched was performed. M.L. is currently employed at Zalicus Inc. No other authors have conflicts.

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FIGURE LEGENDS

Fig. 1. Flowchart of screening procedure. A library of 290 compounds was screened at three doses for activity against MERS-CoV. Seventy two compounds that had activity against MERS-CoV were subsequently screened against both MERS-CoV and SARS-CoV. Twenty-seven compounds showed activity (>50% inhibition) against both viruses, while 33 compounds were only active against MERS-CoV. A 102-compound subset was screened against SARS-CoV leading to six compounds that were only active against SARS-CoV.

Fig. 2. Antiviral activity of chlorpromazine hydrochloride and trifluprom azine hydrochloride. (A). Chemical structures of the compounds. Vero E6 cells were infected with MERS-CoV or SARS-CoV at an m.o.i. of 0.1 or 1, respectively, and treated for 48 h with eight doses of (B). chlorpromazine hydrochloride or (C). trifluprom azine hydrochloride. Antiviral activity is shown in blue and cytotoxicity is shown in red. EC_{50} values are indicated. Results are representative of one experiment (mean ± SEM; n=2).

Fig. 3. Antiviral activity of dasatinib and imatinib mesylate. Vero E6 cells were infected with MERS-CoV or SARS-CoV at an m.o.i. of 0.1 or 1, respectively, and treated for 48 h with eight doses of (A). dasatinib or (B). imatinib mesylate. Antiviral activity is shown in blue and

cytotoxicity is shown in red. EC_{50} values are indicated. Results are representative of one experiment (mean ± SEM; n=2).

Fig. 4. Antiviral activity of gemcitabine hydrochloride and toremifene citrate. Vero E6 cells were infected with MERS-CoV or SARS-CoV at an m.o.i. of 0.1 or 1, respectively, and treated for 48 h with eight doses of (A). gemcitabine hydrochloride or (B). toremifene citrate. Antiviral activity is shown in blue and cytotoxicity is shown in red. EC_{50} values are indicated. Results are representative of one experiment (mean ± SEM; n=2).

Table 1. Compounds with activity against MERS-CoV and/or SARS-CoV.

Pharmaceutical Class	SARS-CoV [a]	MERS-CoV [b]	SARS-CoV and MERS-CoV[c]	Total
Antibacterial agents		1	1	2
Antiparasitic agents		2	4	6
Neurotransmitter inhibitors	2	3	11	16
Estrogen receptor inhibitors		3	2	5
DNA inhibitors		3	1	4
Protein-processing inhibitors		1	3	4
Signaling kinase inhibitors	1		2	3
Cytoskeleton inhibitors		8		8
Lipid, sterol metabolism inhibitors		2	2	4
Anti-inflammatory agents	3			3
Iion channel inhibitors		9		9
Apoptosis inhibitors		1		1
Cathepsin inhibitors			1	1
Total	6	33	27	66

[a]Drugs that showed inhibition (> 50%) against SARS-CoV only and low cytotoxicity (<30%).

[b]Drugs that showed inhibition (> 50%) against MERS-CoV only and low cytotoxicity (<30%).

606 [c]Drugs that showed inhibition (> 50%) against SARS-CoV and MERS-CoV and low cytotoxicity

607 (<30%).

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609 **Table 2. Specific compounds with activity against MERS-CoV and SARS-CoV**

Compound	Pharmaceutical class	MERS-CoV EC_{50}	SARS-CoV EC_{50}
Emetine dihydrochloride hydrate	Antibacterial agent	0.014	0.051
Chloroquine diPhosphate	Antiparasitic agent	6.275	6.538
Hydroxychloroquine sulfate	Antiparasitic agent	8.279	7.966
Mefloquine	Anti-parasitic agent	7.416	15.553
Amodiaquine dihydrochloride dihydrate	Anti-parasitic agent	6.212	1.274
E-64-D	Cathepsin inhibitor	1.275	0.760
Gemcitabine hydrochloride	DNA metabolism inhibitor	1.216	4.957
Tamoxifen citrate	Estrogen receptor inhibitor	10.117	92.886
Toremifene citrate	Estrogen receptor inhibitor	12.915	11.969
Terconazole	Sterol metabolism inhibitor	12.203	15.327
Triparanol	Sterol metabolism inhibitor	5.283	
Anisomycin	Protein-processing inhibitor	0.003	0.191
Cycloheximide	Protein-processing inhibitor	0.189	0.043
Homoharringtonine	Protein-processing inhibitor	0.0718	
Benztropine mesylate	Neurotransmitter inhibitor	16.627	21.611
Fluspirilene	Neurotransmitter inhibitor	7.477	5.963
Thiothixene	Neurotransmitter inhibitor	9.297	5.316
Fluphenazine hydrochloride	Neurotransmitter inhibitor	5.868	21.431
Promethazine hydrochloride	Neurotransmitter inhibitor	11.802	7.545
Astemizole	Neurotransmitter inhibitor	4.884	5.591
Chlorphenoxamine hydrochloride	Neurotransmitter inhibitor	12.646	20.031
Chlorpromazine hydrochloride	Neurotransmitter inhibitor	9.514	12.971
Thiethylperazine maleate	Neurotransmitter inhibitor	7.865	
Trifluprom azine hydrochloride	Neurotransmitter inhibitor	5.758	6.398
Clomipramine hydrochloride	Neurotransmitter inhibitor	9.332	13.238
Imatinib mesylate	Kinase signaling inhibitor	17.689	9.823
Dasatinib	Kinase signaling inhibitor	5.468	2.100

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