Antiviral therapies against Ebola and other emerging viral diseases using existing medicines that block virus entry [version 2; referees: 2 approved]

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Abstract
Emerging viral diseases pose a threat to the global population as intervention strategies are mainly limited to basic containment due to the lack of efficacious and approved vaccines and antiviral drugs. The former was the only available intervention when the current unprecedented Ebolavirus (EBOV) outbreak in West Africa began. Prior to this, the development of EBOV vaccines and anti-viral therapies required time and resources that were not available. Therefore, focus has turned to re-purposing of existing, licenced medicines that may limit the morbidity and mortality rates of EBOV and could be used immediately. Here we test three such medicines and measure their ability to inhibit pseudotype viruses (PVs) of two EBOV species, Marburg virus (MARV) and avian influenza H5 (FLU-H5). We confirm the ability of chloroquine (CQ) to inhibit viral entry in a pH specific manner. The commonly used proton pump inhibitors, Omeprazole and Esomeprazole were also able to inhibit entry of all PVs tested but at higher drug concentrations than may be achieved in vivo. We propose CQ as a priority candidate to consider for treatment of EBOV.

This article is included in the Ebola collection.
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Introduction

Emerging pathogens such as Ebolaviruses (EBOV), Avian Influenza viruses, Severe Acute Respiratory Syndrome (SARS) virus, Middle-East coronavirus (MERS), Chikungunya virus (CHIKV) and Dengue virus pose public health challenges that demand researchers and governments work together to assess their pandemic potential and plan mitigating strategies. Of the five species of EBOV belonging to the Filoviridae (including Zaire ebolavirus (EBOV-Z), Bundibugyo ebolavirus (EBOV-B), Reston ebolavirus, Sudan ebolavirus (EBOV-S) and Tai Forest ebolavirus\(^1\)), EBOV-Z and EBOV-S are responsible for the majority of outbreaks of highly pathogenic haemorrhagic fevers causing high fatality rates\(^2\).

Past outbreaks have been of limited size affecting a local population, however a strain of EBOV-Z is the causative agent of the current outbreak that began in late 2014 and has since become an unprecedented and devastating epidemic\(^3-5\), resulting in over 20,000 suspected cases, of which those confirmed had a case fatality rate of around 60% in the afflicted West African countries (http://apps.who.int/gho/data/view.ebola-sitrep.ebola-summary-20150107?lang=en and http://www.who.int/csr/disease/ebola/situation-reports/en/). Towards the end of 2014 the trend in case numbers reversed in Liberia and the epidemic slowed in Sierra Leone and Guinea, but the virus continues to transit in new geographical areas\(^6\). This epidemic has triggered a significant global health response relying on primary hygiene and other containment measures that have proved successful in limiting the spread of the virus in previous outbreaks. Given the scale of this outbreak and the fear that traditional containment measures may fail to prevent global spread, several vaccines have been fast-tracked into phase I clinical trials\(^7-9\) although even if proved efficacious, the limited supply of sufficient quantities of vaccine will hinder their use in the current situation. For disease treatment, patients suffering a haemorrhagic fever have relied on the clinical management of symptoms (http://www.cdc.gov/hiv/ebola/treatment/). with a handful of patients in this outbreak receiving experimental therapies such as ZMapp, TKM-Ebola, brincidofovir and favipiravir (http://www.nature.com/news/ebola-trials-to-start-in-december-1.16342)\(^10-12\). Alternatively antibody treatment by transfusion therapy using blood or plasma from Ebola virus survivors has been approved\(^11-13,16\); although issues with safety and lack of resources for this method limit its suitability in West Africa today. Having no approved or widely available therapeutics for EBOV, as with many other emerging viral diseases, focus has turned to possible re-purposing of drugs already licensed for other uses by the EMA and FDA. Several clinically approved drugs have been identified by researchers\(^12-20\), including amiodarone, one of the several cationic amphiphiles found to inhibit filovirus entry which is currently being trialled in Sierra Leone\(^21\). However reservations have been expressed about the complications that could be caused by side effects of the drug in EBOV patients. The anti-malarial drug chloroquine (CQ) has also been shown to inhibit EBOV entry and protected mice from EBOV infection\(^14,22\) and has been previously highlighted as a possible drug to treat EBOV infection\(^1\).

The possible difficulties that may arise with use of re-purposed drugs include unforeseen interactions between virus/drug and host causing exacerbation of disease. Therefore it is important to try and understand the mechanism of virus inhibition by such drugs. To this end we re-examined the anti-viral properties of CQ, and show here that it inhibited the pH-dependent endosomal entry of a pseudo-typed virus (PV) bearing EBOV glycoproteins, in the same way as did the potent and specific vacuolar-ATPase (vATPase) inhibitor bafilomycin A1 (BafA1) (a non-medical laboratory compound). We also show that licensed and widely used proton pump inhibitors (PPIs) for treatment of gastric acid reflux, omeprazole (OM) and esomeprazole (ESOM), inhibited PV EBOV entry, likely by their off-target inhibitory activity on endosomal vATPase.

Methods

Cell culture

Human embryonic kidney (293T/17) (ATCC) and Human lung adenocarcinoma epithelial cells (A549) (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS) (Biosera) and 1% Penicillin-Streptomycin (PS) (Invitrogen). The cell lines were maintained at 37°C in a 5% CO\(_2\) atmosphere.

Compounds

Chloroquine diphosphate salt (CQ), bafilomycin A1 from Streptomyces griseus (BafA1), omeprazole (OM) and esomeprazole magnesium hydrate (ESOM) (Sigma) were resuspended as per manufacturer’s instructions and aliquots stored at -20°C: CQ was prepared in sterile dH\(_2\)O; BafA1, OM and ESOM were prepared in sterile DMSO (Sigma).

Plasmid constructs

The Bundibugyo ebolavirus (EBOV-B) envelope glycoprotein (GP) (FJ217161) coding sequence was synthesised (Bio Basic Inc.) and the HA glycoprotein of avian influenza A/turkey/England/50-92/91(H5N1) (FLU-H5) was amplified from the HA plasmid of the H5N1 reverse genetics system\(^31\). Both were sub-cloned into the pCAGGS expression vector. Expression vectors containing the envelope glycoproteins of Zaire Ebolavirus (Mayinga) (EBOV_Z), Marburg virus (Lake Victoria isolate; MARV) and Gibbon Ape Leukemia Virus (GALV) (modified to contain the trans-membrane domain of amphotropic murine leukemia virus (A-MLV) envelope glycoprotein) are described previously\(^34,25\). The Renilla luciferase gene was sub-cloned into pCAGGS expressing vector from a minigene reporter described previously\(^26\).

Generation of pseudotype viruses

The generation of all lentiviral pseudotype viruses was based on the methods detailed previously\(^27-29\). Briefly, 293T/17 cells were seeded into 10cm\(^2\) tissue culture plates (Nunc\(^\text{TM}\) Thermo Scientific). The HIV gag-pol plasmid, pCMV-Δ8.91 and the firefly luciferase reporter construct, pCSFLW, were transfected together with either influenza HA, GALV, EBOV or Marburg GP expression constructs at a ratio of 1:1:5:1 (core:reporter:envelope) using Fugene6 transfection reagent (Promega). At 24 h post-transfection, cells were washed and fresh media applied. For the generation of H5
PVs, 1U exogenous recombinant neuraminidase from Clostridium perfringens (Sigma-Aldrich) was also added 24 h after transfection to effect egress from the producer cells. PV supernatants were harvested at 48 and 72 h post-transfection and passed through a 0.45µm pore filter. EBOV PVs were aliquoted and stored at 4°C; the remaining PVs were stored at -80°C.

**Entry inhibition assay**

293T cells in 10cm² plates were transfected with 15ug of Renilla luciferase expressing plasmid using Lipofectamine 2000 according to manufacturer’s instructions (Life Technologies™). CQ, BafA1, OM and ESOM were serially diluted in 96-well white-bottomed plates (Nunc™ Thermo Scientific) to give the final described concentrations. After 20h the transfected cells were trypsinised and 1×10⁶ cells were added to each well. After 30min cells were transduced with no more than 1×10⁵ RLU of PV per well (estimated from raw RLU values of previously infected 293T cells), and to an equal volume per well. 48 h later supernatant was removed and cells were lysed with 30µl of passive lysis buffer (Promega), and firefly/Renilla luciferase activity measured using a FLUOstar Omega plate reader (BMG Labtech) and the Dual Luciferase assay system (Promega).

**Measurement of intracellular pH**

A549 cells were pre-treated with drug 1 h before 75mM of the pH sensitive Lysotracker® Red DND-99 (Life Technologies™) was added to the media of each well. After 30minutes in growth conditions, cells were analyzed for fluorescence using an Axiovert 40 confocal laser (CFL) microscope and an AxioCam MRc camera (Carl Zeiss).

**Statistical analysis**

PV transduction RLU were normalised to the Renilla value in the corresponding wells. Percent infection of each drug dilution was calculated compared to untreated cells. Two-way ANOVA with Bonferroni’s multiple comparisons test between untreated and treated mean values (α-0.05) was performed to measure statistically significant differences. IC₅₀ values were calculated using nonlinear regression analysis (log[inhibitor] vs normalised response). All manipulation of data was performed on GraphPad Prism 6 (GraphPad software).

**Results**

**Inhibition of pseudotype virus entry by existing FDA-approved drugs**

The envelope glycoproteins of several emerging viruses with high pathogenicity and pandemic potential were used to create lentiviral based pseudotype particles as previously described[1]. PVs were generated bearing the envelope glycoproteins from Zaire viral ZEBOV, EBOV-B, MARV and FLU-H5 entry in a dose dependent manner (Figure 1A and B). The IC₅₀ value of BafA1 was in the mM range for EBOV-Z, EBOV-B, FLU-H5 and MARV and inhibition of entry was statistically significant at the 10mM concentration compared to the untreated control (Table 1). CQ inhibited EBOV-Z, EBOV-B, MARV and FLU-H5 with IC₅₀ of 3.319, 3.585, 3.192 and 10.44µM respectively, and inhibition was statistically significant (Table 1). In contrast, GALV entry was augmented by both BafA1 and CQ above that of the untreated cells to a maximum of 143.83% (3.33nM) and 180.38% (3.33µM) respectively. Both OM and ESOM reduced entry of all PVs tested at 100µM but GALV PV was the least affected (Figure 1C and D). Inhibition of entry for EBOV-Z, EBOV-B, MARV and FLU-H5 PVs by ESOM was significant at 50µM, and GALV PV was not significantly inhibited at this dose (Figure 1D and Table 1).

**Increasing endosome pH as a mechanism of inhibiting virus pH-dependent entry**

BafA1 and CQ are known endosomal acidification inhibitors (BafA1 being a potent and specific vATPase inhibitor and CQ a licensed lysotropic agent)[9]. The effects of OM and ESOM on endosomal acidification have also been previously reported[8,33]. To confirm that endosomal pH was being affected at doses used here, A549 cells were treated with drug for 1 hour before applying LysoTracker® Red DND-99 (LifeTechnologies). A549 cells were chosen here because 293T cells are poorly imaged due to their morphology. The lysotracker probe specifically fluoresces in acidic organelles. Fluorescence was decreased in cells treated with BafA1 and CQ in a dose dependant manner, but was unaffected in cells treated with vehicle alone (Figure 2). OM and ESOM appeared to decrease fluorescence, and therefore increase endosomal pH, only at a concentration of 200µM, higher than that required to inhibit PV entry. Moreover cellular toxicity was observed at this concentration after 24 hours.

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**Inhibition of pseudotype virus entry by existing FDA-approved drugs**

1 Data File

http://dx.doi.org/10.8084/m9.figshare.1294801
Figure 1. Inhibition of pseudotype virus entry by existing FDA-approved drugs. 293T cells previously transfected with a Renilla expression plasmid were treated with differing concentrations of drug before being transduced with PV (carried out in triplicate). Data are the percent of infection compared to untreated cells. EBOV-Z, EBOV-B, MARV, FLU-H5 and GALV inhibition was measured for each drug compound. Cells were harvested and firefly and Renilla activity measured after 48 h incubation. A. Cells were treated with 10, 3.33 and 1.11 nM of BafA1. B. Cells were treated with 30, 10, 3.33 and 1.11 μM of CQ. C and D. Cells were treated with 100, 50 and 25 μM of OM and ESOM, respectively. Statistical analysis of these data are shown in Table 1.

Conclusions and discussion

After attachment to cells, viruses require a mechanism of fusion to deliver the viral genome. Preventing this action by fusion inhibitors has been successful approach for HIV antiviral therapy. Unlike HIV, EBOV and many other viruses are dependent on the naturally low pH of acidic endosomes to activate and trigger fusion by their envelope glycoproteins. In this instance, a ‘fusion inhibitor’ could target the host cell machinery preventing acidification of the endosome, working to inhibit virus entry of several different viruses. Here we have reiterated that cell entry by PVs representing EBOV, FLU-H5 and MARV can be inhibited by increasing the endosome pH using BafA1 and CQ (Figure 1), and this correlates with their ability to prevent the acidification of intracellular organelles (Figure 2).

CQ has shown antiviral activity against several viruses in vitro, including EBOV, influenza, Nipah, Hendra, Dengue and CHIKV. Disappointingly, this antiviral activity has not always translated into efficacy in vivo models or clinical trials, although CQ was effective in a mouse model against EBOV. The variability in in vivo results may depend on study design and strains of virus used. In one study BafA1 treated mice were not protected from influenza infection but treatment with a related compound, SaliPhe, was protective, even though both drugs were potent in vitro. Inhibition of endosome acidification as a target for inhibiting EBOV can be justified by the knowledge that the filoviruses depend on the low pH for two separate steps of their entry pathway. Not only is the fusion by G protein triggered by low pH, but its cleavage into a fusogenic form is carried out by endosomal enzymes cathepsins B and L whose activation is also pH dependent. Some have argued that G protein cleavage by cathepsin is less essential than previously thought and that EBOV species other than Zaire together with closely related MARV do not require cathepsin cleavage for entry. Nonetheless, entry of MARV PVs was still inhibited in our assays suggesting that inhibiting fusion alone is sufficient.
Recently, using computational modelling, Ekins et al. suggested the anti-EBOV mechanism of CQ may be by binding the VP35 protein of EBOV. If this drug had activity on several steps of the replication cycle it may not only be more effective in vivo but it may be even less likely that the virus could mutate to escape inhibition.

At first we were surprised that CQ actually increased entry of GALV PV (Figure 1). However this effect has been noted before for other retroviruses, including A-MLV and HIV-1, and is accounted for by the inhibitory effect of CQ on the autophagy pathway. CQ prevents degradation of phagosomes that contain virus particles and prevents them from otherwise being degraded.

CQ has been used for many years as an anti-malarial drug, although it is now only effective in parts of central America and the Caribbean due to accumulation of drug resistance by the Plasmodium parasite. Interestingly, compounds belonging to the omeprazole family have also been described as having anti-malarial properties in vitro, possibly via their reported ability to target vATPase in the plasma membrane of Plasmodium parasite. Soon after its discovery OM was found to also inhibit intracellular vATPase at µM concentrations as opposed to its licensed target of gastric H+ K+-ATPase against which it is effective at much lower concentrations. Indeed there are a plethora of publications indicating use of OM and ESOM in cancer therapy, as a means to inhibit the characteristic acidic intracellular environment, and thus permit sensitivity to cytotoxic therapies. A role of OM and ESOM has also been noted in the suppression of bone resorption, another physiological process dependent on pH. Given the volume of research suggesting these off target effects depend on an ability to affect intracellular pH, we hypothesised that these drugs would, like CQ and BafA1, inhibit EBOV, MARV and influenza virus pH dependent entry. We used GALV as a control again since its entry is reportedly independent of pH. Indeed, EBOV, FLU-H5 and MARV were inhibited by lower doses of OM or ESOM than GALV (Figure 1 and Table 1). GALV entry was also inhibited at the highest concentration, but we cannot exclude that this was due to a toxic effect that was not measured by the Renilla control we employed here. We did not observe as close a correlation between drug doses that mediated the inhibition of EBOV or influenza PV entry and increase in pH of intracellular vesicles for OM and ESOM as for CQ and BafA1 (Figure 1 and Figure 2). More recently, it has been reported that OM and ESOM altered the localisation of vATPase in the cell as well as the pH of intracellular vesicles and this may explain their ability to inhibit PV entry more potently than the pH changes we observed would suggest.

Inhibition of influenza virus entry to cells by means of inhibiting acidification of endosomes has been known for decades, although no current antivirals for influenza have been licensed on this basis. Some epidemiological evidence from population studies suggests that OM could exert a protective effect against influenza-like illness, but our studies suggest that doses required for potent inhibition might be difficult to achieve without significant toxicity. Despite these drugs being readily available, even without prescription in some countries, the licensed dosing would generate a plasma concentration reportedly 1.59–9.61 µM for ESOM that falls short of the IC50 calculated in this study, although higher doses have been used clinically. Therefore it seems unlikely that OM and ESOM would be a suitable therapy for ebolavirus infection, but more specifically designed vATPase inhibitors may have potential as broad acting antivirals against several emerging viruses in the future. With regard to CQ, the evidence suggests a more promising position for its use against ebolavirus. Standard adult dosing (25 mg/kg) achieves plasma concentration of 2 µM, close to our IC50 value against EBOV PV entry. Protection in the mouse model was previously shown with a 90 mg/kg dosage.

### Table 1. Inhibition of pseudotype viruses by existing FDA-approved drugs.

<table>
<thead>
<tr>
<th>BafA1</th>
<th>Pseudotype virus</th>
<th>IC50 (nM)</th>
<th>Std. Err.</th>
<th>Significance at dose (µM) (vs. untreated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.11 3.33 10</td>
</tr>
<tr>
<td>EBOV-Z</td>
<td>1.213</td>
<td>0.195</td>
<td>ns</td>
<td>* ****</td>
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<tr>
<td>EBOV-B</td>
<td>3.297</td>
<td>0.233</td>
<td>ns ns</td>
<td>***</td>
</tr>
<tr>
<td>MARV</td>
<td>3.538</td>
<td>0.260</td>
<td>ns ns</td>
<td>**</td>
</tr>
<tr>
<td>FLU-H5</td>
<td>3.510</td>
<td>0.282</td>
<td>ns ns</td>
<td>**</td>
</tr>
<tr>
<td>GALV</td>
<td>ns</td>
<td></td>
<td>ns ns ns</td>
<td></td>
</tr>
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</table>

<table>
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<tr>
<th>Chloroquine</th>
<th>Pseudotype virus</th>
<th>IC50 (µM)</th>
<th>Std. Err.</th>
<th>Significance at dose (µM) (vs. untreated)</th>
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<tr>
<td>EBOV-Z</td>
<td>3.319</td>
<td>0.147</td>
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<tr>
<td>EBOV-B</td>
<td>3.585</td>
<td>0.198</td>
<td>ns ns</td>
<td>*** ****</td>
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<tr>
<td>MARV</td>
<td>3.192</td>
<td>0.186</td>
<td>ns ns *</td>
<td>**** ****</td>
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<td>FLU-H5</td>
<td>10.44</td>
<td>0.245</td>
<td>ns ns ns</td>
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<tr>
<td>GALV</td>
<td>ns</td>
<td></td>
<td>ns ns ns</td>
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<table>
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<tr>
<th>Omeprazole</th>
<th>Pseudotype virus</th>
<th>IC50 (µM)</th>
<th>Std. Err.</th>
<th>Significance at dose (µM) (vs. untreated)</th>
</tr>
</thead>
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<tr>
<td>EBOV-Z</td>
<td>ns</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>EBOV-B</td>
<td>50.32</td>
<td>0.234</td>
<td>ns ns</td>
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<td>MARV</td>
<td>52.21</td>
<td>12.290</td>
<td>ns ns</td>
<td>***</td>
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<td>FLU-H5</td>
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<td>0.562</td>
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<td>GALV</td>
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<th>Esomeprazole</th>
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<th>Std. Err.</th>
<th>Significance at dose (µM) (vs. untreated)</th>
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<td>EBOV-Z</td>
<td>50.25</td>
<td>0.163</td>
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<td>EBOV-B</td>
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<td>MARV</td>
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<td>* ****</td>
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<tr>
<td>FLU-H5</td>
<td>50.06</td>
<td>0.160</td>
<td>ns ns</td>
<td>**</td>
</tr>
<tr>
<td>GALV</td>
<td>ns</td>
<td></td>
<td>ns ns ns</td>
<td>****</td>
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</tbody>
</table>

1IC50 values were calculated using non-linear regression analysis (log[inhibitor] vs normalised response)
2Two-way ANOVA with Bonferroni’s multiple comparisons test between untreated and treated mean values (α=0.05)

ns P>0.05, * P≤0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001
Figure 2. Correlation of decreased pH with inhibitory effect on entry. A549 cells were treated with drug for 1 h before 75nM Lysotracker® Red DND-99 was added to each well. DMSO (drug vehicle) only was diluted at 30mM, 3mM, 0.3mM and 0.03mM. (A–D). CQ was diluted 30, 10, 3.33 and 1.11μM (E–H), BafA1 was diluted to 10, 3.33 and 1.11nM (I–L) and OM and ESOM were diluted to 100, 50 and 25μM (M–P) and (Q–T) respectively. The level of fluorescence was imaged by confocal microscopy (×50 magnification).

Using re-purposed drugs to treat outbreaks of emerging diseases must surely be approached with caution. In Ebola patients with severe life-threatening disease it would be important to ensure that any side effects of a therapy did not enhance disease progression, particularly if higher doses of re-purposed drugs, as suggested here, were considered. On the other hand, CQ has been taken prophylactically in a tropical setting for many years to prevent malaria and we suggest that, with little additional need for scale up of production of a new agent, this might represent a useful adjunct to the current antiviral strategies being trialled in West Africa. We envisage that in contacts of EBOV cases, CQ might decrease the viral load that establishes in the early days after virus transmission. Further work in in vivo models including guinea pig and primates should inform about doses and administration regimens.

Data availability
Figshare: Inhibition of pseudotype virus entry by existing FDA-approved drugs. doi: http://dx.doi.org/10.6084/m9.figshare.1294801

Author contributions
Dr Jason Long, Dr Edward Wright and Dr Eleonora Molesti generated the PVs. Jason Long performed the drug entry assay and pH assay. This work was planned by Prof Wendy Barclay, Dr Nigel Temperton and Dr Jason Long. All authors were involved in preparing and revising the manuscript.

Competing interests
No competing interests were disclosed.

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Acknowledgments
The authors wish to thank Caroline Goujon for the kind provision of the GALV construct and Olivier Moncorgé for generating the Renilla expression plasmid.
modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and nonhuman primates. Blood. 2002; 100(3): 823–32.


44. Schomberg K, Matsuyama S, Koubek K, et al.: Role of endosomal cathepsins in...


56. De Milto A, Fais S: Proton pump inhibitors may reduce tumour resistance.


Open Peer Review

Current Referee Status: ✔ ✔

Version 2

Referee Report 29 April 2015

doi:10.5256/f1000research.6568.r8175

Robin A. Weiss
Division of Infection and Immunity, University College London, London, UK

This is an elegant study employing envelope pseudotypes of highly pathogenic viruses which demonstrates that certain inhibitors of low endosomal pH can inhibit viral entry. Because some of these molecules such as Chloroquine have been in clinical use for decades, and are inexpensive, they might tip the balance between survival and death during human infection.

I have no criticism of the experimental work. However, I have been told by a reliable physician who has recently cared for patients with Ebola infection that treatment with Chloroquine offered no clinical benefit. Thus it is possible that an in vitro observation may not translate into a useful treatment in vivo. So one should be wary about the conclusions.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 27 April 2015

doi:10.5256/f1000research.6568.r8424

Sean Ekins
Collaborative Drug Discovery, Burlingame, CA, USA

The work was of particular interest especially in light of several viruses shown to be taken up in this rather non specific way. I would have perhaps also like some discussion of receptor and channel mediated virus uptake - there are several publications in this space. The interplay between such different mechanisms may point to multiple targets or need for combined approaches to block them.

The authors describe amiodarone, but there are many molecules that have been found as ebola replication or pseudoviral entry inhibitors, had they looked at more molecules to see if the pH mechanism was common across them?

I would likely suggest adding repurposing in the title of the article.
The conclusion might benefit from comparison of the chloroquine data with that previously published (higher EC50), potential ocular toxicity etc.

Some discussion as to whether the pH effect is an in vitro specific effect or something of in vivo relevance - would also be worth mention.

This study confirms the previous work on chloroquine and suggestions by others as to its potential utility. This begs the question why it is not used clinically. What other data would be needed to show that chloroquine could be clinically useful?

The study is well designed and reported and adds to the growing literature on chloroquine and its potential as an antiviral.

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.