

# Interferon- $\beta$ and mycophenolic acid are potent inhibitors of Middle East respiratory syndrome coronavirus in cell-based assays

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The Middle East respiratory syndrome coronavirus (MERS-CoV) presents a novel emerging threat to public health worldwide. Several treatments for infected individuals have been suggested including IFN, ribavirin and passive immunotherapy with convalescent plasma. Administration of IFN- $\alpha$ 2b and ribavirin has improved outcomes of MERS-CoV infection in rhesus macaques when administered within 8 h post-challenge. However, detailed and systematic evidence on the activity of other clinically available drugs is limited. Here we compared the susceptibility of MERS-CoV with different IFN products (IFN- $\alpha$ 2b, IFN- $\gamma$ , IFN-universal, IFN- $\alpha$ 2a and IFN- $\beta$ ), as well as with two antivirals, ribavirin and mycophenolic acid (MPA), against MERS-CoV (Hu/Jordan-N3/2012) *in vitro*. Of all the IFNs tested, IFN- $\beta$  showed the strongest inhibition of MERS-CoV *in vitro*, with an IC<sub>50</sub> of 1.37 U ml<sup>-1</sup>, 41 times lower than the previously reported IC<sub>50</sub> (56.08 U ml<sup>-1</sup>) of IFN- $\alpha$ 2b. IFN- $\beta$  inhibition was confirmed in the virus yield reduction assay, with an IC<sub>90</sub> of 38.8 U ml<sup>-1</sup>. Ribavirin did not inhibit viral replication *in vitro* at a dose that would be applicable to current treatment protocols in humans. In contrast, MPA showed strong inhibition, with an IC<sub>50</sub> of 2.87  $\mu$ M. This drug has not been previously tested against MERS-CoV and may provide an alternative to ribavirin for treatment of MERS-CoV. In conclusion, IFN- $\beta$ , MPA or a combination of the two may be beneficial in the treatment of MERS-CoV or as a post-exposure intervention in high-risk patients with known exposures to MERS-CoV.

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## INTRODUCTION

In June 2012, a novel betacoronavirus was isolated from a fatal case of pneumonia with renal failure (Zaki *et al.*, 2012). Owing to the genetic similarities as well as the development of respiratory disease, the new virus was commonly referred to as severe respiratory syndrome coronavirus (SARS)-like virus. The virus was later renamed Middle East respiratory syndrome coronavirus (MERS-CoV). MERS-CoV has been confirmed in 150 cases with 64 deaths to date (as of 4 November 2013; World Health Organization, 2013). Whilst the majority of the cases have occurred in the Kingdom of Saudi Arabia, cases have been identified across the Arabian Peninsula (Qatar, Jordan and United Arab Emirates). Imported cases have also been identified in the UK, Italy, Spain, Germany and Tunisia. Importantly, there are no approved medical countermeasures

for MERS-CoV disease, and thus case management has relied on supportive care, contact tracing, monitoring of close contacts and appropriate infection control (ISARIC, 2013).

Appreciable efforts have been made to identify novel antiviral therapeutics for MERS-CoV. It has been demonstrated that compounds targeting host effectors can be beneficial when administered alone or in combination with antivirals during the course of viral infection (Josset *et al.*, 2010; Ludwig, 2011; Tisoncik *et al.*, 2012). Falzarano *et al.* (2013a, b) recently reported that the administration of IFN- $\alpha$ 2b and ribavirin resulted in synergistic antiviral activities both *in vitro* and *in vivo* in rhesus macaques.

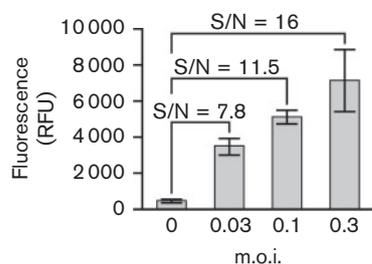
This paper expands on reported studies to evaluate the efficacy of a variety of IFNs and mycophenolic acid (MPA)

for the inhibition of MERS-CoV infection *in vitro* (Chan *et al.*, 2013a, 2013b; de Wilde *et al.*, 2013; Kindler *et al.*, 2013). Similar to ribavirin, MPA has broad antiviral activities. Whilst the mechanism of action for MPA remains under debate, data suggest that it may have both direct antiviral activity and indirect activity through modulation of IFN response activities (Henry *et al.*, 2006; Khan *et al.*, 2011; Leyssen *et al.*, 2005; Morrey *et al.*, 2002; Pan *et al.*, 2012; Smee *et al.*, 2001). Here, we applied a cell-based ELISA screen to test the activity of MPA and the IFN products and compared them with ribavirin and IFN- $\alpha$ 2b *in vitro*.

## RESULTS

A cell-based ELISA was developed to screen candidate antivirals for MERS-CoV. To optimize this assay, Vero E6 cells were inoculated with MERS-CoV (Hu/Jordan-N3/2012) at an m.o.i. of 0.03, 0.1 or 0.3 for 48 h. Viral antigen was detected with an antibody specific to the MERS-CoV viral spike protein S and with an Alexa Fluor 594-conjugated secondary antibody. Data from over 10 experiments indicated that an m.o.i. of 0.1 consistently demonstrated a robust fluorescent signal with signal-to-noise ratios in the range of 6–16 with no visible cell death at 48 h (Fig. 1). Based on these results, an m.o.i. of 0.1 was used in all subsequent MERS-CoV ELISA drug screens.

The MERS-CoV ELISA screen was first used to evaluate four compounds, MPA, ribavirin, IFN- $\alpha$ 2a and IFN- $\beta$ . Subsequently, a set of different IFNs (IFN- $\alpha$ 2b, IFN- $\gamma$ , IFN-universal, IFN- $\alpha$ 2a and IFN- $\beta$ ) was compared in the MERS-CoV ELISA screen. Vero E6 cells were treated with threefold dilutions of MPA, ribavirin, IFN- $\alpha$ 2a or IFN- $\beta$  approximately 1 h prior to infection with MERS-CoV. Following 48 h incubation, the cells were fixed and stained, and nine image fields per well were acquired with an Operetta high-content imaging platform (Fig. 2). Use of



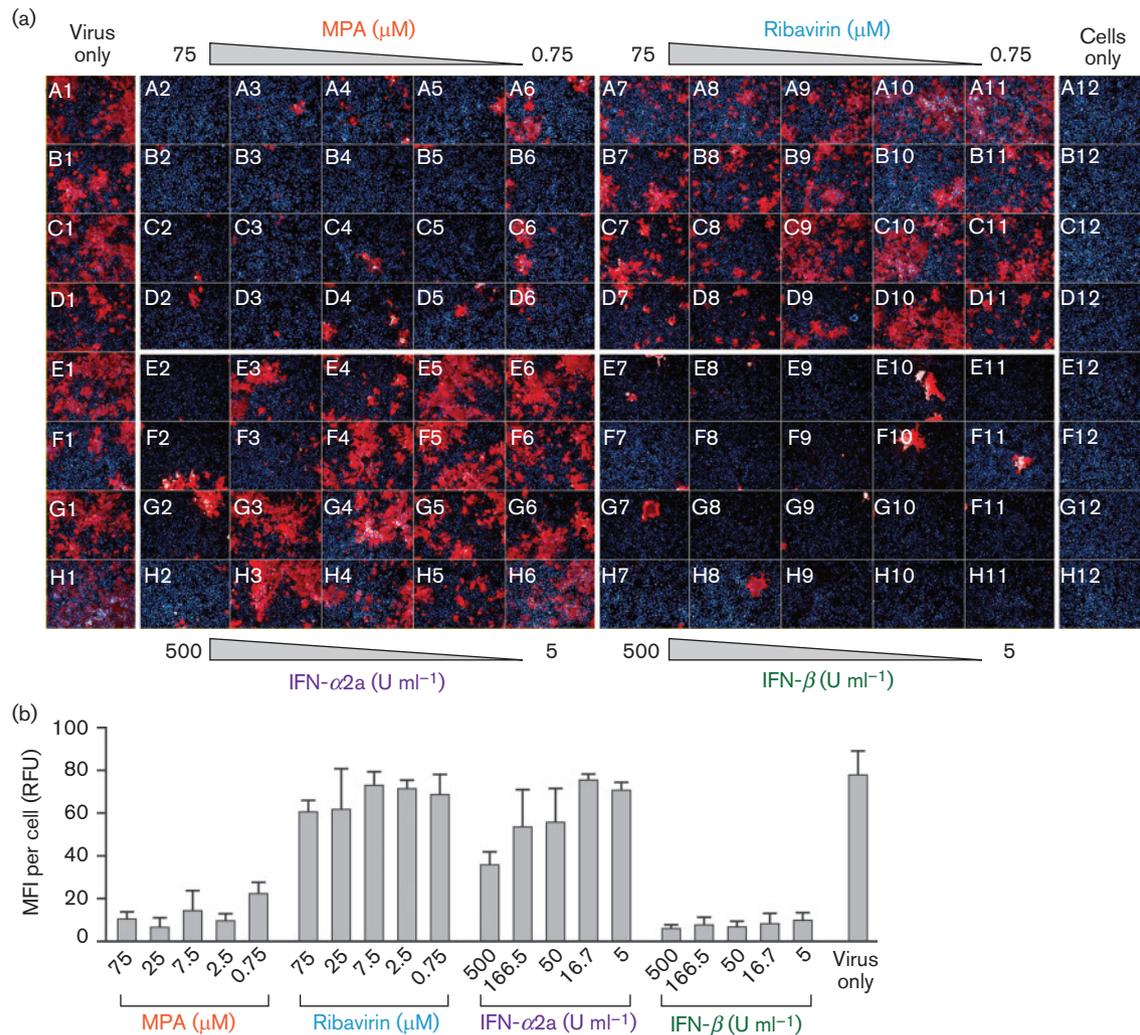
**Fig. 1.** Optimization of the cell-based ELISA screen for MERS antivirals. Vero E6 cells were infected at the indicated m.o.i. to optimize the relative fluorescence signal intensity in arbitrary relative fluorescence units (RFU) at 48 h. Signal-to-noise ratios (S/N) between mock-infected cells (noise; m.o.i. 0) and infected cells (signal; m.o.i. 0.03, 0.1, or 0.3) are shown for the indicated m.o.i. Results shown are representative of one experiment (mean  $\pm$  SD,  $n=4$ ) out of at least 10 experiments.

the Operetta provides the advantage of monitoring different fluorescence parameters within the same well. Hoechst 33342 nuclei staining was used to determine cell numbers in each well to quantify the cytotoxic effect, and Alexa Fluor 594-conjugated secondary antibody bound to a MERS-CoV S protein-specific primary antibody was used to determine the percentage of infected cells per well. In the wells containing virus alone, 46–66% of the cells were positive for viral antigen when left untreated (Fig. 2a, left column). MPA treatment had a significant inhibitory effect on MERS-CoV replication, whilst ribavirin did not show inhibition at the tested concentrations (Figs 2b and 3). Further analysis at lower concentrations confirmed strong inhibition of MERS-CoV replication with MPA at an  $IC_{50}$  of 2.87  $\mu$ M (Fig. 4). Ribavirin exhibited activity only at concentrations of 250  $\mu$ M or higher (Fig. 4). This inhibition of MERS-CoV with MPA is in contrast to the reported inability of MPA to inhibit SARS-CoV infection with MPA (Barnard *et al.*, 2006).

Strong anti-MERS-CoV activity was observed in the lower dose range (starting at 5 U  $ml^{-1}$ , Figs 2b and 3). A detailed comparison of various IFN products demonstrated that antiviral activity of IFN- $\beta$  ( $IC_{50}=1.37$  U  $ml^{-1}$ ) was 16-, 41-, 83- and 117-fold higher than those of IFN- $\alpha$ 2b, IFN- $\gamma$ , IFN-universal type 1 and IFN- $\alpha$ 2a, respectively (Fig. 5). The ability of IFN- $\beta$  to inhibit MERS-CoV growth was confirmed in a virus yield reduction assay (Fig. 6). Vero E6 cells were infected at an m.o.i. of 0.1 and incubated in the presence of IFN- $\beta$  (10–1000 U  $ml^{-1}$ ) for 48 or 72 h. IFN- $\beta$  reduced MERS-CoV yield very effectively with an  $IC_{90}$  and  $IC_{99}$  of 39 and 426 U  $ml^{-1}$ , respectively, at 48 h, approximately ten and four times lower, respectively, than previously reported for IFN- $\alpha$ 2b (Falzarano *et al.*, 2013b). At 72 h, IFN- $\beta$  retained the ability to reduce MERS-CoV yield up to 99.9% at the higher concentrations (100–1000 U  $ml^{-1}$ ) tested.

## DISCUSSION

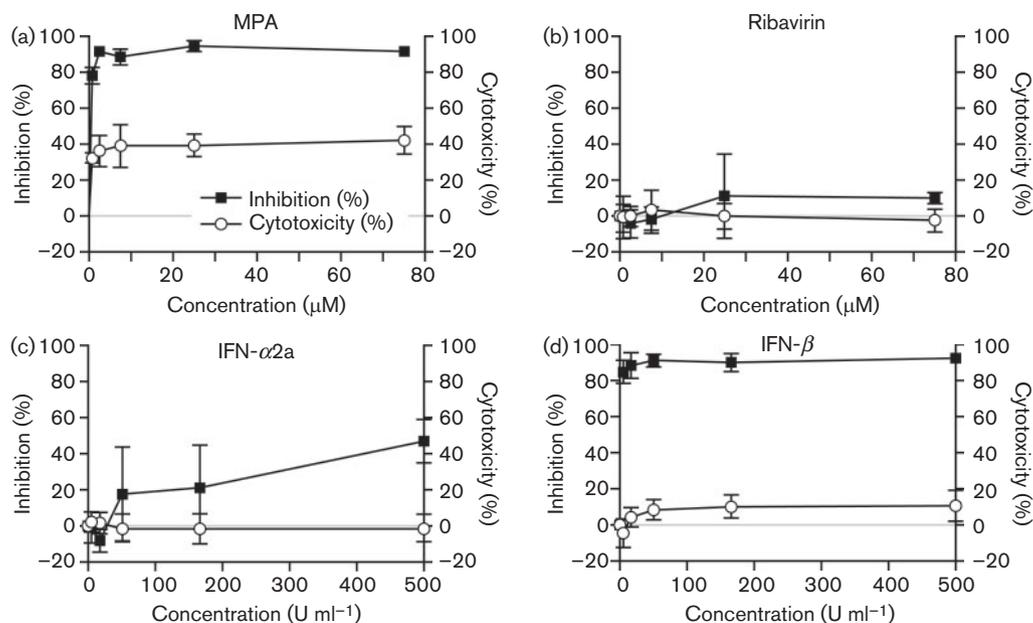
Since the emergence of MERS, several potential treatments for clinical patients have been reviewed and recommended by the International Severe Acute Respiratory and Emerging Infection Consortium (Brown *et al.*, 2013). Whilst neutralizing antibody-based treatments such as convalescent plasma are considered to have the most probable beneficial effect, such plasma is limited in availability. The repurposing of Food and Drug Administration (FDA)-approved drugs typically provides the most viable treatment option during emergency situations if efficacy can be demonstrated. Previous *in vitro* and *in vivo* studies have indicated that IFN- $\alpha$ 2b alone, or in combination with ribavirin, could have a clinical effect if given early in the disease course (de Wilde *et al.*, 2013; Falzarano *et al.*, 2013a, b). Here, we demonstrated that IFN- $\beta$  showed even higher (16 times) biological activity against MERS-CoV infection *in vitro* than IFN- $\alpha$ 2b. In addition, we also demonstrated that another broad-spectrum antiviral,



**Fig. 2.** High content imaging of the MPA, ribavirin, IFN- $\alpha$ 2a and IFN- $\beta$  dose-response in MERS-CoV-infected Vero E6 cells. Vero E6 cells were treated with threefold dilutions of MPA and ribavirin (0.75–75  $\mu\text{M}$ ), and threefold dilutions of IFN- $\alpha$ 2a and IFN- $\beta$  (5–500  $\text{U ml}^{-1}$ ), and subsequently infected at an m.o.i. of 0.1 with MERS-CoV. (a) At 48 h post-inoculation, cells were fixed and stained with antibody to MERS-CoV S protein and Alexa Fluor 594-conjugated secondary antibody. High content imaging analysis (Operetta, Harmony 3.1) was performed to determine the percentage of infected cells per well, and Hoechst 33342 staining was used to determine the number of viable cells in each well. One out of nine acquired fields per well is shown. The left-hand column shows the positive control of cells infected with MERS-CoV without drug treatment. The right-hand column shows the negative control of uninfected viable cells without drug treatment. (b) Quantification of relative fluorescence intensity from fluorescence microscopic images shown in (a). Mean fluorescence intensity (MFI) was measured in relative fluorescence units (RFU) and normalized to the number of cells per well. Results are representative of one experiment (mean  $\pm$  SD,  $n=4$ ). The experiment was repeated at least twice.

MPA, was effective against MERS-CoV infection *in vitro*. Furthermore, MERS-CoV was susceptible to MPA inhibition with an  $\text{IC}_{50}=2.87 \mu\text{M}$ . Importantly, both IFN- $\beta$  and MPA have been approved by the FDA for other indications and currently are in use. As a result, both of these drugs are readily available and can be used off label at the discretion of the clinician. There are also data in the literature to indicate that improved efficacy and potential synergy can be achieved when these drugs are combined.

Similar to ribavirin, there have been a number of proposed mechanisms of action for MPA. Previously, MPA treatment was shown to induce the expression of IFN-stimulated genes, including IFN regulatory factor 1, suggesting that the antiviral activity of MPA is dependent on the modulation of both inosine 5'-monophosphate dehydrogenase activity and IFN-stimulated gene expression (Pan *et al.*, 2012). These data also support the hypothesis that MPA may increase the responsiveness of cells to

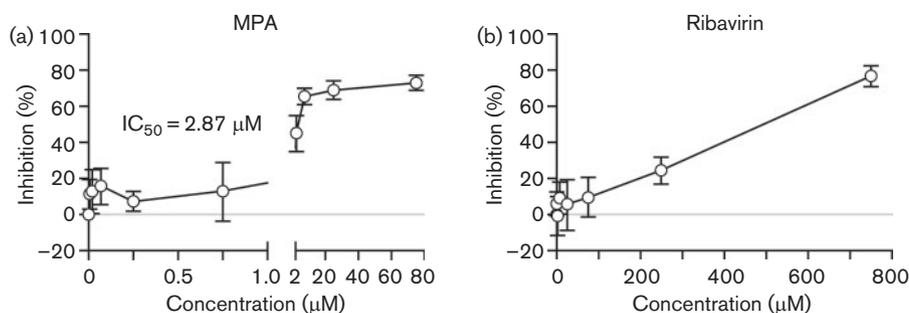


**Fig. 3.** Comparison of test compounds MPA (a), ribavirin (b), IFN- $\alpha$ 2a (c) and IFN- $\beta$  (d) for inhibition of viral replication and cell cytotoxic effects. The MERS-CoV screen was performed as described in Fig. 2. The MFI of infected cells was determined using Harmony 3.1 software and the percentage inhibition of treated wells was normalized to uninfected control wells and measured relative to untreated wells. Viable cell numbers were used to determine the percentage cytotoxic effect in infected/treated wells relative to uninfected/untreated wells. Results are representative of one experiment (mean  $\pm$  SD,  $n=4$ ). The experiment was repeated at least twice.

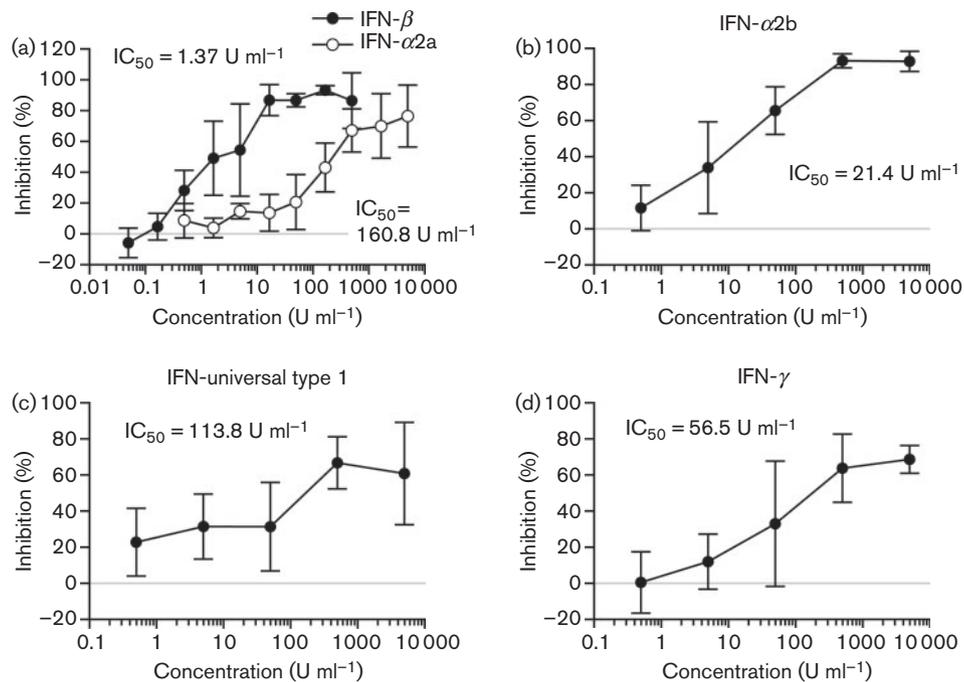
IFN treatment and, when combined with IFN, may act synergistically to reduce viral loads. To account for this possibility, the initial screens presented in this paper were performed in Vero cells. Vero cells, whilst responsive to IFN, cannot produce IFN. As such, the data here suggested that the observed MPA activity is not through induction or sensitization of cells to IFN. Future analysis will assess the mechanism of action for the MPA inhibitory effect on

MERS-CoV infection and possible additive or synergistic effects when combined with IFN.

In conclusion, the data presented here demonstrated that IFN- $\beta$  and MPA, or a combination of the two drugs, should be considered for the treatment of MERS-CoV-infected patients. Previously published reports demonstrating IFN antagonist activity of MERS-CoV support



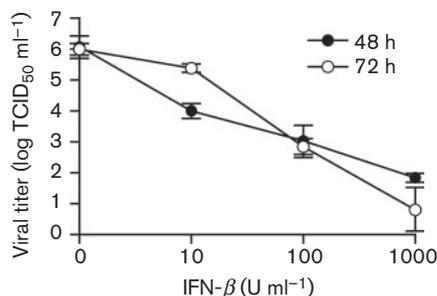
**Fig. 4.** Antiviral activity of MPA and ribavirin. Vero E6 cells were treated with threefold dilutions of MPA (0–75 μM) (a) or ribavirin (0–750 μM) (b) and subsequently infected at an m.o.i. of 0.1 with MERS-CoV. At 48 h post-inoculation, cells were fixed and stained with antibody to MERS-CoV S protein and Alexa Fluor 594-conjugated secondary antibody. Fluorescence was quantified on a plate reader and the percentage inhibition of treated wells was normalized to uninfected control wells and measured relative to untreated wells. Results are representative of one experiment (mean  $\pm$  SD,  $n=4$ ). The experiment was repeated at least twice.



**Fig. 5.** Antiviral activity by various IFN types. Vero E6 cells were treated at indicated concentrations of IFN- $\beta$  and IFN- $\alpha$ 2a (a), IFN- $\alpha$ 2b (b), IFN-universal (c) or IFN- $\gamma$  (d) and subsequently infected at an m.o.i. of 0.1 with MERS-CoV. At 48 h post-inoculation, cells were fixed and stained with antibody to MERS-CoV S protein and Alexa Fluor 594-conjugated secondary antibody. The percentage inhibition of treated wells was normalized to uninfected control wells and measured relative to untreated wells. Results are representative of one experiment (mean  $\pm$  SD,  $n=4$ ). The experiment was repeated at least twice.

the hypothesis that control of the host innate immune response, in particular the IFN response, is critical for survival of the virus. Early intervention with the use of exogenous IFNs alone or in combination with direct antivirals prior to complete subversion of the host's

immune response may provide a viable treatment option. In addition, the data presented here, in combination with the current state of knowledge of MERS-CoV, suggest that exogenous IFNs may also provide an option for intervention in high-risk individuals with known exposure to MERS-CoV.



**Fig. 6.** Reduction in virus yield by IFN- $\beta$ . Vero E6 cells were infected at an m.o.i. of 0.1 with MERS-CoV. At 1 h after inoculation, inoculum was removed, and cells washed and treated with the indicated concentrations of IFN. At 48 and 72 h, supernatants were titrated in the infectivity assay. Results are representative of one experiment (mean  $\pm$  SD,  $n=3$ ). The experiment was repeated at least twice.

## METHODS

**Cell lines and virus.** Vero E6 cell line (ATCC 1568) was maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS. The Jordan strain of MERS-CoV (Hu/Jordan-N3/2012, GenBank accession no. KC776174.1; de Groot *et al.*, 2013), kindly provided by Drs Kanta Subbarao (National Institutes of Health, Bethesda, MD, USA) and Gabriel Defang [Naval Medical Research Unit 3 (NAMRU-3), Cairo, Egypt], was amplified in Vero E6 cells at an m.o.i. of 0.01. On day 4 after infection, when a cytopathic effect was visible, virus-containing supernatants were collected and clarified by centrifugation. MERS-CoV was titrated on Vero E6 cells by plaque assay.

**Cell-based ELISA screen for MERS antiviral agents.** Vero E6 cells were seeded using 40 000 cells in 100  $\mu$ l DMEM plus 10% FBS per well in black-, opaque- or clear-bottomed 96-well plates. Test compounds MPA and ribavirin were obtained from Sigma-Aldrich, and IFN- $\beta$ , IFN- $\alpha$ 2a, IFN- $\alpha$ 2b, IFN- $\gamma$  and a recombinant product based on the consensus sequence of the IFN- $\alpha$  subtypes designated 'universal type 1 IFN' were obtained from PBL. After 24 h, five dilutions of test compounds were added to the cells in 50  $\mu$ l using

a 96-well liquidator (Rainin Instrument). The cell plates were transferred to the containment laboratory to add MERS-CoV (Hu/Jordan-N3/2012) at an m.o.i. of 0.1 in 50 µl DMEM plus 10% FBS approximately 1 h after the addition of the drugs. After 48 h, plates were fixed with 10% neutral-buffered formalin and removed from biocontainment. MERS-CoV was detected with a rabbit polyclonal antibody to the HCoV-EMC/2012 S protein (Sino Biological) followed by staining with Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) antibody (Life Technologies). Nuclei were detected with the Hoechst 33342 dye, which stains DNA. For Alexa Fluor 594, fluorescence was quantified on a plate reader (Infinite M1000 Pro; Tecan US) with an excitation wavelength of 590 nm and an emission wavelength of 617 nm. The Operetta high content imaging system (PerkinElmer) and analysis software (Harmony 3.1) was used to quantify fluorescence of both dyes, Alexa Fluor 594 and Hoechst 33342. Wavelengths of 360–400 and 560–580 nm were used to excite Hoechst 33342 dye and Alexa Fluor 594 dye, respectively. Emission wavelengths of 410–480 and 590–640 nm were used to detect Hoechst 33342 and Alexa Fluor 594 fluorescence, respectively. Operetta software was used to set the threshold for background versus MERS-CoV-positive cells and determine the mean fluorescence intensity (MFI) of MERS-CoV-positive cells in nine fields per well at  $\times 20$  magnification. MFI per cell was determined by normalizing the MFI to the total cell number in each well. The percentage inhibition of treated wells (TREAT) compared with untreated wells (UNTR) was determined using the formula: % inhibition =  $[1 - (\text{TREAT} - \text{normal}) / (\text{UNTR} - \text{normal})] \times 100$ . The signal from treated wells was normalized to uninfected control wells (normal) and measured (as a %) relative to untreated wells. Non-linear regression analysis was performed to calculate  $IC_{50}$  values (GraphPad Software). The MERS-CoV ELISA drug screen was carried out with four replicates for each drug concentration and the assay was repeated at least twice for confirmation. Error bars for dose–response curves represent the SD of four replicates.

**Cytotoxicity assay.** To evaluate the cytotoxicity of the drugs, Vero E6 cells were plated and treated with the drugs at the same concentrations used for detection of MERS-CoV replication inhibition, as described above for the cell-based MERS-CoV ELISA drug screen, but were not infected with virus. At 48 h after drug addition, the plates were analysed using a CellTiter Glo luminescent cell viability assay kit (Promega), and luminescence was read on an Infinite M1000 Pro plate reader. Alternatively, the fluorescent stain Hoechst 33342 (Life Technologies) was used to determine cell numbers in each well to quantify the cytotoxic effect.

**Virus yield reduction assay.** Vero E6 cells were seeded in 12-well plates at 200 000 cells per well. After overnight incubation, the cells were infected in triplicate with MERS-CoV strain Hu/Jordan-N3/2012 at an m.o.i. of 0.1 and incubated at 37 °C for 1 h with shaking every 15 min. The inoculum was removed, the cells were washed with PBS and fresh medium was added containing 10-fold dilutions of IFN- $\beta$  (10–1000 U ml<sup>-1</sup>) for 48 and 72 h. Supernatants were collected and titrated using the infectivity assay. The assay was carried out with three replicates for each drug concentration and was repeated at least twice for confirmation. Error bars of dose–response curves represent the SD of three replicates.

**Infectivity assay.** Vero E6 cells were infected in eight replicates with 10-fold dilutions of supernatants from the virus yield reduction assay. MERS-CoV was absorbed for 1 h, and then removed and replaced with DMEM plus 10% FBS. Cells were incubated at 37 °C, 5% CO<sub>2</sub>, in a humidified environment for 6 days. The cytopathic effect in wells was scored by fixing and staining the cells with crystal violet. The TCID<sub>50</sub> was calculated with Microsoft Excel as described by Reed & Muench (1938).

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