Interferon-β 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-α 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-α2b, IFN-γ, IFN-universal, IFN-α2a and IFN-β), 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-β showed the strongest inhibition of MERS-CoV in vitro, with an IC₅₀ of 1.37 U ml⁻¹, 41 times lower than the previously reported IC₅₀ (56.08 U ml⁻¹) of IFN-α2b. IFN-β inhibition was confirmed in the virus yield reduction assay, with an IC₉₀ of 38.8 U ml⁻¹. 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 IC₅₀ of 2.87 μM. 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-β, 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.

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-α2b 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-x2b 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-x2a and IFN-β. Subsequently, a set of different IFNs (IFN-x2b, IFN-γ, IFN-universal, IFN-x2a and IFN-β) was compared in the MERS-CoV ELISA screen. Vero E6 cells were treated with threefold dilutions of MPA, ribavirin, IFN-x2a or IFN-β 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 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 IC50 of 2.87 μM (Fig. 4). Ribavirin exhibited activity only at concentrations of 250 μM 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-β (IC50=1.37 U ml−1) was 16-, 41-, 83- and 117-fold higher than those of IFN-x2b, IFN-γ, IFN-universal type 1 and IFN-x2a, respectively (Fig. 5). The ability of IFN-β 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-β (10–1000 U ml−1) for 48 or 72 h. IFN-β reduced MERS-CoV yield very effectively with an IC90 and IC99 of 39 and 426 U ml−1, respectively, at 48 h, approximately ten and four times lower, respectively, than previously reported for IFN-x2b (Falzarano et al., 2013b). At 72 h, IFN-β 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-x2b 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-β showed even higher (16 times) biological activity against MERS-CoV infection in vitro than IFN-x2b. In addition, we also demonstrated that another broad-spectrum antiviral,
MPA, was effective against MERS-CoV infection in vitro. Furthermore, MERS-CoV was susceptible to MPA inhibition with an IC50 of 2.87 μM. Importantly, both IFN-β 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...
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-β 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
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.

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 µl 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-α2a, IFN-α2b, IFN-α2c and a recombinant product based on the consensus sequence of the IFN-α 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 µl 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 3342. Wavelengths of 360–400 and 560–580 nm were used to excite Hoechst 3342 dye and Alexa Fluor 594 dye, respectively. Emission wavelengths of 410–480 and 590–640 nm were used to detect Hoechst 3342 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 ×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 = \[
\left(1 - \frac{\text{TREAT}}{\text{normal}}\right) / \left(1 - \frac{\text{UNTR}}{\text{normal}}\right) \times 100
\]
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 IC50 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 3342 (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 to 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-β (10–1000 U ml−1) 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% CO2, in a humidified environment for 6 days. The cytopathic effect in wells was scored by fixing and staining the cells with crystal violet. The TCID50 was calculated with Microsoft Excel as described by Reed & Muench (1938).

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