RESEARCH NOTE

REVISED Antiviral therapies against Ebola and other emerging viral diseases using existing medicines that block virus entry [v2; ref status: indexed, http://f1000r.es/52g]

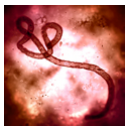
Jason Long[1], Edward Wright[2], Eleonora Molesti[3], Nigel Temperton[3], Wendy Barclay[1]

[1]Section of Virology, St Mary's Campus, Imperial College London, London, W2 1PG, UK
[2]Viral Pseudotype Unit (Fitzrovia), Faculty of Science and Technology, University of Westminster, London, W1W 6UW, UK
[3]Viral Pseudotype Unit, School of Pharmacy, University of Kent, Chatham Maritime, Kent, ME4 4TB, UK

v2 **First published:** 29 Jan 2015, **4**:30 (doi: 10.12688/f1000research.6085.1)
Latest published: 10 Feb 2015, **4**:30 (doi: 10.12688/f1000research.6085.2)

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 channel.

Open Peer Review

Referee Status: ✓ ✓

	Invited Referees 1	2
REVISED version 2 published 10 Feb 2015	✓ report	✓ report
version 1 published 29 Jan 2015		

1 **Sean Ekins**, Collaborative Drug Discovery USA

2 **Robin A. Weiss**, University College London UK

Discuss this article

Comments (0)

Corresponding author: Wendy Barclay (w.barclay@imperial.ac.uk)

How to cite this article: Long J, Wright E, Molesti E *et al.* **Antiviral therapies against Ebola and other emerging viral diseases using existing medicines that block virus entry [v2; ref status: indexed, http://f1000r.es/52g]** *F1000Research* 2015, **4**:30 (doi: 10.12688/f1000research.6085.2)

Grant information: This work was supported by the grants FLUPIG EU FP7 258084 and BBSRC BB/K002465/1.

Competing interests: No competing interests were disclosed.

First published: 29 Jan 2015, **4**:30 (doi: 10.12688/f1000research.6085.1)
First indexed: 29 Apr 2015, **4**:30 (doi: 10.12688/f1000research.6085.2)

REVISED **Amendments from Version 1**

The units for chloroquine in Table 1 have been corrected from nM to µM.

See referee reports

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 2013 and has since become an unprecedented and devastating epidemic[3,4], 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[5]. 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[6–8] 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/vhf/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)[9–12]. 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[17–20], 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[18,22] and has been previously highlighted as a possible drug to treat EBOV infection[11].

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 pseudotyped virus (PV) bearing EBOV glycoproteins, in the same way as did the potent and specific vacuolar-ATPase (vATPase) inhibitor bafilyomycin 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) (ATTC) 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_2O; 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[23]. 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[24,25]. The *Renilla* luciferase gene was sub-cloned into pCAGGS expressing vector from a mini-genome 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^3 tissue culture plates (Nunc™ 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 0.45m 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 10cm^3 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 1×10^4 cells were added to each well. After 30min cells were transduced with no more than 1×10^5 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 75nM of the pH sensitive Lysotracker® Red DND-99 (Life Technologies™) was added to the media of each well[30]. 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 RLUs 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. IC_{50} values were calculated using non-linear 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 exisiting 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[29]. PVs were generated bearing the envelope glycoproteins from Zaire ebolavirus (Mayinga strain) (EBOV-Z), Bundibugyo ebolavirus (EBOV-B), Marburg (Lake Victoria isolate) virus (MARV), H5 HA from a highly pathogenic avian influenza virus A/turkey/England/50-92/91(H5N1) (FLU-H5), and Gibbon Ape Leukaemia virus (GALV). GALV PVs were included because GALV is a virus that does not require acidification of endosomes for its entry into cells. All the PVs generated were shown to transduce 293T cells and firefly luciferase expression from the packaged reporter gene was measured above mock infected cells (non-transduced cells) (Dataset 1).

In order to assess the ability of CQ, BafA1, OM and ESOM to inhibit PV entry, drugs were serially diluted in triplicate in white bottomed 96-well plates. Next, 293T cells transfected 24 hours previously with a *Renilla* luciferase expression plasmid to allow monitoring of cell viability, were added to each well. Appropriately diluted PVs were then added to each dilution, including a no-drug control. After 48 hours incubation, the supernatant was removed and firefly and *Renilla* luciferase RLUs were recorded using the Dual Luciferase Assay System (Promega).

PV RLUs were normalised to the corresponding *Renilla* values, which reduced the edge effect observed in the 96-well plates, and controlled for toxicity of the drugs. Only BafA1 appeared to reduce expression of *Renilla* at the highest concentrations, suggesting cellular toxicity, (Dataset 1) and visible cytopathic effect was not observed in cells treated by CQ, OM and ESOM at the concentrations used in Figure 1.

Both BafA1 and CQ reduced EBOV-Z, EBOV-B, MARV and FLU-H5 entry in a dose dependent manner (Figure 1A and B). The IC_{50} value of BafA1 was in the nM range for EBOV-Z, EBOV-B, FLU-H5 and MARV and inhibition of entry was statistically significant at the 10nM concentration compared to the untreated control (Table 1). CQ inhibited EBOV-Z, EBOV-B, MARV and FLU-H5 with IC_{50} 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)[31]. The effects of OM and ESOM on endosomal acidification have also been previously reported[32,33]. 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.

Inhibition of pseudotype virus entry by existing FDA-approved drugs

1 Data File

http://dx.doi.org/10.6084/m9.figshare.1294801

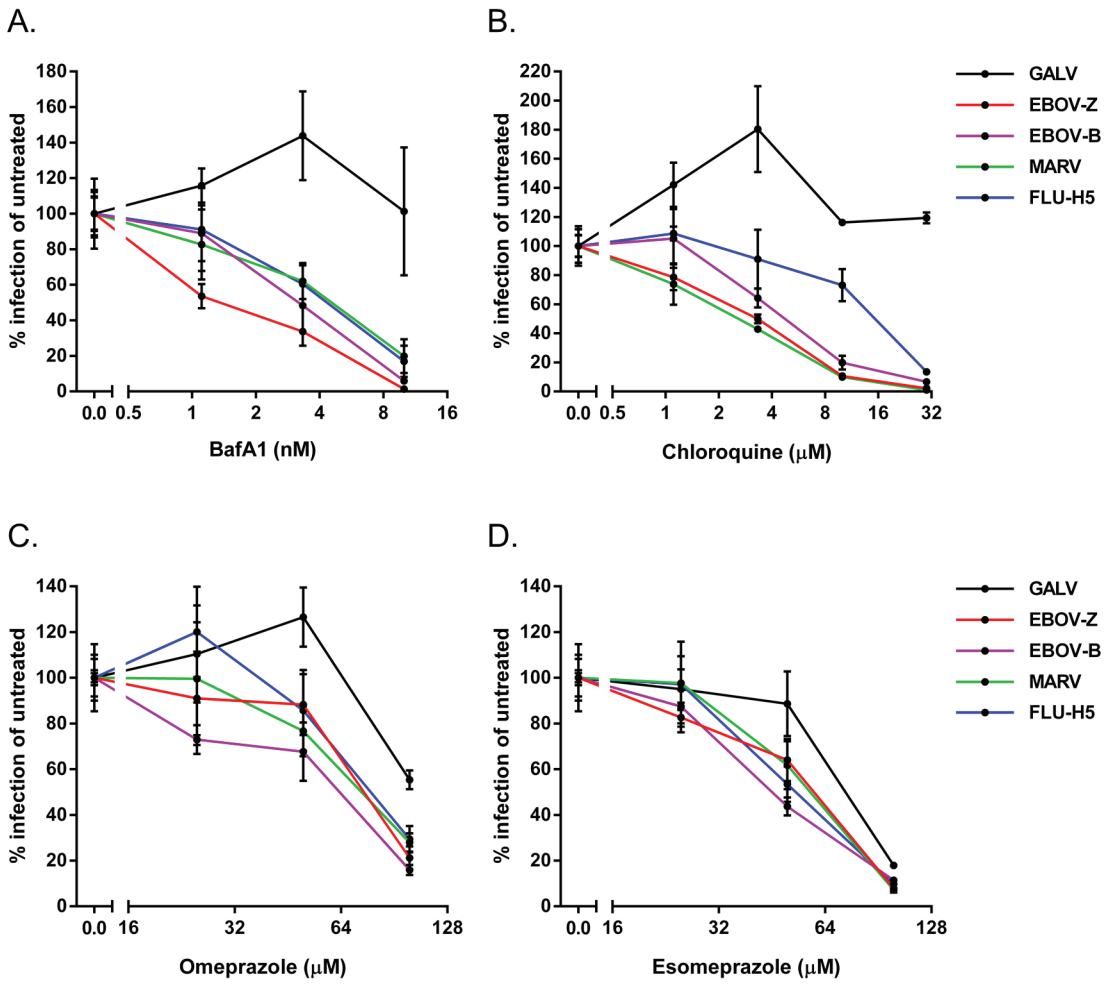


Figure 1. Inhibition of pseudotype virus entry by existing FDA-approved drugs. 293T cells previously transfected with a *Renilla* expression plasmid were treated with differing concentrations of drug before being transduced with PV (carried out in triplicate). Data are the percent of infection compared 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 1.11nM of BafA1. **B.** Cells were treated with 30, 10, 3.33 and 1.11μM of CQ. **C** and **D.** Cells were treated with 100, 50 and 25μM 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[34]. 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[35–37]. 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[18,35,38–42]. 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*[43]. 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[44]. Some have argued that G protein cleavage by cathepsin is less essential than previously thought[45,46] and that EBOV species other than Zaire together with closely related MARV do not require cathepsin cleavage for entry[47,48]. Nonetheless, entry of MARV PVs was still inhibited in our assays suggesting that inhibiting fusion alone is sufficient.

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

Pseudotype virus	IC_{50} (nM)[a]	Std. Err.	Significance at dose (nM) (vs. untreated)[b]			
			1.11	3.33	10	
BafA1						
EBOV-Z	1.213	0.195	ns	*	****	
EBOV-B	3.297	0.233	ns	ns	***	
MARV	3.538	0.260	ns	ns	**	
FLU-H5	3.510	0.282	ns	ns	**	
GALV	ns		ns	ns	ns	
Chloroquine						
Pseudotype virus	IC_{50} (μM)[a]	Std. Err.	Significance at dose (μM) (vs. untreated)[b]			
			1.11	3.33	10	30
EBOV-Z	3.319	0.147	ns	*	****	****
EBOV-B	3.585	0.198	ns	ns	****	****
MARV	3.192	0.186	ns	**	****	****
FLU-H5	10.44	0.245	ns	ns	ns	****
GALV	ns		ns	****	ns	ns
Omeprazole						
Pseudotype virus	IC_{50} (μM)[a]	Std. Err.	Significance at dose (μM) (vs. untreated)[b]			
			25	50	100	
EBOV-Z	ns		ns	ns	***	
EBOV-B	50.32	0.234	ns	ns	***	
MARV	52.21	12.290	ns	ns	***	
FLU-H5	50.78	0.562	ns	ns	**	
GALV	ns		ns	ns	ns	
Esomeprazole						
Pseudotype virus	IC_{50} (μM)[a]	Std. Err.	Significance at dose (μM) (vs. untreated)[b]			
			25	50	100	
EBOV-Z	50.25	0.163	ns	*	****	
EBOV-B	49.89	0.127	ns	***	****	
MARV	50.21	0.174	ns	*	****	
FLU-H5	50.06	0.160	ns	**	****	
GALV	ns		ns	ns	****	

[a]IC_{50} values were calculated using non-linear regression analysis (log[inhibitor] vs normalised response)
[b]Two-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

Recently, using computational modelling, Ekins *et al.* suggested the anti-EBOV mechanism of CQ may be by binding the VP35 protein of EBOV[49]. 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[50–52].

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[53]. 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[54]. 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[32,33]. 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[55–59]. A role of OM and ESOM has also been noted in the suppression of bone resorption, another physiological process dependent on pH[60–62]. 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 BafA, (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[46] 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[63], 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[64], 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 1.59–9.61μM for ESOM that falls short of the IC_{50} calculated in this study, although higher doses have been used clinically[65]. 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 use against ebolavirus. Standard adult dosing (25mg/kg) achieves plasma concentration of 2μM, close to our IC_{50} value against EBOV PV entry. Protection in the mouse model was previously shown with a 90mg/kg dosage[18,66].

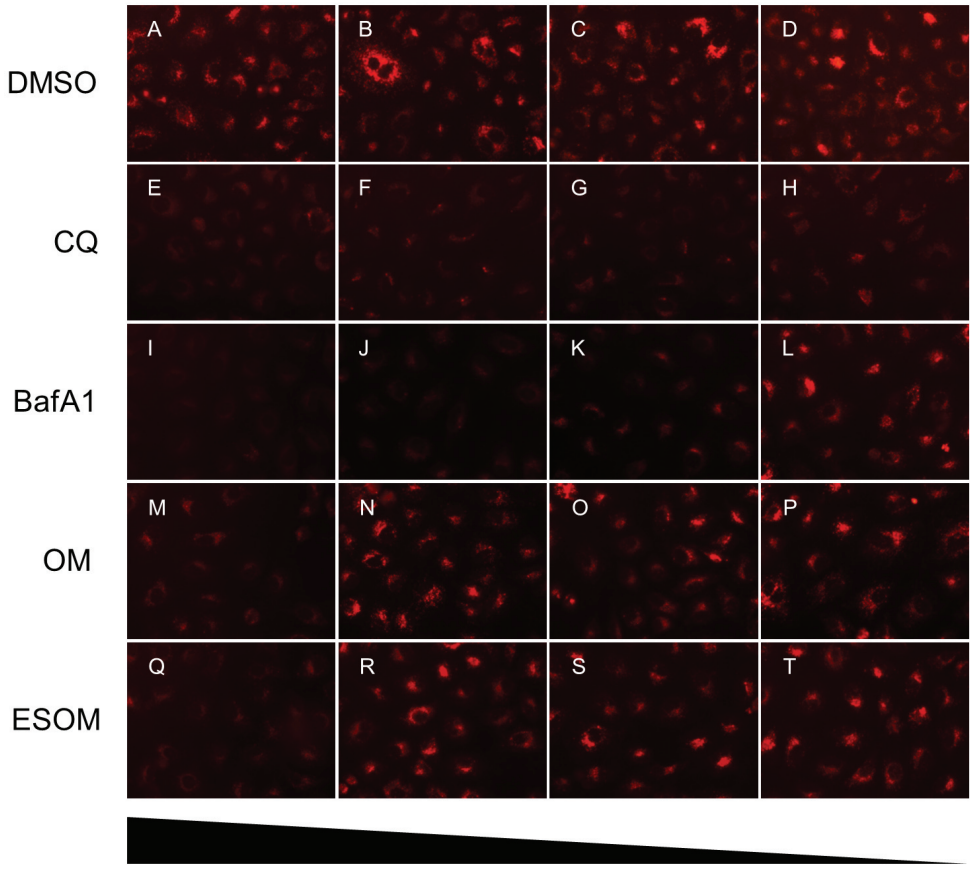


Figure 2. Correlation of decreased pH with inhibitory effect on entry. A549 cells were treated with drug for 1 h before 75nM Lysotracker® Red DND-99 was added to each well. DMSO (drug vehicle) only was diluted at 30mM, 3mM, 0.3mM and 0.03mM. (**A–D**). CQ was diluted 30, 10, 3.33 and 1.11μM (**E–H**), 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 (x50 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[67]

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.

Grant information

This work was supported by the grants FLUPIG EU FP7 258084 and BBSRC BB/K002465/1.

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.

References

1. Li YH, Chen SP: **Evolutionary history of Ebola virus.** *Epidemiol Infect.* 2014; **142**(6): 1138–45.
PubMed Abstract | Publisher Full Text
2. Feldmann H, Geisbert TW: **Ebola haemorrhagic fever.** *Lancet.* 2011; **377**(9768): 849–62.
PubMed Abstract | Publisher Full Text | Free Full Text
3. Baize S, Pannetier D, Oestereich L, *et al.*: **Emergence of Zaire Ebola virus disease in Guinea.** *N Engl J Med.* 2014; **371**(15): 1418–25.
PubMed Abstract | Publisher Full Text
4. Gatherer D: **The 2014 Ebola virus disease outbreak in West Africa.** *J Gen Virol.* 2014; **95**(Pt 8): 1619–24.
PubMed Abstract | Publisher Full Text
5. WHO Ebola Response Team: **West African Ebola Epidemic after One Year - Slowing but Not Yet under Control.** *N Engl J Med.* 2014.
PubMed Abstract | Publisher Full Text
6. Kanapathipillai R, Henao Restrepo AM, Fast P, *et al.*: **Ebola vaccine--an urgent international priority.** *N Engl J Med.* 2014; **371**(24): 2249–51.
PubMed Abstract | Publisher Full Text
7. Kibuuka H, Berkowitz NM, Millard M, *et al.*: **Safety and immunogenicity of Ebola virus and Marburg virus glycoprotein DNA vaccines assessed separately and concomitantly in healthy Ugandan adults: a phase 1b, randomised, double-blind, placebo-controlled clinical trial.** *Lancet.* 2014; S0140-6736(14)62385-0.
PubMed Abstract | Publisher Full Text
8. Ledgerwood JE, DeZure AD, Stanley DA, *et al.*: **Chimpanzee Adenovirus Vector Ebola Vaccine - Preliminary Report.** *N Engl J Med.* 2014.
PubMed Abstract | Publisher Full Text
9. Smither SJ, Eastaugh LS, Steward JA, *et al.*: **Post-exposure efficacy of oral T-705 (Favipiravir) against inhalational Ebola virus infection in a mouse model.** *Antiviral Res.* 2014; **104**: 153–5.
PubMed Abstract | Publisher Full Text
10. Oestereich L, Lüdtke A, Wurr S, *et al.*: **Successful treatment of advanced Ebola virus infection with T-705 (favipiravir) in a small animal model.** *Antiviral Res.* 2014; **105**: 17–21.
PubMed Abstract | Publisher Full Text
11. Bishop BM: **Potential and Emerging Treatment Options for Ebola Virus Disease.** *Ann Pharmacother.* 2015; **49**(2): 196–206.
PubMed Abstract | Publisher Full Text
12. Qiu X, Wong G, Audet J, *et al.*: **Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp.** *Nature.* 2014; **514**(7520): 47–53.
PubMed Abstract | Publisher Full Text | Free Full Text
13. Mupapa K, Massamba M, Kibadi K, *et al.*: **Treatment of Ebola hemorrhagic fever with blood transfusions from convalescent patients. International Scientific and Technical Committee.** *J Infect Dis.* 1999; **179**(Suppl 1): S18–23.
PubMed Abstract | Publisher Full Text
14. Jahrling PB, Geisbert JB, Swearengen JR, *et al.*: **Ebola hemorrhagic fever: evaluation of passive immunotherapy in nonhuman primates.** *J Infect Dis.* 2007; **196**(Suppl 2): S400–3.
PubMed Abstract | Publisher Full Text
15. Gulland A: **First Ebola treatment is approved by WHO.** *BMJ.* 2014; **349**: g5539.
PubMed Abstract | Publisher Full Text
16. Burnouf T, Seghatchian J: **Ebola virus convalescent blood products: where we are now and where we may need to go.** *Transfus Apher Sci.* 2014; **51**(2): 120–5.
PubMed Abstract | Publisher Full Text
17. Gehring G, Rohrmann K, Atenchong N, *et al.*: **The clinically approved drugs amiodarone, dronedarone and verapamil inhibit filovirus cell entry.** *J Antimicrob Chemother.* 2014; **69**(8): 2123–31.
PubMed Abstract | Publisher Full Text
18. Madrid PB, Chopra S, Manger ID, *et al.*: **A systematic screen of FDA-approved drugs for inhibitors of biological threat agents.** *PLoS One.* 2013; **8**(4): e60579.
PubMed Abstract | Publisher Full Text | Free Full Text
19. Nagata T, Lefor AK, Hasegawa M, *et al.*: **Favipiravir: A New Medication for the Ebola Virus Disease Pandemic.** *Disaster Med Public Health Prep.* 2014; 1–3.
PubMed Abstract | Publisher Full Text
20. Kouznetsova J, Sun W, Martínez-Romero C, *et al.*: **Identification of 53 compounds that block Ebola virus-like particle entry via a repurposing screen of approved drugs.** *Emerg Microbes Infect.* 2014; **3**: e84.
Publisher Full Text
21. Turone F: **Doctors trial amiodarone for Ebola in Sierra Leone.** *BMJ.* 2014; **349**: g7198.
PubMed Abstract | Publisher Full Text
22. Wool-Lewis RJ, Bates P: **Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines.** *J Virol.* 1998; **72**(4): 3155–60.
PubMed Abstract | Free Full Text
23. Howard W, Hayman A, Lackenby A, *et al.*: **Development of a reverse genetics system enabling the rescue of recombinant avian influenza virus A/Turkey/England/50-92/91 (H5N1).** *Avian Dis.* 2007; **51**(1 Suppl): 393–5.
PubMed Abstract | Publisher Full Text
24. Sandrin V, Boson B, Salmon P, *et al.*: **Lentiviral vectors pseudotyped with a 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.
PubMed Abstract | Publisher Full Text
25. Salvador B, Sexton NR, Carrion R Jr, *et al.*: **Filoviruses utilize glycosaminoglycans for their attachment to target cells.** *J Virol.* 2013; **87**(6): 3295–304.
PubMed Abstract | Publisher Full Text | Free Full Text
26. Moncorgé O, Mura M, Barclay WS: **Evidence for avian and human host cell factors that affect the activity of influenza virus polymerase.** *J Virol.* 2010; **84**(19): 9978–86.
PubMed Abstract | Publisher Full Text | Free Full Text
27. Temperton NJ, Hoschler K, Major D, *et al.*: **A sensitive retroviral pseudotype assay for influenza H5N1-neutralizing antibodies.** *Influenza Other Respi Viruses.* 2007; **1**(3): 105–12.
PubMed Abstract | Publisher Full Text
28. Wright E, Temperton NJ, Marston DA, *et al.*: **Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: a cross-species comparison.** *J Gen Virol.* 2008; **89**(Pt 9): 2204–13.
PubMed Abstract | Publisher Full Text | Free Full Text
29. Mather ST, Wright E, Scott SD, *et al.*: **Lyophilisation of influenza, rabies and Marburg lentiviral pseudotype viruses for the development and distribution of a neutralisation -assay-based diagnostic kit.** *J Virol Methods.* 2014; **210C**: 51–8.
PubMed Abstract | Publisher Full Text
30. Chikte S, Panchal N, Warnes G: **Use of LysoTracker dyes: a flow cytometric study of autophagy.** *Cytometry A.* 2014; **85**(2): 169–78.
PubMed Abstract | Publisher Full Text
31. Dean RT, Jessup W, Roberts CR: **Effects of exogenous amines on mammalian cells, with particular reference to membrane flow.** *Biochem J.* 1984; **217**(1): 27–40.
PubMed Abstract
32. Fellenius E, Berglindh T, Sachs G, *et al.*: **Substituted benzimidazoles inhibit gastric acid secretion by blocking ($H^+ + K^+$)ATPase.** *Nature.* 1981; **290**(5802): 159–61.
PubMed Abstract | Publisher Full Text
33. Elander B, Fellenius E, Leth R, *et al.*: **Inhibitory action of omeprazole on acid formation in gastric glands and on H^+,K^+-ATPase isolated from human gastric mucosa.** *Scand J Gastroenterol.* 1986; **21**(3): 268–72.
PubMed Abstract | Publisher Full Text
34. Lalezari JP, Eron JJ, Carlson M, *et al.*: **A phase II clinical study of the long-term safety and antiviral activity of enfuvirtide-based antiretroviral therapy.** *AIDS.* 2003; **17**(5): 691–8.
PubMed Abstract
35. Freiberg AN, Worthy MN, Lee B, *et al.*: **Combined chloroquine and ribavirin treatment does not prevent death in a hamster model of Nipah and Hendra virus infection.** *J Gen Virol.* 2010; **91**(Pt 3): 765–72.
PubMed Abstract | Publisher Full Text
36. Ooi EE, Chew JS, Loh JP, *et al.*: ***In vitro* inhibition of human influenza A virus replication by chloroquine.** *Virol J.* 2006; **3**: 39.
PubMed Abstract | Publisher Full Text
37. Nuckols JT, McAuley AJ, Huang YJ, *et al.*: **pH-Dependent entry of chikungunya virus fusion into mosquito cells.** *Virol J.* 2014; **11**(1): 215.
PubMed Abstract | Publisher Full Text
38. Pallister J, Middleton D, Crameri G, *et al.*: **Chloroquine administration does not prevent Nipah virus infection and disease in ferrets.** *J Virol.* 2009; **83**(22): 11979–82.
PubMed Abstract | Publisher Full Text
39. De Lamballerie X, Boisson V, Reynier JC, *et al.*: **On chikungunya acute infection and chloroquine treatment.** *Vector Borne Zoonotic Dis.* 2008; **8**(6): 837–9.
PubMed Abstract | Publisher Full Text
40. Tricou V, Minh NN, Van TP, *et al.*: **A randomized controlled trial of chloroquine for the treatment of dengue in Vietnamese adults.** *PLoS Negl Trop Dis.* 2010; **4**(8): e785.
PubMed Abstract | Publisher Full Text
41. Paton NI, Lee L, Xu Y, *et al.*: **Chloroquine for influenza prevention: a randomised, double-blind, placebo controlled trial.** *Lancet Infect Dis.* 2011; **11**(9): 677–83.
PubMed Abstract | Publisher Full Text
42. Vigerust DJ, McCullers JA: **Chloroquine is effective against influenza A virus *in vitro* but not *in vivo*.** *Influenza Other Respi Viruses.* 2007; **1**(5–6): 189–92.
PubMed Abstract | Publisher Full Text
43. Müller KH, Kainov DE, El Bakkouri K, *et al.*: **The proton translocation domain of cellular vacuolar ATPase provides a target for the treatment of influenza A virus infections.** *Br J Pharmacol.* 2011; **164**(2): 344–57.
PubMed Abstract | Publisher Full Text | Free Full Text
44. Chandran K, Sullivan NJ, Felbor U, *et al.*: **Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection.** *Science.* 2005; **308**(5728): 1643–5.
PubMed Abstract | Publisher Full Text
45. Schornberg K, Matsuyama S, Kabsch K, *et al.*: **Role of endosomal cathepsins in**

entry mediated by the Ebola virus glycoprotein. *J Virol.* 2006; **80**(8): 4174–8.
PubMed Abstract | Publisher Full Text | Free Full Text

46. Brecher M, Schornberg KL, Delos SE, *et al.*: **Cathepsin cleavage potentiates the Ebola virus glycoprotein to undergo a subsequent fusion-relevant conformational change.** *J Virol.* 2012; **86**(1): 364–72.
PubMed Abstract | Publisher Full Text | Free Full Text

47. Marzi A, Reinheckel T, Feldmann H: **Cathepsin B & L are not required for Ebola virus replication.** *PLoS Negl Trop Dis.* 2012; **6**(12): e1923.
PubMed Abstract | Publisher Full Text | Free Full Text

48. Gnirss K, Kühl A, Karsten C, *et al.*: **Cathepsins B and L activate Ebola but not Marburg virus glycoproteins for efficient entry into cell lines and macrophages independent of TMPRSS2 expression.** *Virology.* 2012; **424**(1): 3–10.
PubMed Abstract | Publisher Full Text

49. Ekins S, Freundlich JS, Coffee M: **A common feature pharmacophore for FDA-approved drugs inhibiting the Ebola virus [v2; ref status: indexed, http://f1000r.es/4wt].** *F1000Res.* 2014; **3**: 277.
Publisher Full Text

50. Rutz M, Metzger J, Gellert T, *et al.*: **Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner.** *Eur J Immunol.* 2004; **34**(9): 2541–50.
PubMed Abstract | Publisher Full Text

51. Hart OM, Athie-Morales V, O'Connor GM, *et al.*: **TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production.** *J Immunol.* 2005; **175**(3): 1636–42.
PubMed Abstract | Publisher Full Text

52. Shintani T, Klionsky DJ: **Autophagy in health and disease: a double-edged sword.** *Science.* 2004; **306**(5698): 990–5.
PubMed Abstract | Publisher Full Text | Free Full Text

53. White NJ, Pukrittayakamee S, Hien TT, *et al.*: **Malaria.** *Lancet.* 2014; **383**(9918): 723–35.
Pubmed Abstract | Publisher Full Text

54. Saliba KJ, Kirk K: **pH regulation in the intracellular malaria parasite, *Plasmodium falciparum*. H($^+$) extrusion via a V-type H($^+$)-ATPase.** *J Biol Chem.* 1999; **274**(47): 33213–9.
PubMed Abstract | Publisher Full Text

55. Luciani F, Spada M, De Milito A, *et al.*: **Effect of proton pump inhibitor pretreatment on resistance of solid tumors to cytotoxic drugs.** *J Natl Cancer Inst.* 2004; **96**(22): 1702–13.
PubMed Abstract | Publisher Full Text

56. De Milito A, Fais S: **Proton pump inhibitors may reduce tumour resistance.** *Expert Opin Pharmacother.* 2005; **6**(7): 1049–54.
PubMed Abstract | Publisher Full Text

57. Perut F, Avnet S, Fotia C, *et al.*: **V-ATPase as an effective therapeutic target for sarcomas.** *Exp Cell Res.* 2014; **320**(1): 21–32.
PubMed Abstract | Publisher Full Text

58. Avnet S, Di Pompo G, Lemma S, *et al.*: **V-ATPase is a candidate therapeutic target for Ewing sarcoma.** *Biochim Biophys Acta.* 2013; **1832**(8): 1105–16.
PubMed Abstract | Publisher Full Text

59. De Milito A, Iessi E, Logozzi M, *et al.*: **Proton pump inhibitors induce apoptosis of human B-cell tumors through a caspase-independent mechanism involving reactive oxygen species.** *Cancer Res.* 2007; **67**(11): 5408–17.
PubMed Abstract | Publisher Full Text

60. Mizunashi K, Furukawa Y, Katano K, *et al.*: **Effect of omeprazole, an inhibitor of H^+,K($^+$)-ATPase, on bone resorption in humans.** *Calcif Tissue Int.* 1993; **53**(1): 21–5.
PubMed Abstract | Publisher Full Text

61. Tuukkanen J, Väänänen HK: **Omeprazole, a specific inhibitor of H^+-K^+-ATPase, inhibits bone resorption *in vitro*.** *Calcif Tissue Int.* 1986; **38**(2): 123–5.
PubMed Abstract | Publisher Full Text

62. Sheraly AR, Lickorish D, Sarraf F, *et al.*: **Use of gastrointestinal proton pump inhibitors to regulate osteoclast-mediated resorption of calcium phosphate cements *in vivo*.** *Curr Drug Deliv.* 2009; **6**(2): 192–8.
PubMed Abstract | Publisher Full Text

63. Yoshimura A, Kuroda K, Kawasaki K, *et al.*: **Infectious cell entry mechanism of influenza virus.** *J Virol.* 1982; **43**(1): 284–93.
PubMed Abstract | Free Full Text

64. Gasparini R, Lai PL, Casabona F, *et al.*: **Do the omeprazole family compounds exert a protective effect against influenza-like illness?** *BMC Infect Dis.* 2014; **14**: 297.
PubMed Abstract | Publisher Full Text | Free Full Text

65. Lind T, Rydberg L, Kylebäck A, *et al.*: **Esomeprazole provides improved acid control vs. omeprazole In patients with symptoms of gastro-oesophageal reflux disease.** *Aliment Pharmacol Ther.* 2000; **14**(7): 861–7.
PubMed Abstract | Publisher Full Text

66. Maitland K, Williams TN, Kotecka BM, *et al.*: **Plasma chloroquine concentrations in young and older malaria patients treated with chloroquine.** *Acta Trop.* 1997; **66**(3): 155–61.
PubMed Abstract | Publisher Full Text

67. Long JS, Wright E, Molesti E, *et al.*: **Inhibition of pseudotype virus entry by existing FDA-approved drugs.** *Figshare.* 2014.
Data Source

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.

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.

Competing Interests: No competing interests were disclosed.

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.

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.

Competing Interests: No competing interests were disclosed.