Chloroquine inhibited Ebola virus replication in vitro but failed to protect against infection and disease in the in vivo guinea pig model

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Ebola virus (EBOV) is highly pathogenic, with a predisposition to cause outbreaks in human populations accompanied by significant mortality. Owing to the lack of approved therapies, screening programmes of potentially efficacious drugs have been undertaken. One of these studies has demonstrated the possible utility of chloroquine against EBOV using pseudotyped assays. In mouse models of EBOV disease there are conflicting reports of the therapeutic effects of chloroquine. There are currently no reports of its efficacy using the larger and more stringent guinea pig model of infection. In this study we have shown that replication of live EBOV is impaired by chloroquine in vitro. However, no protective effects were observed in vivo when EBOV-infected guinea pigs were treated with chloroquine. These results advocate that chloroquine should not be considered as a treatment strategy for EBOV.

INTRODUCTION

Ebolavirus is a genus of the family Filoviridae and includes five species [Bundibugyo virus, Reston virus, Sudan virus, Tai Forest virus and Ebola virus (EBOV)]. Ebola virus (formerly designated Zaire ebolavirus) is the prototype species (Adams & Carstens, 2012; Kuhn et al., 2010) and has been responsible for the large outbreak of EBOV disease in parts of West Africa which was first recognized in March 2014 (Baize et al., 2014). EBOV is the most virulent species, with a case mortality of up to 90%, whilst the Reston strain is virtually non-pathogenic in humans (Mahanty & Bray, 2004). Owing to the ongoing infection and threat of further outbreaks in the absence of approved and proven therapeutics or vaccines, there has been increased international, political, humanitarian and scientific momentum to identify treatment strategies. Using drugs already licensed for other conditions, which have well-established safety and pharmacokinetic profiles in patients, along with manufacturing and distribution networks, would allow the rapid implementation of novel therapies for EBOV disease. Chloroquine, a widely used antimalarial prophylactic, is one such compound that has arisen from these repurposing drug screens for EBOV (Kouznetsova et al., 2014; Long et al., 2015; Madrid et al., 2013).

Chloroquine has broad-spectrum activity against a range of bacterial, fungal and viral infections (Rolain et al., 2007). In vitro antiviral activity has been demonstrated against many medically important viruses, including human immunodeficiency virus 1 (Savarino et al., 2001), severe acute respiratory syndrome coronavirus (Keyaerts et al., 2004; Vincent et al., 2005), dengue virus (Farias et al., 2014), chikungunya virus (Delogu & de Lamballerie, 2011; Khan et al., 2010), arenaviruses (Pichinde, Mopeia and Lassa) (Glushakova & Lukashevich, 1989), henipaviruses (Hendra and Nipah) (Freiberg et al., 2010; Porotto et al., 2009), Crimean-Congo hemorrhagic fever virus (Ferraris et al., 2015), rabies virus (Tsang & Superti, 1984), poliovirus (Kronenberger et al., 1991), influenza virus (Ooi et al., 2006; Paton et al., 2011) and EBOV (Falzarano et al., 2015; Madrid et al., 2013). There are several proposed mechanisms of action for the antiviral activity of chloroquine. Owing to its lysosomotropic and weak base properties, chloroquine could interfere with endosomal fusion (Tricou et al., 2010). This low pH environment may affect the efficiency of the virus maturation process (Randolph et al., 1990) and the recognition of viral antigen by plasmacytoid dendritic cells, which occurs through a Toll-like receptor-dependent pathway that requires endosomal acidification (Diebold et al., 2004). Other proposed effects on the immune system include increasing the export of soluble antigens into the cytosol of dendritic cells (Accapezzato et al., 2005) and attenuating the inflammatory cytokine response (Jang et al., 2006).

For EBOV, the in vitro antiviral activity of chloroquine has previously been demonstrated using an approach based on a lentivirus pseudotyped with EBOV glycoprotein spikes to infect tissue culture cells. In this study, virus infectivity was determined to be inhibited at the level of virus entry
and linked to the downregulation of the acidification of virus-containing endosomes and subsequently membrane fusion (Long et al., 2015). A low pH is required for two steps of the filovirus entry pathway: fusion by the G protein and its cleavage into a fusogenic form by endosome enzymes cathepsins B and L (Chandran et al., 2005). Chloroquine is known to limit the acidification process of endosomes (Nujic et al., 2012). Other effects of chloroquine have also been suggested, including its ability to bind the EBOV VP35 protein in computer models (Ekins et al., 2014) and notably its effects on the production of several cytokines, including TNF-α, IL-6 and IFN-γ (Savarino et al., 2003), for example the activation blocking of plasmacytoid dendritic cells and MyD88 signalling (Martinson et al., 2010). The role of cytokines in EBOV infection is well recognized, with immune pathology being a feature of fatal disease progression (Wauquier et al., 2010).

To further study the effects of chloroquine against EBOV, an in vitro assay was conducted using a human cell line and in vivo studies were conducted using the well characterized guinea pig model.

## RESULTS

### Chloroquine reduced EBOV replication in MRC-5 cells

For in vitro studies a human cell line was used, MRC-5, which has previously been employed for EBOV infection studies (Garcia-Dorival et al., 2014) and is host-matched for the species in which an intervention is required. At 1 h post-infection with 500 TCID₅₀ per well of a 96-well plate, medium was replaced with fresh medium containing chloroquine. After 3 days, viral RNA levels in the supernatants were quantified and expressed as cycle threshold (Cₜ) values (Table 1). Results demonstrated that the Cₜ values increased, indicative of a reduction in viral replication. When tested at 4.7 and 0.47 μM, the results were statistically significant (P=0.119 and P=0.0292, respectively; Mann–Whitney statistical test); however, at the lower concentration of 0.094 μM, results were not statistically different (P=0.1050, Mann–Whitney statistical test). To confirm the MRC-5 data, the study was repeated with a higher concentration of EBOV (5000 TCID₅₀ per well) and samples harvested 2 days post-infection. Data showed that chloroquine treatment still resulted in lower levels of viral replication as observed by reduced levels of viral RNA in the supernatant (Table 2), although results were not statistically significant (P=0.0809, 0.0809, 0.0809 and 0.6625 for 10, 3.33, 1.11 and 0.37 μM, respectively; Mann–Whitney statistical test). Uninfected cells treated with chloroquine at the same concentrations and tested with EBOV did not result in significant cytotoxicity as assessed by microscopic examination of cell monolayers and subsequent staining of formalin-fixed cell monolayers with crystal violet for visual inspection of cell adherence levels (data not shown).

### Chloroquine did not protect against Ebola disease in EBOV-infected guinea pigs

Twelve guinea pigs were challenged with EBOV, and 6 h afterwards a group of six were administered 33.75 mg kg⁻¹ chloroquine orally. Treatment continued on a twice daily schedule and survival of chloroquine-treated animals was compared with that of six untreated controls. The dose used was based on 90 mg kg⁻¹ being shown to be efficacious against EBOV disease in mice (Madrid et al., 2013), and employing a conversion factor of 0.375 for guinea pigs from guidance provided by the US Food and Drug Administration based on body surface area (Center for Drug Evaluation and Research, 2005). A significantly poorer outcome for treated animals was observed (P=0.001, log-rank survival analysis) (Fig. 1a). The chloroquine treatment also resulted in a more rapid loss in body weight than for untreated control animals (Fig. 1b). Increases in body temperature occurred to a similar extent in both chloroquine-treated and untreated animals to day 5; however, thereafter body temperatures began to decrease back to baseline levels in the treated animals (Fig. 1c). Clinical signs were measured twice a day and a numerical value was assigned to each sign and recorded to aid analysis. Chloroquine resulted in a rapid progression of severity of illness after 5 days post-challenge (Fig. 1d).

In a separate study, guinea pigs were scheduled to be treated with chloroquine at 33.75 mg kg⁻¹ via the intravenous

### Table 1. Differences in Cₜ values of viral RNA levels after incubation of EBOV in the presence of chloroquine at 4.7, 0.47 and 0.094 μM on MRC-5 cells for 3 days

<table>
<thead>
<tr>
<th>Chloroquine concentration (μM)</th>
<th>4.7</th>
<th>0.47</th>
<th>0.094</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values represent mean change in Cₜ compared with untreated samples ± SD.</td>
<td>3.81 ± 0.41</td>
<td>3.06 ± 0.30</td>
<td>1.99 ± 0.67</td>
</tr>
</tbody>
</table>

### Table 2. Differences in Cₜ values of viral RNA levels after incubation of EBOV in the presence of chloroquine at 10, 3.33, 1.11 and 0.37 μM on MRC-5 cells for 2 days

<table>
<thead>
<tr>
<th>Chloroquine concentration (μM)</th>
<th>10</th>
<th>3.33</th>
<th>1.11</th>
<th>0.37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values represent mean change in Cₜ compared with untreated samples ± SD.</td>
<td>0.53 ± 0.26</td>
<td>0.45 ± 0.27</td>
<td>0.24 ± 0.05</td>
<td>0.08 ± 0.17</td>
</tr>
</tbody>
</table>
route, since oral administration was not well tolerated in the guinea pigs, with some animals regurgitating the compound repeatedly prior to swallowing. However, the first two animals that received intravenous chloroquine died within 30 s of administration, so the study was aborted.

Chloroquine treatment had no effect on viraemia in EBOV-infected guinea pigs

At day 8 post-challenge, a sample of blood was taken from animals remaining in the study to assess viraemia. Only two animals from the chloroquine-treated group remained alive, along with all six untreated animals. There was no statistically significant difference in viral RNA levels between the chloroquine-treated and untreated groups \((P=0.0668, \text{Mann–Whitney statistical test})\), although the data indicated higher levels of viral RNA in the chloroquine-treated group (Fig. 2a). Blood was also sampled at necropsy, when animals met humane clinical end points or were culled at the scheduled end of the study. Viral RNA levels at the time of euthanasia were not significantly different between the two groups \((P=0.0656, \text{Mann–Whitney statistical test})\) (Fig. 2b).

Similar severity of histological changes was present in chloroquine-treated guinea pigs and untreated controls

Spleen and liver samples were assessed for histological changes and severity of lesions was recorded. In addition, the presence of viral antigen in these organs was analysed by immunohistochemical staining (Table 3). Lesions associated with EBOV infection were observed in the liver and spleen of all animals treated with chloroquine and in five of the six untreated animals. Viral antigen was present in all samples from the chloroquine-treated

Fig. 1. Survival and clinical signs of EBOV-challenged guinea pigs treated with oral chloroquine compared with untreated animals. (a) Survival analysis between chloroquine-treated animals and untreated animals. (b) Weight changes showing percentage difference compared with the day of challenge. (c) Temperature difference in animals compared with baseline taken on the day of challenge. (d) Clinical score of animals post-challenge. (b–d) Mean results are shown of animals still surviving in all groups, with error bars denoting SE. Group sizes of six guinea pigs were used.
group and five out of six animals in the untreated group. None was found in animal 98 563, which survived until the scheduled end of the study. Immunostaining showed diffuse positivity of infected cells throughout the spleen of chloroquine-treated and untreated animals (Fig. 3a, b, respectively). In the liver, the virus was generally observed in larger foci throughout the samples in both chloroquine-treated and untreated animals (Fig. 3c, d, respectively). The histological findings confirm that chloroquine failed to exert any protective effects on Ebola disease pathogenesis in EBOV-challenged guinea pigs.

**DISCUSSION**

In the present study, the results demonstrate that chloroquine reduced the viral replication of EBOV in vitro, adding to the body of evidence of its broad-spectrum antiviral activity when tested on mammalian cells. The current results show activity in the human cell line used, MRC-5. However, the data confirm findings from recent reports, which have demonstrated antiviral activity of chloroquine against EBOV using an African Green monkey cell line, Vero E6, infected with a transgenic EBOV expressing GFP (Falzarano et al., 2015; Madrid et al., 2013). Analysis using a quantitative reverse transcriptase PCR (RT-PCR) approach enabled a rapid readout, which was preferable for initial screening assays at Containment Level 4 (CL-4) and has been used previously (Falzarano et al., 2015). In the present studies, chloroquine was added 1 h post-infection of the cells, in line with similar studies looking at the effects of the drug on EBOV (Falzarano et al., 2015). Time-of-addition experiments with human and avian influenza viruses demonstrated that the inhibitory effect of chloroquine was maximal when the drug had been added at the time of infection and was lost 2 h post-infection (Di Trani et al., 2007).

In the MRC-5 cells, the maximum reduction in viral RNA levels observed was approximately $3C_\text{r}$, which is estimated to be equivalent to 1-log, based on in-house assay validation data using TCID$_{50}$ assay and those of others using dilution of RNA (Towner et al., 2004). Using a pseudotype murine leukemia virus expressing EBOV glycoprotein, 200 µM chloroquine reduced the titre to 0.01 % of the untreated control (Wool-Lewis & Bates, 1998). The highest concentration of chloroquine used for the in vitro assays was 10 µM, as higher concentrations caused a cytopathic effect in the MRC-5 cells. This concentration was at a similar level to other reported studies assessing chloroquine against Ebola. Using a lentivirus-based pseudotype approach, inhibition of viral entry from EBOV glycoprotein-coated viruses was confirmed, with an IC$_{50}$ of 3.319 µM (Long et al., 2015). A repurposing screen of drugs approved by the US Food and Drug Administration, based on an Ebola virus-like particle entry assay,

Fig. 2. Viral RNA levels in blood in EBOV-challenged guinea pigs treated with chloroquine compared with untreated controls. (a) Samples taken at day 8 post-challenge. Means values are plotted with the error bar denoting se. (b) Samples taken at the time of necropy, when animals met humane clinical end points, except for animal 98 583, which was culled at the scheduled end of the study on day 18 post-challenge.
Table 3. Summary of severities from histological analysis of guinea pig spleen and liver samples from EBOV-challenged guinea pigs treated with chloroquine or untreated

<table>
<thead>
<tr>
<th>Group 1: chloroquine</th>
<th>Group 2: untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal identification (day of necropsy)</td>
<td>Animal identification (day of necropsy)</td>
</tr>
<tr>
<td>98604 (8)</td>
<td>98578 (6)</td>
</tr>
<tr>
<td>98562 (8)</td>
<td>98582 (6)</td>
</tr>
<tr>
<td>99119 (11)</td>
<td>9870 (12)</td>
</tr>
<tr>
<td>98563 (8)</td>
<td>98517 (11)</td>
</tr>
<tr>
<td>98585 (10)</td>
<td>98564 (18)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Lesion</th>
<th>Animal identification (day of necropsy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Hepatocyte vacuolation</td>
<td>Min, mild, minimal</td>
</tr>
<tr>
<td></td>
<td>Portal inflammation</td>
<td>Mod, mild</td>
</tr>
<tr>
<td></td>
<td>Focal necrosis</td>
<td>Mod, mild</td>
</tr>
<tr>
<td></td>
<td>Focal mineralization</td>
<td>WNL, mild</td>
</tr>
<tr>
<td>Spleen</td>
<td>Splenic red pulp congestion</td>
<td>Mild, minimal</td>
</tr>
<tr>
<td></td>
<td>Scattered single-cell necrosis</td>
<td>Mod, mild</td>
</tr>
<tr>
<td></td>
<td>Splenic white pulp</td>
<td>Min, mild</td>
</tr>
<tr>
<td></td>
<td>Presence of viral antigen</td>
<td>Yes, yes</td>
</tr>
</tbody>
</table>

| Min, minimal; Mod, moderate; Mkd, marked; WNL, within normal limits. |

Testing chloroquine using the stringent and well-established guinea pig model indicated no evidence of protective effect after oral administration starting at 6 h post-challenge. Six animals treated with chloroquine and five untreated control animals had generally minimal to marked changes in the liver and spleen, with the presence of viral antigen as demonstrated by immunostaining. These findings are in line with recent reports in other animal models of EBOV; in mice the treatment had no significant effect on survival, and in hamsters the experiment was terminated owing to acute toxicity of the drug after intraperitoneal injection, typically within 30 min post-treatment (Falzarano et al., 2015). To overcome this toxicity, in one study a lower dose of 50 mg kg\(^{-1}\) chloroquine was used in addition to delivery of broad-spectrum antimicrobials (doxycycline and azithromycin), with the hypothesis that reperfusion injury of the gut might be involved, leading to bacterial sepsis (Falzarano et al., 2015). Nevertheless, despite the hamsters tolerating this dosing regime there were no positive effects on survival (Falzarano et al., 2015). When previously tested in mice, chloroquine was shown to exert a protective effect, although 100 % survival was not observed (Madrid et al., 2013). In this latter study, chloroquine was administered 4 h before challenge; thus, it might be postulated that treatment with chloroquine prior to challenge is required in order to demonstrate any positive effects. However, differences between the two studies exist with regard to the toxicity of the chloroquine in uninfected mice; whereas Madrid et al. (2013) reported that all mice survived a twice daily repeat dosing at 90 mg kg\(^{-1}\) for a period of 8 days, Falzarano et al. (2015) reported that two of three mock-challenged mice did not survive because of chloroquine treatment alone, given at the same concentration of 90 mg kg\(^{-1}\); intraperitoneally but on one occasion. The same mouse strain and a similar age range were used in the two studies, so these discrepancies remain unexplained.

Owing to the poor results of the oral delivery of chloroquine, intravenous delivery of chloroquine was attempted to determine whether direct inoculation into the circulation would provide benefit. However, when the chloroquine was delivered intravenously, it resulted in rapid death. Severe toxicity of intravenous chloroquine delivery was shown in human volunteers given 300 mg doses (about 4.5–5 mg kg\(^{-1}\)) in an infusion over 25 min, where plasma levels rose rapidly to 1000 ng ml\(^{-1}\) and every volunteer developed side effects, which included dizziness, demonstrating that chloroquine was effective, with an IC\(_{50}\) of 15.3 µM (Kouznetsova et al., 2014). Likewise, a vesicular stomatitis virus pseudotype approach showed 50 % effective concentration (EC\(_{50}\)) values of 4.7 and 16 µM for chloroquine entry and replication, respectively (Madrid et al., 2013), and EC\(_{50}\) was shown to be 1.77 µg ml\(^{-1}\) using a live Ebola virus (Falzarano et al., 2015). These concentrations fall within the clinically achievable ranges in plasma during malaria therapy, which varies from 1.6 to 12.5 µmol l\(^{-1}\) (Ducharme & Farinotti, 1996).
diplopia, difficulty in swallowing, muscle weakness, nausea and tiredness (Gustafsson et al., 1983). In another clinical study, doses of 3 mg kg$^{-1}$ were delivered intravenously, diluted with 200 ml 0.9 % saline, and infused over 15 min; although the patients did not report any side effects they were confined to bed for a few hours before and after the injection (Aderounmu et al., 1986).

The finding that chloroquine worked in vitro yet failed to provide evidence of any effect in vivo has been observed with other viruses. A similar observation was shown with influenza A virus, with in vitro activity reported yet minimal effects on influenza infection using in vivo models with mice and ferrets (Vigerust & McCullers, 2007). Chloroquine was further shown not to prevent infection with influenza in a human clinical study (Paton et al., 2011). Additionally, despite in vitro activity of chloroquine against chikungunya virus (Delogu & de Lamballerie, 2011), a double-blind placebo-controlled randomized trial failed to demonstrate any positive effects for treatment of acute chikungunya infections (De Lamballerie et al., 2008). These findings of in vitro activity in the absence of in vivo effects are also observed with Nipah and Hendra viruses, with decreasing survival time in the hamster model compared with untreated controls for both viruses (Freiberg et al., 2010) and being ineffective in the ferret model of Nipah virus infection when delivered 24 h before or 10 h after challenge (Pallister et al., 2009). For dengue virus, positive in vivo effects were seen in the aotus monkey model (Farias et al., 2015), but in a human clinical trial chloroquine did not reduce the duration of viraemia and presence of viral NS1 antigen in the blood of dengue patients (Tricou et al., 2010). In a mouse model of coronavirus infection, chloroquine was shown to be effective; however, the model was newborn mice, with chloroquine acquired transplacentally or via maternal milk (Keyaerts et al., 2009). Possible reasons for the failure of in vitro results to translate into in vivo efficacy could be narrow therapeutic indices, poor penetration into specific tissues and strain effects between viruses (Savarino, 2011).

Overall, the present study provides further evidence to show chloroquine is not a promising therapy for EBOV and confirms the importance of performing studies in relevant animal models of infection. This was a disappointing result, as repurposing of chloroquine would have offered a rapid access route to help treat EBOV patients and aid with efforts at tackling the devastation that EBOV causes affected communities. However, further impetus should now be used to assess other potential therapeutic options.

**METHODS**

*In vitro virus assay.* Chloroquine phosphate (Selleckchem) was diluted to 5 mM with sterile water before further dilution to the required concentration with Eagle’s minimum essential medium (Sigma). Concentrations were made at double the final dilution to take into account an equal volume of virus suspension to be added. MRC-5 cells (obtained from the European Collection of Cell Cultures,
UK) were seeded into 96-well plates. Within the CL-4 laboratory, medium was removed from the inner wells of the 96-well plates. Owing to edge-effects, the outer wells were left with medium added. Five replicates were used per dilution.

For the first in vitro screen, EBOV suspension [strain ME718, recently renamed 1976/Yambuku-Ecran (Kuhn et al., 2014)] was added at a concentration of approximately 500 TCID50 per well to triplicate wells per chloroquine dilution, with the remaining two wells having medium alone added to assess for cytotoxicity without the presence of EBOV. Based on results from growth of EBOV in different cell lines, the supernatants from MRC-5 cells were harvested on day 3 post-infection. One hundred and forty microlitres from each well was added to 560 µl AVL buffer (Qiagen) for RNA extraction and PCR assay.

To confirm activity of chloroquine against EBOV, screening assays were repeated for effects against EBOV using MRC-5 cells. However, changes to the previous method comprised of a 10-fold higher viral inoculum being used (approx. 5000 TCID50 per well) and samples were harvested after 2 days.

**RNA extraction and PCR assay.** Samples for RNA extraction were prepared in Qiagen Buffer RLT and the vessel was transferred into MagNA Pure 96 (Roche) compatible 96-well sample plates at CL-3 for thermal inactivation at 60 °C for 30 min prior to removal to CL-2 for RNA purification. Extraction of RNA was performed using the MagNA Pure 96 small-volume RNA kit (Roche), a magnetic bead-based method of RNA separation. In brief, plates were loaded onto the MagNA Pure 96 automated extraction robot and RNA was eluted in 60 µl nuclease-free water. Target amplification was performed using primers to EBOV glycoprotein as described in Trombley et al. (2010) and the Fast Virus quantitative RT-PCR kit (Qiagen). Analysis was performed using an ABI 7500 (Applied Biosystems) with the following cycling conditions: 50 °C for 10 min, 95 °C for 3 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Temperature cycling was set to maximum ramp speed and data were acquired and analysed using the ABI 7500 on-board software with a threshold set to 0.05. C_T values from the PCR assay were used to give a consistent reading of the EBOV RNA levels in the samples.

**Animals.** Female adult Dunkin–Hartley guinea pigs weighing 250–350 g (Harlan Laboratories) were used for animal infection studies. Animals were supplied with catheters inserted into the jugular vein to allow access to the intravenous route to reduce hazards of working at high biological containment (Dowall et al., 2013). For animal procedures, guinea pigs were anaesthetized with 1.5 to 2 % isoflurane in oxygen in an induction chamber until full sedation was achieved. Animals infected with EBOV were housed within an isolator under climate-control conditions in a CL-4 room. Food and sterile water were available ad libitum. All procedures were undertaken according to the UK Animals (Scientific Procedures) Act 1986. A group size of six met a power of 0.8 and alpha at 0.05.

EBOV stock generated from virus that had been passaged in guinea pigs to cause lethality (Dowall et al., 2014) was diluted in sterile PBS to prepare the relevant dose of 10^5 TCID50 virus in a 0.2 ml volume. Surplus inoculation was made to confirm viable virus concentration via back titration in Vero E6 cells. Guinea pigs were sedated and inoculated subcutaneously with the virus suspension in the lower right quadrant of the back before being returned to their cages and monitored for adverse effects caused by the injection of the anaesthetic until they had fully recovered. Chloroquine was administered 6 h post-challenge. Chloroquine diphosphate (Sigma K) was dissolved in sterile water to give a concentration that equated to 33.75 mg kg^-1 in a volume of 1 ml for oral delivery and administered via oral gavage (first study) or intravenously through the catheter (second study).

Animals were uniquely identified, weighed and had temperatures recorded daily via an indwelling temperature chip. Clinical signs were monitored at least twice daily and a numerical score assigned for analysis: 0 (normal), 2 (ruffled fur), 3 (lethargy, pinched, hunched, wasp-waisted), 5 (laboured breathing, rapid breathing, inactive) and 10 (immobile). A set of humane clinical end points was defined (20 % weight loss, or 10 % weight loss and a clinical symptom) that indicated that the animal must be euthanized to prevent unnecessary suffering. Animals were humanely euthanized by intraperitoneal or intravenous injection of 200 mg ml^-1 sodium pentobarbital. Necropsies were performed within a flexible-film isolator in the animal CL-4 facility.

**Viral load.** Blood samples taken at 8 days post-challenge or at necropsy were stored in RNAProtect animal blood tubes (Qiagen). Samples were mixed and stored at −80 °C until processing and to lyse red blood cells. Blood solution (200 µl) was transferred to 600 µl RLT buffer (Qiagen) for RNA extraction and PCR.

**Histology.** Samples of liver and spleen were placed in 10 % neutral-buffered formalin for at least 21 days and processed routinely to paraffin wax. Sections were cut at 3–5 µm, stained with haematoxylin and eosin and examined microscopically. For immunohistochemistry, formalin-fixed, paraffin-embedded sections of spleen and liver, cut at 3–5 µm, were stained for Ebola viral antigen using the Leica BondMax (Leica Biosystems) and the Leica Bond Polymer Refine Detection kit (Leica Biosystems). An antigen retrieval step was included for 10 min using the Bond Enzyme Pretreatment kit enzyme 3 (three drops). A rabbit polyclonal, anti-EBOV VP40 antibody (IBT Bioservices) (dilution 1:2000) was incubated with the slides for 60 min. 3’,3’-Diaminobenzidine chromogen and haematoxylin counterstains were used to visualize the slides.

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