



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



ELSEVIER

BIAM  
British Infection Association

[www.elsevierhealth.com/journals/jinf](http://www.elsevierhealth.com/journals/jinf)



# Broad-spectrum antivirals for the emerging Middle East respiratory syndrome coronavirus

Jasper F.W. Chan<sup>a,b,c,e</sup>, Kwok-Hung Chan<sup>b,e</sup>,  
Richard Y.T. Kao<sup>a,b,c,e</sup>, Kelvin K.W. To<sup>a,b,c</sup>, Bo-Jian Zheng<sup>a,b,c</sup>,  
Clara P.Y. Li<sup>b</sup>, Patrick T.W. Li<sup>b</sup>, Jun Dai<sup>b</sup>, Florence K.Y. Mok<sup>b</sup>,  
Honglin Chen<sup>a,b,c</sup>, Frederick G. Hayden<sup>d</sup>,  
Kwok-Yung Yuen<sup>a,b,c,\*</sup>

<sup>a</sup> State Key Laboratory of Emerging Infectious Diseases, The University of Hong Kong, Hong Kong, China

<sup>b</sup> Department of Microbiology, The University of Hong Kong, Hong Kong, China

<sup>c</sup> Research Centre of Infection and Immunology, The University of Hong Kong, Hong Kong, China

<sup>d</sup> Division of Infectious Diseases and International Health, Department of Medicine, University of Virginia School of Medicine, Charlottesville, VA, USA

Accepted 27 September 2013

Available online 3 October 2013

## KEYWORDS

Coronavirus;  
Middle East;  
Mycophenolic acid;  
Interferon;  
Ribavirin;  
Antiviral

**Summary Objectives:** Middle East respiratory syndrome coronavirus (MERS-CoV) has emerged to cause fatal infections in patients in the Middle East and traveler-associated secondary cases in Europe and Africa. Person-to-person transmission is evident in outbreaks involving household and hospital contacts. Effective antivirals are urgently needed.

**Methods:** We used small compound-based forward chemical genetics to screen a chemical library of 1280 known drugs against influenza A virus in Biosafety Level-2 laboratory. We then assessed the anti-MERS-CoV activities of the identified compounds and of interferons, nelfinavir, and lopinavir because of their reported anti-coronavirus activities in terms of cytopathic effect inhibition, viral yield reduction, and plaque reduction assays in Biosafety Level-3 laboratory.

**Results:** Ten compounds were identified as primary hits in high-throughput screening. Only mycophenolic acid exhibited low EC<sub>50</sub> and high selectivity index. Additionally, ribavirin and interferons also exhibited *in-vitro* anti-MERS-CoV activity. The serum concentrations achievable at therapeutic doses of mycophenolic acid and interferon-β1b were 60–300 and 3–4 times higher than the concentrations at which *in-vitro* anti-MERS-CoV activities were demonstrated, whereas

\* Corresponding author. Carol Yu Centre for Infection, Department of Microbiology, The University of Hong Kong, Queen Mary Hospital, 102, Pokfulam Road, Pokfulam, Hong Kong, China. Tel.: +852 22554892; fax: +852 28551241.

E-mail address: [kyyuen@hkucc.hku.hk](mailto:kyyuen@hkucc.hku.hk) (K.-Y. Yuen).

<sup>e</sup> The authors contributed equally to the manuscript.

that of ribavirin was ~2 times lower. Combination of mycophenolic acid and interferon- $\beta$ 1b lowered the EC<sub>50</sub> of each drug by 1–3 times.

**Conclusions:** Interferon- $\beta$ 1b with mycophenolic acid should be considered in treatment trials of MERS.

© 2013 The British Infection Association. Published by Elsevier Ltd. All rights reserved.

## Introduction

A novel lineage C betacoronavirus, previously known as human coronavirus EMC/2012 and later renamed as Middle East respiratory syndrome coronavirus (MERS-CoV), has emerged in the Arabian Peninsula since April 2012 to cause a "severe acute respiratory syndrome (SARS)-like" disease in 136 laboratory-confirmed cases with 58 fatalities in 9 countries in the Middle East, Europe, and North Africa as of 4 October 2013.<sup>1–5</sup> Animal-to-human transmission has been suspected in view of MERS-CoV's close phylogenetic relatedness to other lineage C betacoronaviruses found in bats in Hong Kong, Mexico, Europe, and Africa,<sup>6–13</sup> and its broad species tropism in various animal cell lines including those of bats, primates, pigs, civets, and rabbits.<sup>14,15</sup> Recently, a serological study of major livestock suggested dromedary camels to be a possible host based on the high prevalence of MERS-CoV neutralizing antibodies in dromedary camels from Oman.<sup>16</sup> However, targeted studies are needed to confirm this finding and its possible relevance to human cases of MERS-CoV infection as most cases did not have contact with camels and the virus has not been isolated in animals yet. The epidemic continues to evolve with recent outbreaks occurring among epidemiologically-linked household contacts in the Kingdom of Saudi Arabia, the United Kingdom, Italy, and Tunisia, and hospital contacts in the Kingdom of Saudi Arabia, Jordan, the United Kingdom, and France providing evidence for MERS-CoV's potential for person-to-person transmission.<sup>17–23</sup>

Unlike most other human coronavirus infections which are generally mild, most patients with MERS have suffered from rapidly progressive pneumonia with some also developing acute renal failure, hepatic dysfunction, gastrointestinal upset, pericarditis, disseminated intravascular coagulation, and/or cytopenias.<sup>2,24</sup> The resulting crude mortality rate of nearly 50% in documented cases far exceeded those seen in all other human coronavirus infections including SARS despite aggressive supportive treatment including extracorporeal membrane oxygenation in some of the MERS cases. While mild and asymptomatic cases have been recognized,<sup>2,19,24</sup> these recent case clusters signify a global health threat especially in view of the unusual clinical severity of MERS, travel of infected persons to other countries and influx of religious pilgrims to the Kingdom of Saudi Arabia, and the lack of proven effective specific antiviral treatment.

After our initial success in applying chemical genetics in probing novel targets and compounds for antiviral development,<sup>25</sup> we started looking for broad-spectrum antiviral compounds that may be active against both influenza A viruses and coronaviruses, the two viral pathogens responsible for causing the recent 2009 pandemic and large-scale epidemics.<sup>9</sup> While neuraminidase inhibitors such as oseltamivir and zanamivir remain effective against most

seasonal and avian influenza A viruses,<sup>26–30</sup> proven antiviral therapeutic options for coronavirus infections is lacking. Given the limited time available to develop novel anti-MERS-CoV agents in this evolving epidemic, we attempted to provide an alternative solution by identifying potential broad-spectrum antiviral agents against MERS-CoV and influenza A viruses by a small compound-based forward chemical genetics approach using chemical libraries consisting of 1280 drug compounds already marketed or having reached clinical trials in the United States, Europe, or Asia (Microsource Discovery Systems, USA).<sup>25</sup> We then assessed the anti-MERS-CoV activities of the identified drug compounds in cell culture by cytopathic effect (CPE) inhibition, viral yield reduction, and plaque reduction assay (PRA) assays, as well as drug cytotoxicity.

## Materials and methods

### Viruses

A clinical isolate of MERS-CoV was kindly provided by R. Fouchier, A. Zaki, and colleagues.<sup>3</sup> The isolate was amplified by one additional passage in Vero cells to make working stocks of the virus ( $4 \times 10^5$  TCID<sub>50</sub>/ml). All experimental protocol involving live MERS-CoV isolate followed the standard operating procedures of the approved Biosafety Level-3 facility as we previously described.<sup>31</sup> The influenza A/WSN/1933 (H1N1) virus was expanded in chick embryo as we previously described.<sup>25</sup>

### Chemical reagents and high-throughput screening (HTS)

A total of 1280 pre-existing drug compounds (Microsource Discovery Systems) were screened against influenza A/WSN/1933 (H1N1) virus. High-throughput screening (HTS) was carried out in a fully automated Beckman Coulter Core System (Beckman Coulter, USA) integrated with a Kendro robotics CO<sub>2</sub> incubator (Thermo Fisher Scientific) at Chemical Genetics Unit, Department of Microbiology, Research Center of Infection and Immunology, Li Ka-shing Faculty of Medicine, the University of Hong Kong as we previously described with modifications.<sup>25</sup> Briefly, compounds were added in 96-well microtitre plates (TPP) in duplicate with a final concentration of 10  $\mu$ M or 100  $\mu$ M and 20,000 Madin–Darby canine kidney (MDCK) cells per well in 100  $\mu$ l complete Eagle's minimal essential medium (EMEM) supplemented with 1% heat-inactivated FBS. Cells were then inoculated at an MOI of 0.01 with influenza A/WSN/1933 (H1N1) virus for detection of broad-spectrum antivirals. After infection, the plates were incubated at 37 °C with 5% CO<sub>2</sub> and monitored daily using a Leica DM inverted light microscope for virus-induced CPE. Drugs that

gave full protection of MDCK cells (no CPE) were selected for further evaluation with MERS-CoV in a Biosafety Level-3 laboratory.

The cytotoxicity of selected drug (Ribavirin: 1600–0.1  $\mu\text{g}/\text{ml}$ ; IntronA 75,000–4.58 IU/ml; Avonex: 75,000–4.58 IU/ml; Rebif: 250,000–15.26 IU/ml; Betaferon: 50,000–3.05 IU/ml; MMF: 32–0.25  $\mu\text{g}/\text{ml}$ ) was determined by thiazolyl blue tetrazolium bromide (MTT) assay according to manufacturer's instructions. The endpoint was the 50% effective cytotoxic concentration ( $\text{TC}_{50}$ ).

### MERS-CoV CPE inhibition assay

The drug compounds identified as primary hits showing a  $\text{EC}_{50}$  of less than or equal to 50  $\mu\text{M}$  and a selectivity index of more than 100 were diluted with serum free MEM and added to confluent Vero cells in 96-well culture plates in triplicate for 2 h at 37 °C. After incubation, the drug-containing media was removed, and MERS-CoV at 0.0001 MOI was added together with fresh drug-compound media to each well containing approximately 60,000 cells. Following 1 h adsorption at 37 °C, the virus-compound mixture was removed and the cells were washed 2 times with MEM to remove unbound virus. Subsequently, media with antiviral compounds were added to the cells for further incubation for 72 h at 37 °C in a 5%  $\text{CO}_2$  humidified environment. CPE was examined by inverted light microscopy, and 50  $\mu\text{l}$  of supernatant was collected for virus quantification, as we previously described with modifications.<sup>14</sup> Thereafter, 50  $\mu\text{l}$  of serum free MEM and 10  $\mu\text{l}$  of 5 mg/ml MTT solution (prepared in 1  $\times$  PBS, filtered) were added to the wells. The monolayers were incubated as above for 4 h (away from light). Finally, 100  $\mu\text{l}$  of 10% SDS with 0.01 M HCl was added and further incubated at 37 °C with 5%  $\text{CO}_2$  overnight. The activity was read at  $\text{OD}_{570}$  with reference wavelength at  $\text{OD}_{640}$ . The interferon and non-interferon drug compound with the lowest 50% effective inhibitory concentration ( $\text{EC}_{50}$ ) and highest selectivity index were selected for combination studies using the CPE inhibition assay.

### MERS-CoV virus yield reduction and plaque reduction assays

For the drug compounds with antiviral activity in the MTT assay, further evaluation by quantitative virus yield reduction and plaque reduction assays (PRA) was performed. Virus yield quantification was performed by quantitative RT-PCR using total nucleic acid extracted from culture supernatants of the Vero cells infected by MERS-CoV on day 3 post-infection as we previously described.<sup>14</sup>

PRA was performed as we previously described with modifications.<sup>32</sup> Briefly, it was performed in duplicate in 24-well tissue culture plates (TPP). The Vero cells were seeded at 1  $\times$  10<sup>5</sup> cells/well in MEM (Invitrogen) with 10% FBS on the day before carrying out the assay. After 16–24 h incubation, 70–100 plaque-forming units (PFU) of MERS-CoV virus were added to the cell monolayer with or without the addition of drug compounds and the plates further incubated for 2 h at 37 °C in 5%  $\text{CO}_2$  atmosphere before removal

of unbound viral particles by aspiration of the media and washing once with MEM. Monolayers were then overlaid with media containing 1% low melting agarose (Cambrex) in MEM and appropriate concentrations of drug compounds and incubated as above for 72 h. Next, the wells were fixed with 10% formaldehyde (BDH) overnight. After removal of the agarose plugs, the monolayers were stained with 0.7% crystal violet (BDH) and the plaques counted. The percentage of plaque inhibition relative to the control (without the addition of compound) plates was determined for each drug compound concentration. The  $\text{EC}_{50}$  and the 50% cellular cytotoxicity concentration ( $\text{CC}_{50}$ ) were calculated using Sigma plot (SPSS) in an Excel add-in ED50V10. The PRA were carried out in triplicate and repeated twice for confirmation.

## Results

### High-throughput screening (HTS)

Ten drugs compounds, namely mycophenolic acid, flufenamic acid, tolfenamic acid, meclofenamate sodium, mefenamic acid, ribavirin, mercaptopurine, pyrimethamine, emetine, and estradiol were identified as primary hits with protective results in chemical library screening against influenza A/WSN/1933 (H1N1) virus (Table 1). Neuraminidase inhibitors were not identified because they were not included in the chemical library. Amantadine was not identified because the virus strain had an M2 gene mutation (S31N) conferring drug resistance. Using both  $\text{EC}_{50}$  and  $\text{TC}_{50}$  as the hit selection criteria, only mycophenolic acid exhibited a low  $\text{EC}_{50}$  of <10  $\mu\text{M}$  with a high selectivity index of >100. Mercaptopurine, which is a competitive, selective, and reversible inhibitor of the SARS-CoV papain-like protease,<sup>33</sup> demonstrated a high  $\text{EC}_{50}$  of 26.5 and low selectivity index of 4.

### MERS-CoV CPE inhibition assay

In addition to mycophenolic acid (Sigma–Aldrich, USA), ribavirin (Tianxin Pharmaceutical, China), Intron A (recombinant interferon- $\alpha$ 2b, Schering-Plough, USA), Avonex (recombinant interferon- $\beta$ 1a, Biogen Idec, Denmark), Rebif (recombinant interferon- $\beta$ 1a, Merck Serono, Italy), Betaferon (recombinant interferon- $\beta$ 1b, Bayer Schering Pharma, Germany), Imukin (recombinant interferon- $\gamma$ 1b, Boehringer Ingelheim, Germany), nelfinavir mesylate hydrate (Agouron Pharmaceuticals, USA), and lopinavir (Abbott, USA) were also tested in the MTT assays because of their documented *in vitro* anti-SARS-CoV activities in previous reports.<sup>34–37</sup> Among them, only mycophenolic acid, ribavirin, Intron A, Avonex, Rebif, and Betaferon showed anti-MERS-CoV activity at the tested concentrations (Table 2). CPE was completely absent in Vero cells infected with MERS-CoV on day 3 post-infection at concentrations of  $\geq 0.063$   $\mu\text{g}/\text{ml}$  for mycophenolic acid and  $\geq 100$   $\mu\text{g}/\text{ml}$  for ribavirin, and was decreased but not absent in the tested concentrations of Intron A, Avonex, Rebif, or Betaferon (Table 3). Combination studies showed that the  $\text{EC}_{50}$  of mycophenolic acid was lowered by 1.7–2.8 times in the presence of 6.25–12.5 IU/ml of

**Table 1** Drug compounds identified as primary hits with protective results in chemical library screening against influenza A/WSN/1933 (H1N1) virus.

Drug	EC <sub>50</sub> (μM) <sup>a</sup>	TC <sub>50</sub> (μM) <sup>a</sup>	Selectivity index	Bioactivity	Serum concentration (μg/ml) [oral dose]
Mycophenolic acid	0.24	170.00	708.00	Anti-neoplastic	Mycophenolate mofetil: 10–50 [1 g] Mycophenolate sodium: 26.1 [720 mg]
Flufenamic acid	6.30	79.16	12.60	Anti-inflammatory, analgesic	6–20 [200 mg]
Tolfenamic acid	7.94	64.00	8.00	Anti-inflammatory, analgesic	4.1 [300 mg]
Mefenamic acid	50.00	200.00	4.00	Anti-inflammatory, analgesic	10 [1 g]
Meclofenamate sodium	45.00	100.00	2.00	Anti-inflammatory, antipyretic	4.8 [100 mg tds]
Ribavirin	20.00	168.00	8.00	Antiviral	2.2 [4 weeks of 600 mg bd]
Mercaptopurine	26.50	100.00	4.00	Anti-neoplastic, purine anti-metabolite	0.09 [50 mg/m <sup>2</sup> ]
Pyrimethamine	3.10	5.40	1.80	Anti-malarial	0.55 [1500/75 mg of sulfadoxine/pyrimethamine]
Emetine	14.70	17.00	1.50	Inhibits RNA, DNA, and protein synthesis	0.001 [30 ml of syrup ipecac]
Estradiol	20.00	75.00	3.00	Estrogen	Not available

<sup>a</sup> Values represent activity against influenza A/WSN/1933 (H1N1) virus in MDCK cells.

Betaferon, and that the EC<sub>50</sub> of Betaferon was lowered by 1.1–1.8 times in the presence of 0.016–0.063 μg/ml of mycophenolic acid (Table 2).

### MERS-CoV virus yield reduction

The mean baseline viral load in the cell culture supernatants without drugs was 12.110 ± 0.003 log<sub>10</sub> copies/ml. There was a 50% reduction in viral load as compared to the baseline in cell culture supernatants inoculated with each of the six drugs (Fig. 1). There was a >2-log reduction in viral load in cell culture supernatants inoculated with mycophenolic acid, ribavirin, Rebif, and Betaferon. There was >1-log reduction in the viral load in cell culture

supernatants at 40 IU/ml of Betaferon and >3-log reduction at the highest concentration of 50,000 IU/ml (Fig. 1c). The largest reduction in viral load at clinically relevant drug levels was a nearly 4-log reduction at 16 μg/ml of mycophenolic acid.

### MERS-CoV PRA

Mycophenolic acid, ribavirin, and Rebif achieved 100% plaque reduction at concentrations of 6.4 μg/ml, 400 μg/ml, and 62,500 IU/ml respectively (Figs. 2 and 3). The maximum percentages of plaque reduction achieved by Intron A, Avonex, and Betaferon were 76.2% at 70,000 IU/ml, 70.2% at 5000 IU/ml, and 66.6% at

**Table 2** Inhibitory effect of mycophenolic acid, ribavirin, and interferons on MERS-CoV replication in Vero cell yield reduction assay.

Drug	EC <sub>50</sub>	EC <sub>90</sub>	EC <sub>99</sub>	CC <sub>50</sub>	Selectivity index <sup>a</sup>
Mycophenolic acid (μg/ml)					
Alone	0.17 ± 0.03	2.61 ± 0.34	4.86 ± 0.57	>32	>195.12
With 6.25 IU/ml Betaferon	0.10 ± 0.01				
With 12.5 IU/ml Betaferon	0.06 ± 0.01				
Ribavirin (μg/ml)	9.99 ± 2.97	107.06 ± 11.24	183.17 ± 11.97	>1600	>152.98
Intron A (IU/ml)	6709.79 ± 1747.97	184015.75 ± 90145.01	371242.78 ± 255482.32	>75,000	>11.73
Avonex (IU/ml)	5073.33 ± 7333.86	179949.17 ± 138588.37	708919.75 ± 840503.36	>75,000	>35.19
Rebif (IU/ml)	480.54 ± 183.85	2473.86 ± 576.35	3599.06 ± 778.81	15,625	27.08
Betaferon (IU/ml)					
Alone	17.64 ± 1.09	93.31 ± 10.07	135.70 ± 15.96	3125	249.09
With 0.016 μg/ml of mycophenolic acid	16.09 ± 4.09				
With 0.063 μg/ml of mycophenolic acid	9.80 ± 0.53				

<sup>a</sup> Selectivity index defined as ratio of CC<sub>50</sub>/EC<sub>50</sub>.

**Table 3** MERS-CoV-induced cytopathic effects in Vero cells on day 3 post-infection at different concentrations of mycophenolic acid, ribavirin, and interferons.

Drug concentration	Test 1	Test 2	Test 3
<b>Mycophenolic acid (<math>\mu\text{g/ml}</math>)</b>			
0.001	4+	4+	4+
0.004	4+	4+	4+
0.016	1+	1+	1+
0.063	–	–	–
0.250	–	–	–
1.000	–	–	–
4.000	–	–	–
16.000	–	–	–
<b>Ribavirin (<math>\mu\text{g/ml}</math>)</b>			
0.098	4+	4+	4+
0.390	4+	4+	4+
1.560	4+	4+	4+
6.250	4+	4+	4+
25.000	2+	2+	1+
100.000	–	1+	1+
400.000	–	–	–
1600.000	–	–	–
<b>Intron A (IU/ml)</b>			
4.578	4+	4+	4+
18.311	4+	4+	4+
73.242	4+	4+	4+
292.969	4+	4+	4+
1171.875	4+	4+	4+
4687.500	4+	4+	4+
18,750.000	3+	4+	4+
75000.000	–	1+	–
<b>Avonex (IU/ml)</b>			
4.578	4+	4+	4+
18.311	4+	4+	4+
73.242	4+	4+	4+
292.969	4+	4+	4+
1171.875	4+	4+	4+
4687.500	1+	2+	4+
18,750.000	T	3+	3+
75,000.000	–	T	T
<b>Rebif (IU/ml)</b>			
15.260	4+	4+	4+
61.040	4+	4+	4+
244.140	4+	4+	4+
976.560	3+	3+	3+
3906.250	1+	1+	2+
15,625.000	T	1+	3+
62,500.000	T	T	T
250,000.000	T	T	T
<b>Betaferon (IU/ml)</b>			
3.050	4+	4+	4+
12.210	4+	4+	4+
48.830	1+	1+	2+
195.310	1+	1+	1+
781.250	T	T	T
3125.000	T	T	T

**Table 3 (continued)**

Drug concentration	Test 1	Test 2	Test 3
12,500.000	T	T	T
50,000.000	T	T	T

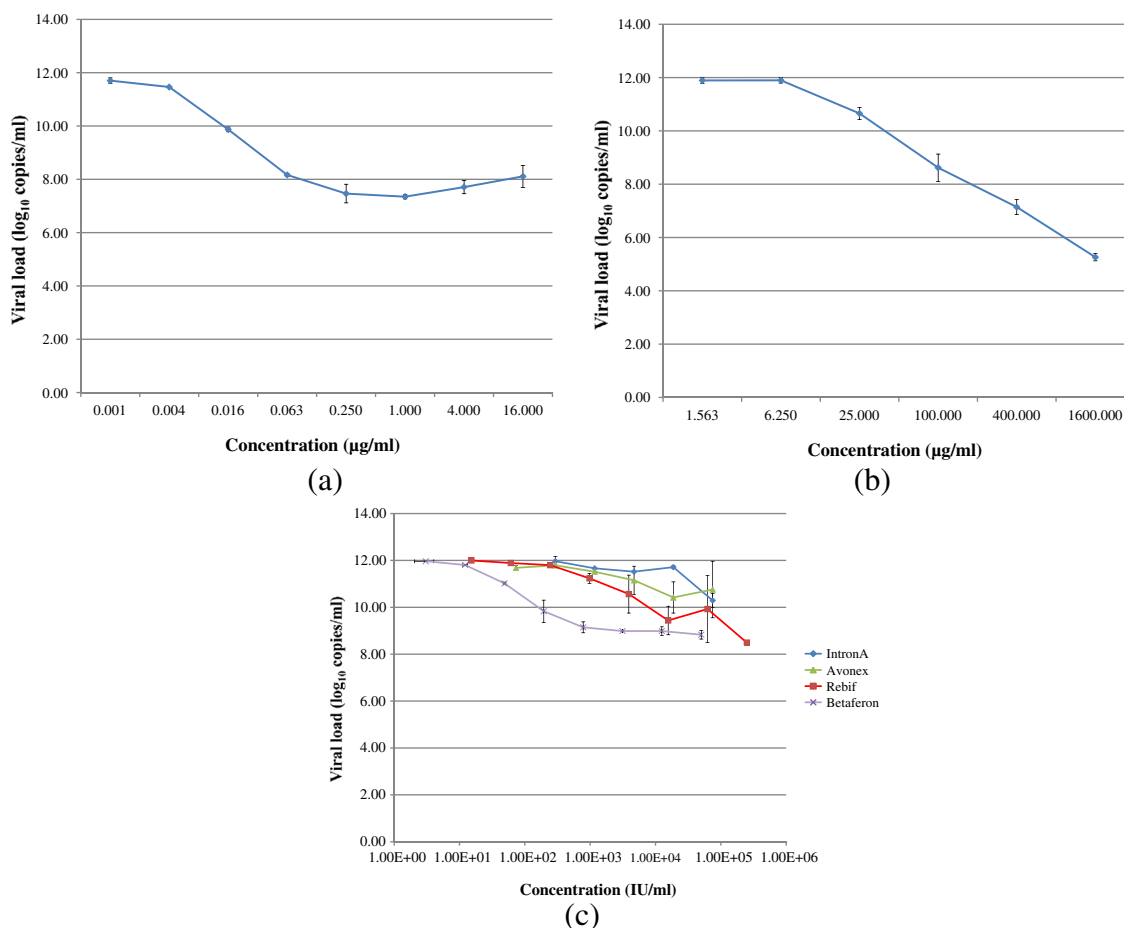
Remarks: –, negative; 1+ is defined as 1%–25% involvement; 2+ is defined as >25%–50% involvement; 3+ is defined as >50%–75% involvement; 4+ is defined as >75% involvement; T, drug-induced toxic effects in Vero cells.

400 IU/ml respectively (Fig. 3). In PRA, Betaferon achieved 40–50% plaque reduction at 40 IU/ml (Fig. 3c).

## Discussion

Novel antiviral targets for SARS coronavirus and influenza A virus have been identified previously using small compound-based forward chemical genetics approaches similar to ours.<sup>25,38,39</sup> In this study, we identified ten compounds among approved drugs with as primary hits in chemical library screening that possess antiviral activities. Some may offer potential therapies in the evolving MERS-CoV epidemic. Influenza A/WSN/1933 (H1N1) virus, instead of MERS-CoV, was used for initial screening because its manipulation did not require a Biosafety Level III laboratory. Other human betacoronaviruses such as HCoV-OC43 and HCoV-HKU1 were not used because of their slow replication and low viral titres in cell culture.<sup>14</sup> Among the 10 identified drug compounds, only mycophenolic acid exhibited an  $\text{EC}_{50}$  of <10  $\mu\text{M}$ , which is a common cut-off value for lead compound detection, and a high selective index of >100. Additionally, we tested other agents reported to have *in vitro* activities against SARS-CoV and/or MERS-CoV.<sup>32,34,40</sup> Imukin (interferon- $\gamma$ 1b) and the HIV protease inhibitors, nelfinavir mesylate hydrate and lopinavir, showed suboptimal  $\text{EC}_{50}$  in the initial CPE inhibition assay and were therefore not further evaluated. Together with mycophenolic acid, four other drug compounds in five preparations, namely ribavirin, Intron A, Avonex, Rebif, and Betaferon, showed *in vitro* anti-MERS-CoV activity of varying magnitude across four assays.

Mycophenolic acid is a selective, non-competitive, and reversible inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH). It inhibits the proliferation of T and B lymphocytes and production of immunoglobulins by depletion of the lymphocyte guanosine and deoxyguanosine nucleotide pools.<sup>41</sup> Its major clinical indication is prevention of graft rejection in solid organ and haematopoietic stem cell transplantations. In addition to potent immunosuppressive activity, mycophenolic acid also has broad activity *in vitro* and/or in animal models against different viruses including West Nile,<sup>42</sup> Japanese encephalitis,<sup>43</sup> yellow fever,<sup>44</sup> dengue,<sup>45</sup> Chikungunya,<sup>46</sup> and possibly hepatitis B viruses.<sup>47</sup> Furthermore, it inhibited the *in vitro* and *in vivo* replication of hepatitis C virus by augmentation of interferon-stimulated gene expression and depletion of guanosine.<sup>48,49</sup> Combination treatment with interferon- $\alpha$  showed additive effects on interferon-stimulated gene expression and enhanced interferon-induced luciferase reporter activity.<sup>48</sup> As for coronaviruses, mycophenolic acid



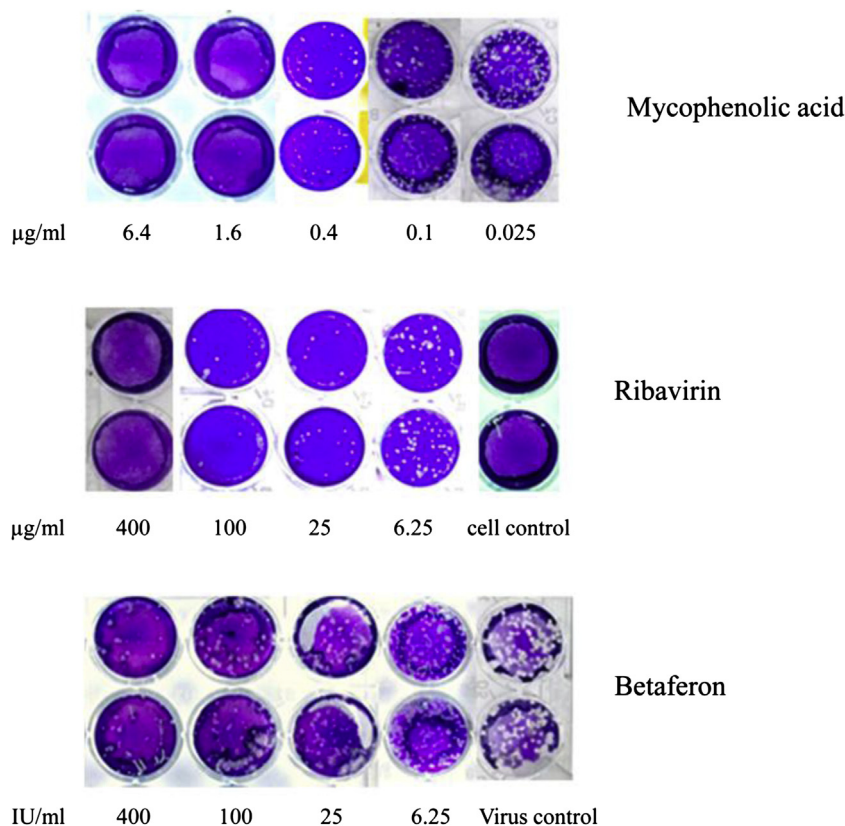
**Figure 1** Viral load quantified by RT-PCR in Vero cells on day 3 after infection by MERS-CoV and inoculation with different drug compounds: (a) mycophenolic acid, (b) ribavirin, (c) interferons (Intron A, Avonex, Rebif, and Betaferon).

was found to be ineffective against SARS-CoV in an animal model, although it did not significantly increase the viral load in the lungs of SARS-infected BALB/c mice as ribavirin did.<sup>50</sup> We are unaware of data on its activity against other human coronaviruses. Our study is the first to demonstrate the anti-coronavirus activity of mycophenolic acid against the novel MERS-CoV.

In addition to mycophenolic acid, our *in vitro* findings indicated that ribavirin, interferon- $\alpha$ , and interferon- $\beta$  had anti-MERS-CoV activities *in vitro*. In the case of SARS-CoV, their antiviral activities in *in vitro* susceptibility tests had been conflicting.<sup>34</sup> None of them were tested systemically in large-scale randomized controlled trials and the results from clinical trials involving their use in SARS were often confounded with the concomitant use of corticosteroids.<sup>51,52</sup> Although their clinical use in MERS-CoV infection has not been described, a recent study found that ribavirin had *in vitro* anti-MERS-CoV activity at very high concentrations which was potentiated when given together with interferon- $\alpha$ 2b.<sup>40</sup> Another study showed that MERS-CoV is 50–100 times more sensitive to pegylated interferon- $\alpha$  than SARS-CoV in Vero cells, which is possibly related to the lineage-specific genetic differences between the two coronaviruses with MERS-CoV lacking the homolog of the SARS-CoV ORF6 protein responsible for the blockade of interferon-induced nuclear translocation of phosphorylated

transcription factor STAT1.<sup>53</sup> Furthermore, the delayed and aberrant induction of inflammatory cytokines and chemokines by MERS-CoV might support the use of adjunctive immuno-modulatory treatment combined with antivirals in patients with MERS.<sup>54,55</sup> Among the four preparations of interferons tested, Betaferon exhibited the lowest EC<sub>50</sub> of 17.64 IU/ml, which was below the mean peak serum concentration of 40 IU/ml after a subcutaneous dose of 16 million IU or an intravenous dose of 0.2 million to 64 million IU.<sup>56</sup> Although the other preparations of interferons also demonstrated *in vitro* anti-MERS-CoV activities, their EC<sub>50</sub> were generally above the peak serum concentrations achievable with usual therapeutic dosing. Combination treatment consisting of mycophenolic acid and Betaferon resulted in a 1.7–2.8-fold reduction in the EC<sub>50</sub> of mycophenolic acid in Vero cells with 6.25–12.5 IU/ml of Betaferon, and 1.1–1.8-fold reduction in the EC<sub>50</sub> of Betaferon in Vero cells with 0.016–0.063 µg/ml of mycophenolic acid. Our finding may provide the basis for combination mycophenolic acid and Betaferon in future clinical trials.

Compared with ribavirin and interferons, mycophenolic acid exhibits a number of attributes that support its practical use in MERS-CoV infection. It is commonly available in two forms, the prodrug mycophenolate mofetil and the salt mycophenolate sodium, and could be given orally



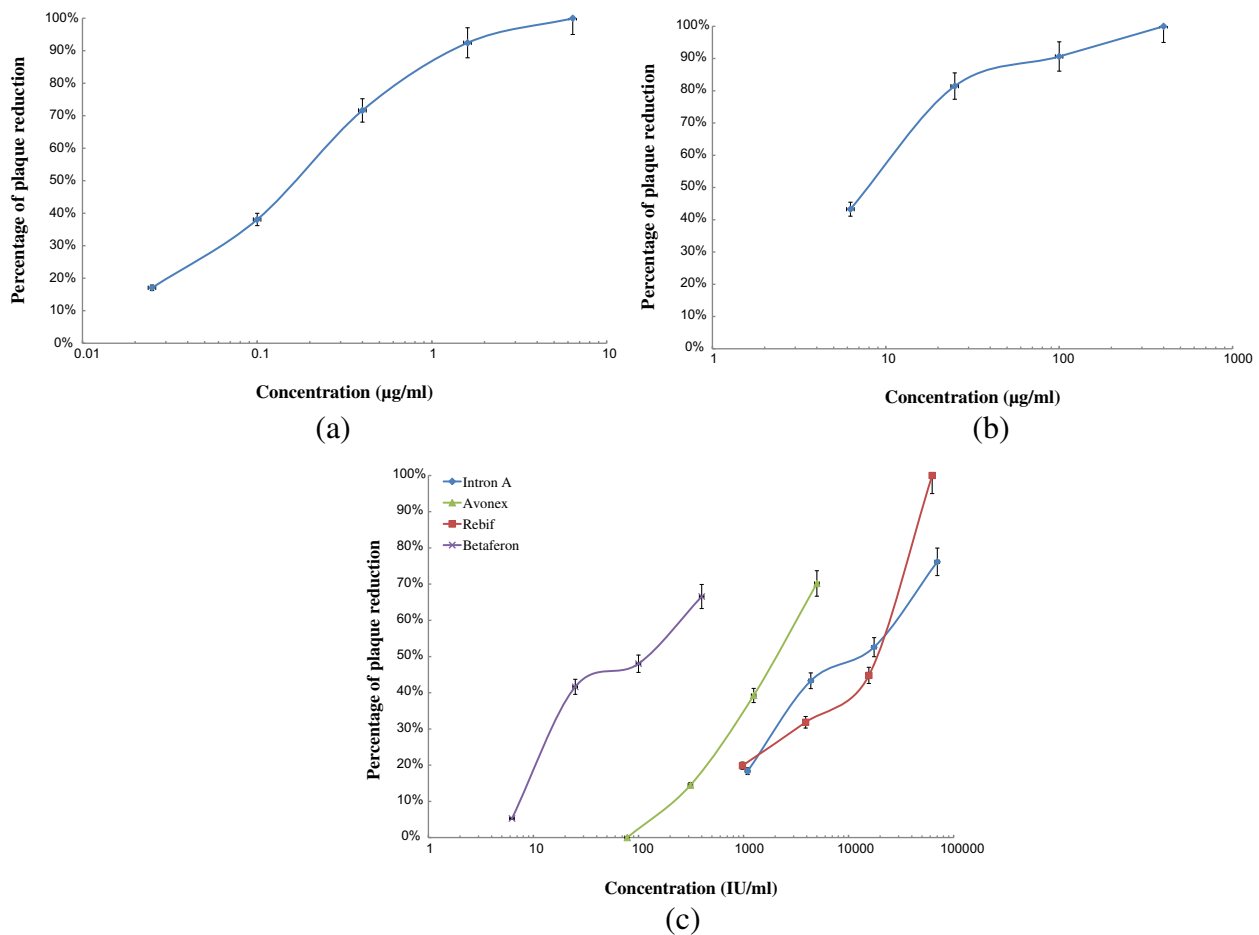
**Figure 2** Photos of plaque reduction assay of mycophenolic acid, ribavirin, and Betaferon.

or parenterally. The serum concentration of mycophenolic acid peaks at around 10–50 µg/ml after a 1000 mg oral dose of mycophenolate mofetil or 26.1 µg/ml after a 720 mg oral dose of mycophenolate sodium. These far exceeds its EC<sub>50</sub> of 0.17 µg/ml and is 60–300 times higher than the concentrations at which the replication of MERS-CoV is inhibited in cell culture and PRA.<sup>57</sup> With average plasma elimination half-lives of 17.9 h and 16.6 h after a 1000 mg oral dose and 1500 mg intravenous dose of mycophenolate mofetil respectively,<sup>41</sup> the usual regimens consisting of 1000 mg twice daily oral or 1500 mg twice daily intravenous mycophenolate mofetil would be sufficient to achieve levels well above the EC<sub>50</sub> throughout the dosing interval. In contrast, the EC<sub>50</sub> of ribavirin for MERS-CoV between 9.99 and 41.45 µg/ml is just marginally effective in some cell lines and greatly exceeds the drug's serum concentration with usual oral doses. Peak concentrations with high intravenous doses may reach approximately 24 µg/ml in humans, but steady-state requires at least 4 weeks to achieve.<sup>40,58</sup> Furthermore, the use of ribavirin, and hence also its combination with interferon-α2b, may be limited in the clinical setting, because a significant proportion of patients with MERS-CoV infection have developed acute renal failure often requiring renal replacement therapy.<sup>2,24</sup> It has been suggested that systemic ribavirin should best be avoided in patients with a creatinine clearance of <50 ml/min because of the increased risk of haemolytic anaemia.<sup>59</sup> Although mycophenolic acid may also be associated with acute renal impairment, the dosage adjustment in such a

setting is generally well established.<sup>41</sup> The potent *in vitro* anti-MERS-CoV activity of mycophenolic acid may allow it to be used as a monotherapy if concomitant interferon is not available or tolerated by the patient. Finally, drug level monitoring for mycophenolate mofetil is generally available in most tertiary hospitals which are the usual referral centers for cases of severe MERS-CoV infections requiring intensive care facilities such as extracorporeal membrane oxygenation. However, the risk of immunosuppression at the onset of adaptive immune responses or polarization towards a deleterious Th1 response by mycophenolic acid needs to be considered.<sup>60</sup> One possible approach is a short course of mycophenolate mofetil combined with an interferon, particularly interferon-β1b, to provide synergistic antiviral and immune-enhancing effects against MERS-CoV. These options should be considered for study in randomized control clinical trials for this highly fatal disease.

There are a number of limitations in our study. Firstly, the cytotoxicity assay likely underestimated more subtle effects of candidate compounds on host cell growth and metabolism. For example, ribavirin inhibits replication of uninfected MDCK cells at concentrations of 10 µg/ml and above but does not cause overt cytotoxicity until much higher concentrations are reached.<sup>61,62</sup> Secondly, we used Vero cells alone to study the antiviral activity of ribavirin. Vero cells have been described as being comparatively resistant to ribavirin due to their inefficient conversion of the drug into its mono- and tri-phosphate forms.<sup>63</sup> However, we decided not to perform the experiment using





**Figure 3** Effects of (a) mycophenolic acid, (b) ribavirin, and (c) interferons (Intron A, Avonex, Rebif, and Betaferon) on MERS-CoV replication in Vero cells.

another cell line as this has been done in another recent report using Vero and LLC-MK2 cell lines which also demonstrated anti-MERS-CoV activity of high ribavirin concentrations similar to our findings.<sup>40</sup> It would be important to extend these *in vitro* studies to human respiratory epithelial cell systems and explants.

To optimize treatment options for MERS-CoV infection, further studies on the anti-MERS-CoV activities of other potential anti-coronavirus agents which have been previously identified for SARS-CoV should be undertaken. Replication of many coronaviruses including SARS-CoV and MERS-CoV requires proteolytic processing of the replicase polyprotein by two viral cysteine proteases, a chymotrypsin-like protease (3CLpro) and a papain-like protease (PLpro). However, the protease inhibitors such as nelfinavir and lopinavir were not found to be active in our *in vitro* study. Helicase inhibitors are another group of agents with *in vitro* anti-SARS-CoV activities but their anti-MERS-CoV activities remain undetermined.<sup>39,64</sup> Inhalational nitric oxide was used as rescue therapy for SARS and might be useful for treating MERS-CoV infection if organic nitric oxide donors such as S-nitro-N-acetylpenicillamine also show anti-MERS-CoV activity.<sup>65,66</sup> Antiviral peptides or neutralizing antibodies designed against heptad repeat region 2 of S2 which may inhibit membrane fusion and cell entry of SARS-CoV could theoretically be

harnessed for MERS-CoV since the S2 region shared significant homology amongst betacoronaviruses.<sup>67–70</sup> Other agents with *in vitro* anti-SARS-CoV activities such as glycyrrhizin, baicalin, reserpine, aescin, valinomycin, niclosamide, aurointricarboxylic acid, mizoribine, indomethacin, chloroquine, and experimental agents like small interfering RNA (siRNA) and inhibitors targeting the binding interface between the S1 domain and receptor *in vivo*, should also be evaluated.<sup>34,35,71</sup> We did not test these agents in this study because most of them have the problems of either not being commercially available or having therapeutic levels that are not easily achievable clinically. Recently, cyclophilin inhibitors, such as cyclosporine which is available commercially, have also been reported to exhibit anti-MERS-CoV and anti-coronavirus activity in cell culture and viral load studies.<sup>53,72</sup> Further evaluation of its potential therapeutic effects of these commercially available agents with *in vitro* activity should be conducted in randomized clinical trials as good animal models for MERS are not widely available at this stage.<sup>73</sup>

## Acknowledgements

We thank Dr. Herman Tse for technical advice. This work is partly supported by the donations of Hui Hoy and Chow Sin

Lan Charity Fund Limited, the Consultancy Service for Enhancing Laboratory Surveillance of Emerging Infectious Disease of the Department of Health, Hong Kong Special Administrative Region, China, and the National Science and Technology Major Project of China (grant 2012ZX10004-213-002).

## Conflict of interests

The authors have no financial or any other conflicts of interest regarding the contents of the investigations.

## References

- Chan JF, Li KS, To KK, Cheng VC, Chen H, Yuen KY. Is the discovery of the novel human betacoronavirus 2c EMC/2012 (HCoV-EMC) the beginning of another SARS-like pandemic? *J Infect* 2012;**65**:477–89.
- Chan JF, Lau SK, Woo PC. The emerging novel Middle East respiratory syndrome coronavirus: the “knowns” and “unknowns”. *J Formos Med Assoc* 2013;**112**:372–81.
- Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* 2012;**367**:1814–20.
- de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, et al. Middle East respiratory syndrome coronavirus (MERS-CoV); announcement of the coronavirus study group. *J Virol* 2013;**87**:7790–2.
- World Health Organization. *Global alert and response: Middle East respiratory syndrome coronavirus (MERS-CoV) – update – as of 4 October 2013*. Geneva: WHO; 2013. [http://www.who.int/csr/don/2013\\_10\\_04/en/index.html](http://www.who.int/csr/don/2013_10_04/en/index.html) [accessed 05.10.13].
- Woo PC, Wang M, Lau SK, Xu H, Poon RW, Guo R, et al. Comparative analysis of twelve genomes of three novel group 2c and group 2d coronaviruses reveals unique group and subgroup features. *J Virol* 2007;**81**:1574–85.
- Woo PC, Lau SK, Li KS, Tsang AK, Yuen KY. Genetic relatedness of the novel human lineage C betacoronavirus to Tylonycteris bat coronavirus HKU4 and Pipistrellus bat coronavirus HKU5. *Emerg Microbe Infect* 2012;**1**:e35. <http://dx.doi.org/10.1038/emi.2012.45>.
- Lau SK, Li KS, Tsang AK, Lam CS, Ahmed S, Chen H, et al. Genetic characterization of Betacoronavirus lineage C viruses in bats revealed marked sequence divergence in the spike protein of Pipistrellus bat coronavirus HKU5 in Japanese pipistrelle: implications on the origin of the novel Middle East Respiratory Syndrome Coronavirus. *J Virol* 2013;**87**:8638–50.
- Chan JF, To KK, Tse H, Jin DY, Yuen KY. Interspecies transmission and emergence of novel viruses: lessons from bats and birds. *Trends Microbiol* 2013;**21**:544–55.
- van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, et al. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. *MBio* 2012;**3**: pii: e00473-12.
- Annan A, Baldwin HJ, Corman VM, Klose SM, Owusu M, Nkrumah EE, et al. Human betacoronavirus 2c EMC/2012-related viruses in bats, Ghana and Europe. *Emerg Infect Dis* 2013;**19**:456–9.
- Anthony SJ, Ojeda-Flores R, Rico-Chávez O, Navarrete-Macias I, Zambrana-Torrel CM, Rostal MK, et al. Coronaviruses in bats from Mexico. *J Gen Virol* 2013;**94**:1028–38.
- Lu L, Liu Q, Du L, Jiang S. Middle East respiratory syndrome coronavirus (MERS-CoV): challenges in identifying its source and controlling its spread. *Microbes Infect* 2013;**15**:625–9.
- Chan JF, Chan KH, Choi GK, To KK, Tse H, Cai JP, et al. Differential cell line susceptibility to the emerging novel human betacoronavirus 2c EMC/2012: implications on disease pathogenesis and clinical manifestation. *J Infect Dis* 2013;**207**:1743–52.
- Müller MA, Raj VS, Muth D, Meyer B, Kallies S, Smits SL, et al. Human coronavirus EMC does not require the SARS-coronavirus receptor and maintains broad replicative capability in mammalian cell lines. *MBio* 2012;**3**: pii: e00515-12.
- Reusken CB, Haagmans BL, Müller MA, Gutierrez C, Meyer B, Muth B, et al. Middle East respiratory syndrome coronavirus neutralizing serum antibodies in dromedary camels: a comparative serological study. *Lancet Infect Dis* 2013;**13**:859–66.
- Centers for Disease Control and Prevention (CDC). Update: severe respiratory illness associated with middle East respiratory syndrome coronavirus (MERS-CoV) – Worldwide, 2012–2013. *MMWR Morb Mortal Wkly Rep* 2013;**62**:480–3.
- Guery B, Poissy J, ElMansouf L, Séjoruné C, Ettahar N, Lemaire X, et al. Clinical features and viral diagnosis of two cases of infection with Middle East Respiratory Syndrome coronavirus: a report of nosocomial transmission. *Lancet* 2013;**381**:2265–72.
- Memish ZA, Zumla AI, Al-Hakeem RF, Al-Rabeeh AA, Stephens GM. Family cluster of middle East respiratory syndrome coronavirus infections. *N Engl J Med* 2013;**369**:587.
- Assiri A, McGeer A, Perl TM, Price CS, Al Rabeeh AA, Cummings DA, et al. Hospital outbreak of middle East respiratory syndrome coronavirus. *N Engl J Med* 2013;**369**:407–16.
- Omrani AS, Matin MA, Haddad Q, Al-Nakhli D, Memish ZA, Albarrak AM. A family cluster of Middle East Respiratory Syndrome Coronavirus infections related to a likely unrecognized asymptomatic or mild case. *Int J Infect Dis* 2013;**17**:e668–72.
- Mailles A, Blanckaert K, Chaud P, van der Werf S, Lina B, Caro V, et al. First cases of middle East respiratory syndrome coronavirus (MERS-CoV) infections in France, investigations and implications for the prevention of human-to-human transmission, France, May 2013. *Euro Surveill* 2013;**18**: pii: 20502.
- Drosten C, Seilmaier M, Corman VM, Hartmann W, Scheible G, Sack S, et al. Clinical features and virological analysis of a case of Middle East respiratory syndrome coronavirus infection. *Lancet Infect Dis* 2013;**13**:745–51.
- Assiri A, Al-Tawfiq JA, Al-Rabeeh AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, et al. Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. *Lancet Infect Dis* 2013;**13**:752–61.
- Kao RY, Yang D, Lau LS, Tsui WH, Hu L, Dai J, et al. Identification of influenza A nucleoprotein as an antiviral target. *Nat Biotechnol* 2010;**28**:600–5.
- Cheng VC, To KK, Tse H, Hung IF, Yuen KY. Two years after pandemic influenza A/2009/H1N1: what have we learned? *Clin Microbiol Rev* 2012;**25**:223–63.
- To KK, Ng KH, Que TL, Chan JM, Tsang KY, Tsang AK, et al. Avian influenza A H5N1 virus: a continuous threat to humans. *Emerg Microbes Infect* 2012;**1**:e25.
- Chen Y, Liang W, Yang S, Wu N, Gao H, Sheng J, et al. Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. *Lancet* 2013;**381**:1916–25.
- To KK, Chan JF, Chen H, Li L, Yuen KY. The emergence of influenza A (H7N9) sixteen years after influenza A(H5N1) in humans: a tale of two cities. *Lancet Infect Dis* 2013;**13**:809–21.
- Yu L, Wang Z, Chen Y, Ding W, Jia H, Chan JF, et al. Clinical, virological, and histopathological manifestations of fatal human infections by avian influenza A(H7N9) virus. *Clin Infect Dis* 2013 [Epub ahead of print].

31. Zheng BJ, Chan KW, Lin YP, Zhao GY, Chan C, Zhang HJ, et al. Delayed antiviral plus immunomodulator treatment still reduces mortality in mice infected by high inoculum of influenza A/H5N1 virus. *Proc Natl Acad Sci USA* 2008;105:8091–6.
32. Chu CM, Cheng VC, Hung IF, Wong MM, Chan KH, Chan KS, et al. Role of lopinavir/ritonavir in the treatment of SARS: initial virological and clinical findings. *Thorax* 2004;59:252–6.
33. Chou CY, Chien CH, Han YS, Prebanda MT, Hsieh HP, Turk B, et al. Thiopurine analogues inhibit papain-like protease of severe acute respiratory syndrome coronavirus. *Biochem Pharmacol* 2008;75:1601–9.
34. Cheng VC, Lau SK, Woo PC, Yuen KY. Severe acute respiratory syndrome coronavirus as an agent of emerging and reemerging infection. *Clin Microbiol Rev* 2007;20:660–94.
35. Chen F, Chan KH, Jiang Y, Kao RY, Lu HT, Fan KW, et al. In vitro susceptibility of 10 clinical isolates of SARS coronavirus to selected antiviral compounds. *J Clin Virol* 2004;31:69–75.
36. Cinatl J, Morgenstern B, Bauer G, Chandra P, Rabenau H, Doerr HW. Treatment of SARS with human interferons. *Lancet* 2003;362:392–4.
37. de Lang A, Osterhaus AD, Haggmans BL. Interferon-gamma and interleukin-4 downregulate expression of the SARS coronavirus receptor ACE2 in Vero E6 cells. *Virology* 2006;353:474–81.
38. Kao RY, To AP, Ng LW, Tsui WH, Lee TS, Tsoi HW, et al. Characterization of SARS-CoV main protease and identification of biologically active small molecule inhibitors using a continuous fluorescence-based assay. *FEBS Lett* 2004;576:325–30.
39. Kao RY, Tsui WH, Lee TS, Tanner JA, Watt RM, Huang JD, et al. Identification of novel small-molecule inhibitors of severe acute respiratory syndrome-associated coronavirus by chemical genetics. *Chem Biol* 2004;11:1293–9.
40. Falzarano D, de Wit E, Martellaro C, Callison J, Munster VJ, Feldmann H. Inhibition of novel  $\beta$  coronavirus replication by a combination of interferon- $\alpha$ 2b and ribavirin. *Sci Rep* 2013;3:1686.
41. Villarroel MC, Hidalgo M, Jimeno A. Mycophenolate mofetil: an update. *Drugs Today (Barc)* 2009;45:521–32.
42. Morrey JD, Smee DF, Sidwell RW, Tseng C. Identification of active antiviral compounds against a New York isolate of West Nile virus. *Antiviral Res* 2002;55:107–16.
43. Sebastian L, Madhusudana SN, Ravi V, Desai A. Mycophenolic acid inhibits replication of Japanese encephalitis virus. *Chemotherapy* 2011;57:56–61.
44. Leyssen P, Balzarini J, De Clercq E, Neyts J. The predominant mechanism by which ribavirin exerts its antiviral activity in vitro against flaviviruses and paramyxoviruses is mediated by inhibition of IMP dehydrogenase. *J Virol* 2005;79:1943–7.
45. Diamond MS, Zachariah M, Harris E. Mycophenolic acid inhibits dengue virus infection by preventing replication of viral RNA. *Virology* 2002;304:211–21.
46. Khan M, Dhanwani R, Patro IK, Rao PV, Parida MM. Cellular IMPDH enzyme activity is a potential target for the inhibition of Chikungunya virus replication and virus induced apoptosis in cultured mammalian cells. *Antiviral Res* 2011;89:1–8.
47. Pan Q, van Vuuren AJ, van der Laan LJ, Peppelenbosch MP, Janssen HL. Antiviral or proviral action of mycophenolic acid in hepatitis B infection? *Hepatology* 2012;56:1586–7.
48. Pan Q, de Ruiter PE, Metselaar HJ, Kwekkeboom J, de Jonge J, Tilanus HW, et al. Mycophenolic acid augments interferon-stimulated gene expression and inhibits hepatitis C Virus infection in vitro and in vivo. *Hepatology* 2012;55:1673–83.
49. Ye L, Li J, Zhang T, Wang X, Wang Y, Zhou Y, et al. Mycophenolate mofetil inhibits hepatitis C virus replication in human hepatic cells. *Virus Res* 2012;168:33–40.
50. Barnard DL, Day CW, Bailey K, Heiner M, Montgomery R, Lauridsen L, et al. Enhancement of the infectivity of SARS-CoV in BALB/c mice by IMP dehydrogenase inhibitors, including ribavirin. *Antiviral Res* 2006;71:53–63.
51. Wong SS, Yuen KY. The management of coronavirus infections with particular reference to SARS. *J Antimicrob Chemother* 2008;62:437–41.
52. Cheng VC, Tang BS, Wu Ak, Chu CM, Yuen KY. Medical treatment of viral pneumonia including SARS in immunocompetent adult. *J Infect* 2004;49:262–73.
53. de Wilde AH, Ray VS, Oudshoorn D, Besterbroer TM, van Nieuwkoop S, Limpens RW, et al. MERS-coronavirus replication induces severe in vitro cytopathology and is strongly inhibited by cyclosporin A or interferon-alpha treatment. *J Gen Virol* 2013;94:1749–60.
54. S.K. A Lau, Lau CC, Chan KH, Li CP, Chen H, Jin DY, et al. Delayed induction of proinflammatory cytokines and suppression of innate antiviral response by the novel Middle East respiratory syndrome coronavirus: implications for pathogenesis and treatment. *J Gen Virol* 2013 [Epub ahead of print].
55. J. B Zhou, Chu H, Li C, Wong BH, Cheng ZS, Poon VK, et al. Active MERS-CoV replication and aberrant induction of inflammatory cytokines and chemokines in human macrophages: implications for pathogenesis. *J Infect Dis* 2013 [Epub ahead of print].
56. Product Information: Betaferon® Single use pack drug (AUST R 83309). <http://www.bayerresources.com.au/resources/uploads/PI/file9314.pdf> [accessed 18.06.13].
57. Johnston A, He X, Holt DW. Bioequivalence of enteric-coated mycophenolate sodium and mycophenolate mofetil: a meta-analysis of three studies in stable renal transplant recipients. *Transplantation* 2006;82:1413–8.
58. Koren G, King S, Knowles S, Phillips E. Ribavirin in the treatment of SARS: a new trick for an old bug? *CMAJ* 2003;168:1289–92.
59. Brochot E, Castelain S, Duverlie G, Capron D, Nguyen-Khac E, François C. Ribavirin monitoring in chronic hepatitis C therapy: anaemia versus efficacy. *Antiviral Ther* 2010;15:687–95.
60. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. *Immunopharmacology* 2000;47:85–118.
61. Hayden FG, Cote KM, Douglas Jr RG. Plaque inhibition assay for drug susceptibility testing of influenza viruses. *Antimicrob Agents Chemother* 1980;17:865–70.
62. Hayden FG, Douglas Jr RG, Simons R. Enhancement of activity against influenza viruses by combinations of antiviral agents. *Antimicrob Agents Chemother* 1980;18:536–41.
63. Shah NR, Sunderland A, Grdzlishvili VZ. Cell type mediated resistance of vesicular stomatitis virus and Sendai virus to ribavirin. *PLoS One* 2010;5:e11265.
64. Adedeji AO, Singh K, Calcaterra NE, DeDiego ML, Enjuanes L, Weiss S, et al. Severe acute respiratory syndrome coronavirus replication inhibitor that interferes with the nucleic acid unwinding of the viral helicase. *Antimicrob Agents Chemother* 2012;56:4718–28.
65. Chen L, Liu P, Gao H, Sun B, Chao D, Wang F, et al. Inhalation of nitric oxide in the treatment of severe acute respiratory syndrome: a rescue trial in Beijing. *Clin Infect Dis* 2004;39:1531–5.
66. Akerström S, Mousavi-Jazi M, Klingström J, Leijon M, Lundkvist A, Mirazimi A. Nitric oxide inhibits the replication cycle of severe acute respiratory syndrome coronavirus. *J Virol* 2005;79:1966–9.
67. Chan KH, Chan JF, Tse H, Chen H, Lau CC, Cai JP. Cross-reactive antibodies in convalescent SARS patients' sera against the emerging novel human coronavirus EMC (2012) by both immunofluorescent and neutralizing antibody tests. *J Infect* 2013;67:130–40.
68. Jiang S, Lu L, Du L, Debnath AK. A predicted receptor-binding and critical neutralizing domain in S protein of the novel human coronavirus HCoV-EMC. *J Infect* 66: 464–6.

69. Du L, Zhao G, Kou Z, Ma C, Sun S, Poon VK, et al. Identification of a receptor-binding domain in the s protine of the novel human coronavirus middle East respiratory syndrome coronavirus as an essential target for vaccine development. *J Virol* 2013;**87**:9939–42.
70. Zheng BJ, Guan Y, Hez ML, Sun H, Du L, Zheng Y, et al. Synthetic peptides outside the spike protein heptad repeat regions as potent inhibitors of SARS-associated coronavirus. *Antivir Ther* 2005;**10**:393–403.
71. Raj VS, Mou H, Smits SL, Dekkers DH, Müller MA, Dijkman R, et al. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature* 2013;**495**:251–4.
72. Tanaka Y, Sato Y, Sasaki T. Suppression of coronavirus replication by cyclophilin inhibitors. *Viruses* 2013;**5**:1250–60.
73. Munster VJ, de Wit E, Feldmann H. Pneumonia from human coronavirus in a macaque model. *N Engl J Med* 2013;**368**:1560–2.