ACS Infectious Diseases

Article

pubs.acs.org/journal/aidcbc

Evaluation of Ebola Virus Inhibitors for Drug Repurposing

Peter B. Madrid,[*,†] Rekha G. Panchal,[‡,∥] Travis K. Warren,[‡,∥] Amy C. Shurtleff,[‡] Aaron N. Endsley,[†] Carol E. Green,[†] Andrey Kolokoltsov,[§,Δ] Robert Davey,[§,Π] Ian D. Manger,[†] Lynne Gilfillan,[†,Θ] Sina Bavari,[‡] and Mary J. Tanga[†]

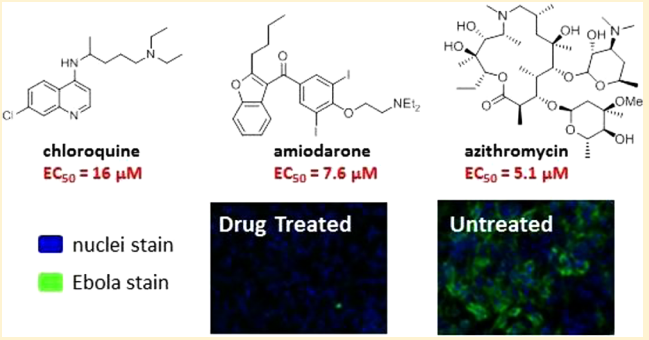
[†]Biosciences Division, SRI International, 333 Ravenswood Avenue, Menlo Park, California 94025, United States

[‡]U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, 1425 Porter Street, Frederick, Maryland 21702, United States

[§]University of Texas Medical Branch at Galveston, 301 University Boulevard, Galveston, Texas 77555, United States

Supporting Information

ABSTRACT: A systematic screen of FDA-approved drugs was performed to identify compounds with in vitro antiviral activities against Ebola virus (EBOV). Compounds active (>50% viral inhibition and <30% cellular toxicity) at a single concentration were tested in dose−response assays to quantitate the antiviral activities in replication and viral entry assays as well as cytotoxicity in the Vero cell line used to conduct these assays. On the basis of the approved human dosing, toxicity/tolerability, and pharmacokinetic data, seven of these in vitro hits from different pharmacological classes (chloroquine (CQ), amiodarone, prochlorperazine, benztropine, azithromycin, chlortetracycline, and clomiphene) were evaluated for their in vivo efficacy at a single dose and were administered via either intraperitoneal (ip) or oral route. Initially, azithromycin (100 mg/kg, twice daily, ip), CQ (90 mg/kg, twice daily, ip), and amiodarone (60 mg/kg, twice daily, ip) demonstrated significant increases in survival in the mouse model. After repeat evaluation, only CQ was found to reproducibly give significant efficacy in the mouse model with this dosing regimen. Azithromycin and CQ were also tested in a guinea pig model of EBOV infection over a range of doses, but none of the doses increased survival, and drug-related toxicity was observed at lower doses than in the mouse. These results show the benefits and specific challenges associated with drug repurposing and highlight the need for careful evaluation of approved drugs as rapidly deployable countermeasures against future pandemics.



KEYWORDS: *Ebola, viral inhibition, cellular toxicity, chloroquine, drug repurposing*

The 2014 Ebola virus (EBOV) outbreak created an immediate need for rapidly deployable medical treatments and prophylaxis options. Currently there are no FDA-approved drugs or vaccines for the treatment or prophylaxis of infection from EBOV. However, given the high mortality associated with EBOV infections, experimental therapies with limited proof-of-concept data for efficacy and safety are currently being evaluated in infected patients under expanded use authorizations. In general, new candidate treatments suffer from unproven clinical efficacy, insufficient safety data, and a lack of availability. Consequently, even if safety and efficacy requirements are met for these experimental treatments, it would remain a significant challenge to produce sufficient material to meet the treatment needs of patients in West Africa.

Given the long timelines required for drug development, a more rapid development pathway involves the repurposing of existing therapeutics with protective activities against EBOV. Furthermore, it is possible for physicians to prescribe the usage of existing therapeutics for off-label indications, particularly in situations in which there is a low risk-to-benefit ratio. A major advantage of drug repurposing is that the safety profiles for the drugs are already known, and it is more likely that a sufficient supply of drug is available for immediate use. Therefore, the main remaining challenges for repurposing approved drugs as therapeutics or prophylactic treatments during EBOV infection are to demonstrate in vitro and in vivo efficacy and to determine optimal dosing regimens.

Our group and others have led efforts to test FDA-approved drugs for activities as inhibitors of biological threat agents, including EBOV.[1−3] Through these efforts, several classes of compounds have been identified with both in vitro and in vivo activities against EBOV. The most promising results from these efforts were the identification of compounds that protected mice from a lethal exposure to EBOV. The antimalarial drug chloroquine (CQ) provided up to 80% survival rates when given at 90 mg/kg doses with twice daily ip administration,

Received: March 7, 2015
Published: April 27, 2015

DOI: 10.1021/acsinfecdis.5b00030
ACS Infect. Dis. 2015, 1, 317−326

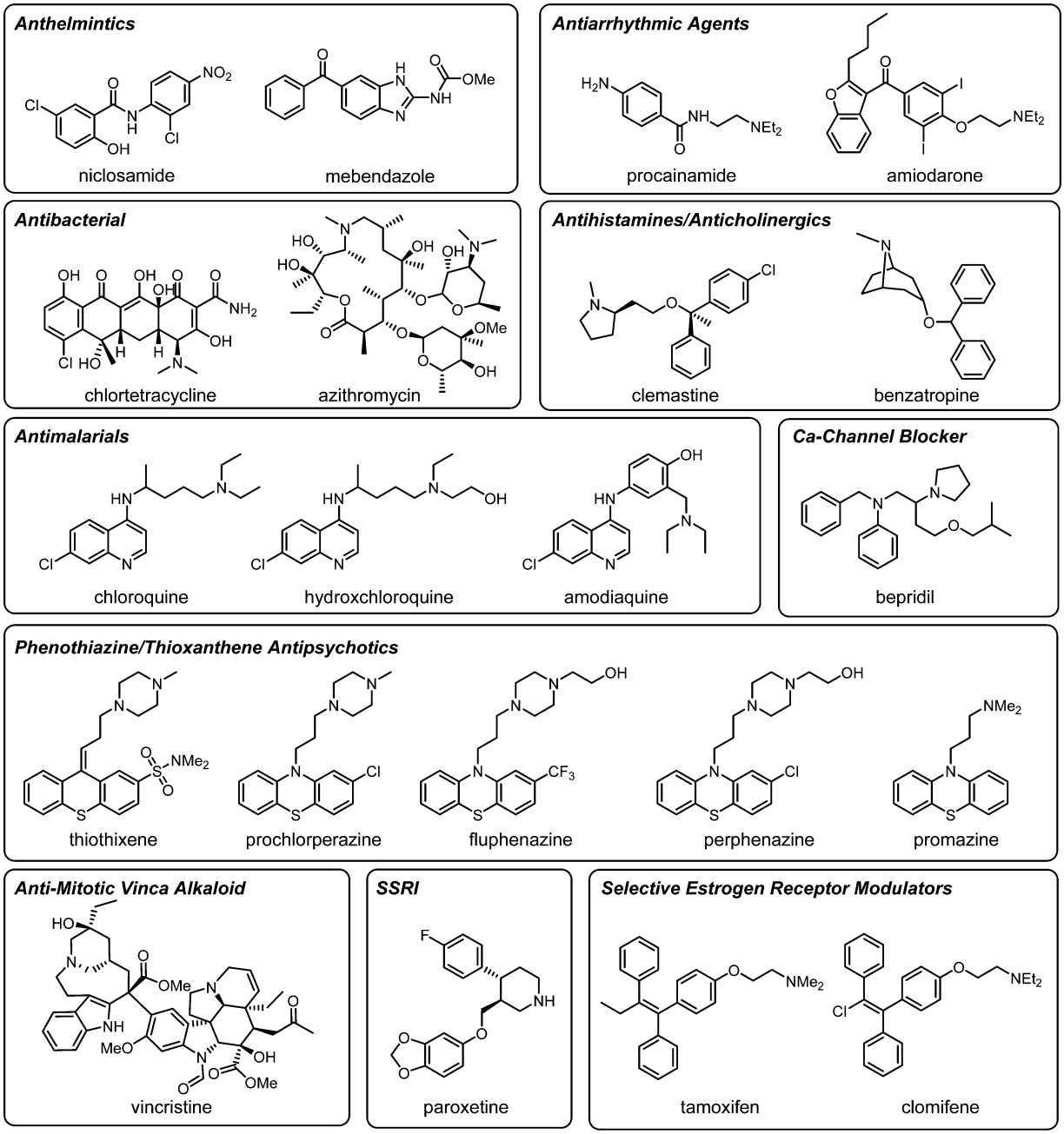


Figure 1. Structures and approved indications for in vitro EBOV inhibitors.

whereas all vehicle control group animals succumbed to infection.[2] The selective estrogen modulators (SERMs) clomiphene and toremifene have also been reported to achieve in vivo efficacy with survival rates of 90 and 50%, respectively.[1]

In the present work, we have re-evaluated some of the data generated from this program and specifically analyzed the in vitro inhibitors of EBOV replication for their drug-repurposing prospects either as prophylaxis or for treatment of EBOV infection in a high-risk environment.

RESULTS AND DISCUSSION

Approved Drugs with Anti-EBOV Activity. The focus of our original drug-repurposing effort was to identify FDA-approved drugs with broad-spectrum antiviral and/or antibacterial activities. Compounds with activity against a single bioterrorism agent were relegated to a lower priority ranking; however, in light of the current EBOV outbreak, we reanalyzed all compounds with confirmed in vitro activities and organized them by structural and therapeutic class (Figure 1). All of these compounds were identified out of an initial screen against EBOV at concentrations of either 10 or 50 μM, depending on cellular toxicity,[2] and then confirmed in a full dose−response assay to quantitate their potency on the basis of EC_{50} values (Table 1).

The EBOV replication inhibitors were also tested in a viral entry assay using pseudotyped virus that expresses the glycoprotein genes from EBOV Zaire displayed on a vesicular stomatitis virus (VSV) core (Table 1). This assay provided a mechanistic context for the inhibition of EBOV replication and served to further confirm the activities. Some of the inhibitors showed significantly greater potency in the pseudotyped EBOV entry assay than the EBOV replication assay (e.g., amiodarone,

DOI: 10.1021/acsinfecdis.5b00030
ACS Infect. Dis. 2015, 1, 317−326

Table 1. Activities of in Vitro EBOV Inhibitors in EBOV Replication, Entry, and Cytotoxicity Assays

compound	EBOV replication EC_{50} (μM)	EBOV entry EC_{50}[a] (μM)	toxicity CC_{50} Vero cells (μM)	replication selectivity index (CC_{50}/EC_{50})	class
niclosamide	1.5	<10[a]	>50	>33	anthelmintic
mebendazole	3.3	<50[a]	>50	>15	anthelmintic
amiodarone	7.6	0.81	40	5.2	antiarrythmic agent
procainamide	15	6.8	45	3	antiarrythmic agent
azithromycin	5.1[c]	1.3	>130	>25.5	antibiotic
chlortetracycline	24	<50[a]	>50	>2.1	antibiotic
clemastine	5.2	ND[b]	38	7.3	antihistamine
benztropine	9.2	14	>50	>5.4	antihistamine/ anticholinergic
chloroquine	16	4.7	>50	>3.1	antimalarial/ antirheumatic
hydroxychloroquine	22	9.5	>50	>2.2	antimalarial/ antirheumatic
amodiaquine	34	2.6	>50	>1.4	antimalarial
bepridil	4.9	24	>50	>10	calcium channel blocker
vincristine	21	<10[a]	>50	>2.4	mitotic inhibitor
thiothixene	3.4	ND[b]	22	6.5	thioxanthene antipsychotic
prochlorperazine	11	1.9	43	3.9	phenothiazine antipsychotic
fluphenazine	12	ND[b]	34	2.8	phenothiazine antipsychotic
perphenazine	12	ND[b]	>50	>4.2	phenothiazine antipsychotic
promazine	21	ND[b]	>50	>2.4	phenothiazine antipsychotic
paroxetine	27	<10[a]	>50	>1.8	SSRI
tamoxifen	3	ND[b]	38	13	SERM
clomiphene	11	1.3	>50	>4.5	SERM

[a]Not all compounds were tested in a full dose response for the EBOV entry assay. Values specified with a "less than" sign were tested only at 10 and 50 μM concentrations, and the specified values indicate a concentration at which at least 50% of viral entry was inhibited with <20% toxicity. [b]ND, not determined. These compounds were tested at 10 and 50 μM concentrations and were considered false positives as they showed cellular toxicity at these concentrations and thereby resulted in inhibition of viral entry. [c]Assay performed in HeLa cells.

Table 2. Human Pharmacokinetic Parameters for Approved Dosing Routes of in Vitro EBOV Inhibitors[a]

compound	terminal half-life ($t_{1/2}$)	bioavailability (F)	route	typical dose	C_{max}
niclosamide	NA[b]	very low (<10%)	po	1–2 g	NA
mebendazole	1 h	low (<25%)	po	100	100 mg; BID 30 ng/mL
amiodarone	3–80 h	moderate(22–86%)	po	800–1600 mg/day	400 mg; 0.6 μg/mL
procainamide	3–4 h	high (>80%)	iv/im/po	500 mg	500 mg; 2.5 μg/mL
azithromycin	2–4 days	moderate (37%)	po/iv	250–1000 mg	500 mg; 0.5 μg/mL
chlortetracycline[c]	5.6–9 h	moderate (30%)	po/iv	NA	NA
clemastine	20 h	moderate (40%)	po	1–3 mg	0.6 ng/mL/mg
benztropine	NA	NA	po	0.5–2 mg	1.5 mg; 2.5 ng/mL
chloroquine[4]	3–6 days	high (>75%)	po	1 g, then 500 mg	600 mg; 1.8 μM
hydroxychloroquine[5]	30–40 days	high (>70%)	po	400–600 mg/day	200 mg; 46 ng/mL
amodiaquine	5 h	NA	po	800 mg, then 400 mg/day	600 mg; 30 ng/mL[d]
bepridil[e]	26–64 h	high (>90%)	po	200–400 mg	NA
vincristine	19–155 h	NA	iv	≤2 mg	NA
thiothixene	34 h	NA	po/im	6–60 mg daily	NA
prochlorperazine	3–5 h	low (<25%)	po	5–10 mg every 8 h	50 mg; 4 ng/mL
fluphenazine	7–14 days	very low (<10%)	po	2.5–10 mg every 6–8 h	12 mg; 1–2 ng/mL
perphenazine	9–12 h	moderate (40%)	po	4–24 mg	NA
promazine	9 h	low (<25%)	po	100 mg every 6 h	NA
paroxetine	21 h	high (>90%)	po	10–40 mg	NA
tamoxifen	5–7 days	high (>90%)	po	10–20 mg	20 mg; 40 ng/mL
clomiphene	8–22 days	high (>90%)	po	50 mg	50 mg; 12 ng/mL

[a]All data are from published product package inserts unless otherwise referenced. [b]NA, not available. We were unable to locate these values from references. [c]Chlortetracycline is primarily used in veterinary medicine and no longer used in humans. [d]The drug is rapidly metabolized to a desethyl derivative that is responsible for the majority of its antimalarial activity and may act similarly to the parent drug.[6] [e]Bepridil is discontinued in the United States.

DOI: 10.1021/acsinfecdis.5b00030
ACS Infect. Dis. 2015, 1, 317–326

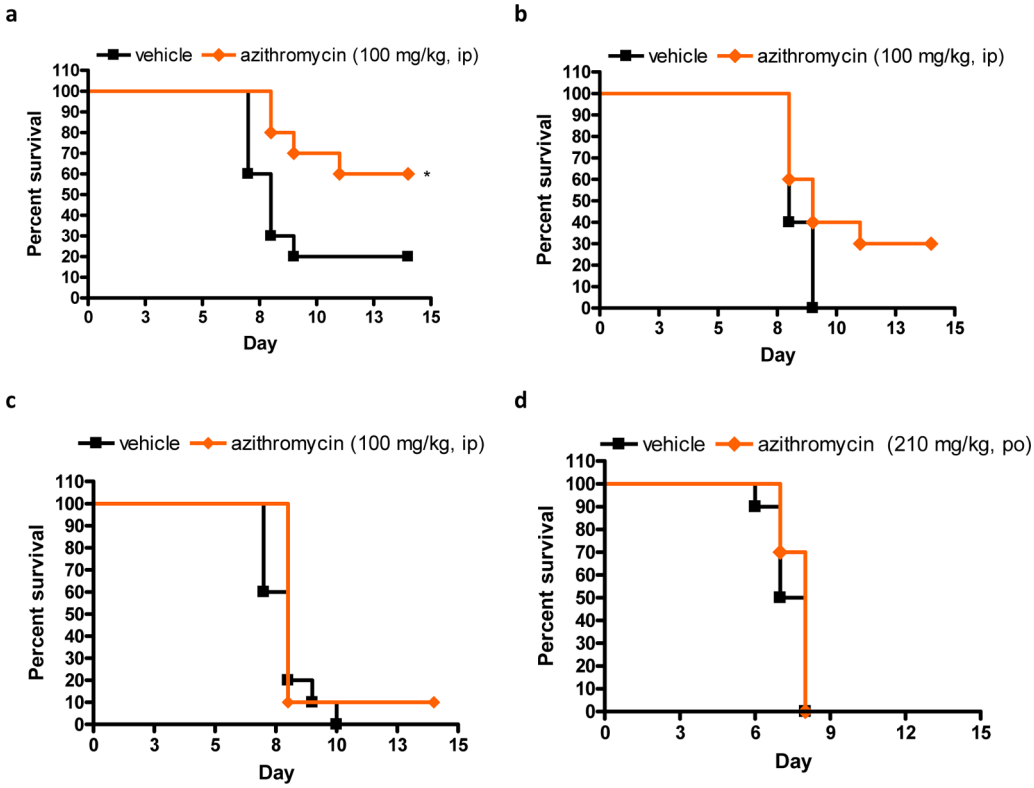


Figure 2. Kaplan–Meier survival curves for azithromycin efficacy studies in mice. (a) Azithromycin (100 mg/kg, ip, twice daily) gave 60% overall survival (p = 0.02). (b) Repeat of azithromycin (100 mg/kg, ip, twice daily) gave 30% overall survival (p = 0.06). (c) Third study of azithromycin (100 mg/kg, ip, twice daily) showed no significant increase in survival. (d) Azithromycin tested with oral dosing (210 mg/kg, po, once daily) gave no increase in survival.

amodiaquine, clomiphene). Other compounds demonstrated greater cytotoxicity in the EBOV entry assay, making it difficult to assess whether the observed antiviral activity was due to entry inhibition (e.g., clemastine, thiothixene, fluphenazine, perphenazine, promazine, tamoxifen).

To further distinguish the antiviral activity from cellular toxicity, all potent compounds were evaluated in parallel for cytoxicity in uninfected Vero cells and a 50% cytotoxicity concentration (CC_{50}) was determined on the basis of the dose response curves (Table 1). Evaluation of antiviral activity together with cytotoxicity is critical because compounds with marginal cytotoxicity can appear to elicit antiviral activity when cell viability is used as the assay readout. The ratio of the 50% cytotoxicity concentration (CC_{50}) and the 50% effective concentration (EC_{50}) was calculated as a selectivity index (SI) (Table 1). Many of the compounds were not tested at high enough concentration to accurately determine an SI value, but compounds with anti-EBOV activities <3-fold greater than their cytotoxicities are much less likely to have utility as an antiviral compound in vivo.

Dosing and Pharmacokinetic Considerations. In addition to the in vitro activities of the EBOV inhibitors, dosing and pharmacokinetic parameters can be analyzed for each of the compounds as part of an assessment for potential repurposing (Table 2). At this early stage, it is difficult to quantitatively predict whether sufficient exposure levels are achievable for each compound, but a qualitative assessment can be performed by looking at the approved doses, bioavailability (F), and most importantly the peak plasma concentration (C_{max}). These data are difficult to use to unequivocally eliminate compounds because it may be possible to safely dose patients at much higher doses that are typical for the approved indications, but mining these data from the literature is difficult. It is very apparent from this analysis that many of the CNS-active drugs (i.e., antipyschotics, antihistamines, SSRIs) are approved and administered at doses of <50 mg, making them unlikely to achieve exposure concentrations near their antiviral EC_{50} values. For example, if the typical plasma volume of an average adult (70 kg) is estimated to be 3 L, then a completely bioavailable compound could achieve only a theoretical C_{max} of just above 16 ng/mL. In addition, escalation of doses may lead to off-target negative CNS effects for these brain-penetrating compounds. Drugs dosed via injection are slightly less desirable, due to the inconvenience of this route for multiple dosing, as well as possible increased risk of EBOV transmission. In general, antiviral compound efficacy is a function of time drug concentration and/or exposure spends above an efficacy threshold; therefore, short half-life drugs with poor bioavailability may be difficult to optimize for EBOV treatment or prophylaxis.

In Vivo Efficacy Screening in Mice. To evaluate the in vivo efficacy, a small subset of the EBOV inhibitors were screened at a single dose in the mouse model of EBOV infection.[2,7] The seven compounds were tested in several different studies, and hence the Kaplan–Meier survival curves are shown for individual studies with appropriate vehicle controls (Figures 2–4 and Table 3). It is also important to note that the majority of the studies were performed with ip compound dosing, although some studies were also performed using oral (po) dosing. The statistical significance of any increase in survival was evaluated using a log rank test in comparison to the vehicle control.

DOI: 10.1021/acsinfecdis.5b00030
ACS Infect. Dis. 2015, 1, 317–326

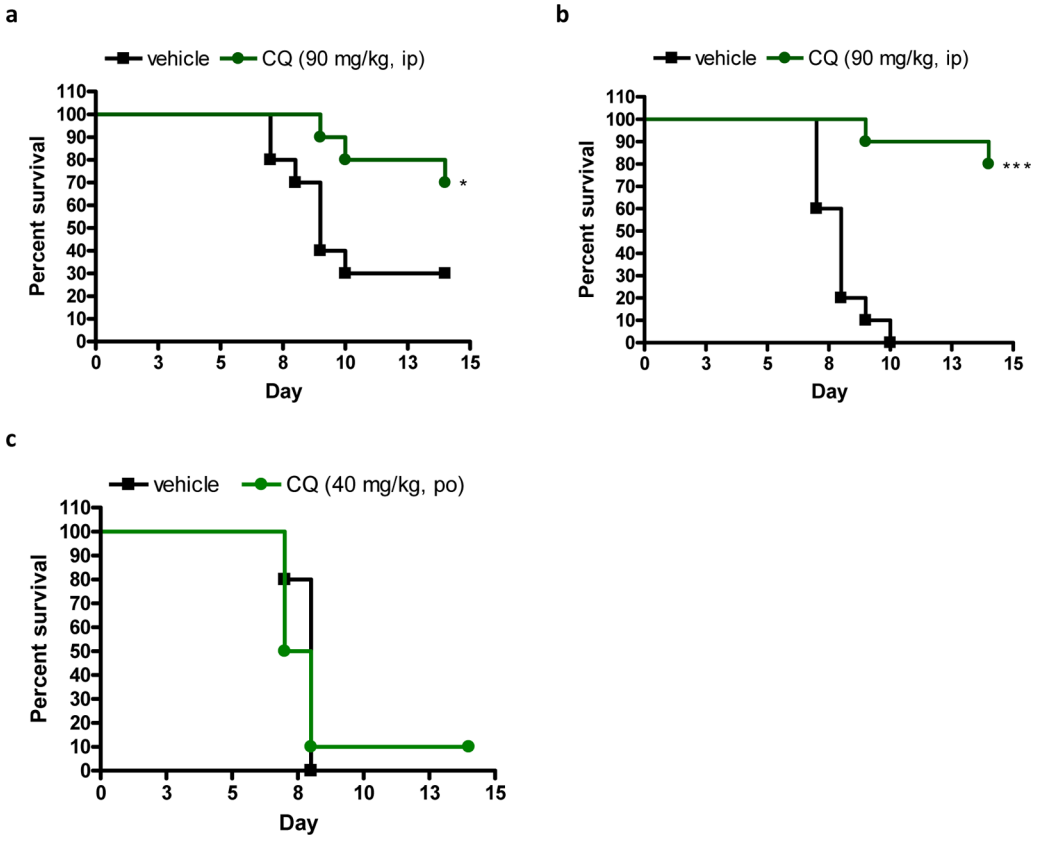


Figure 3. Kaplan–Meier survival curves for CQ efficacy studies in mice. (a) CQ (90 mg/kg, ip, twice daily) gave 70% overall survival (p = 0.037). (b) Repeat of CQ (90 mg/kg, ip, twice daily) gave 80% overall survival (p < 0.0001). (c) CQ tested with oral dosing (40 mg/kg, po, once daily) gave no increase in survival.

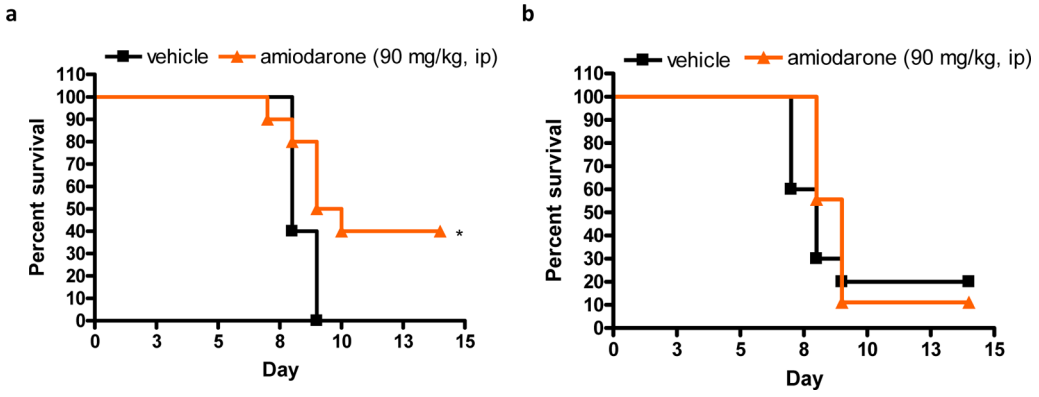


Figure 4. Kaplan–Meier survival curves for amiodarone efficacy studies in mice. (a) Amiodarone (90 mg/kg, ip, twice daily) gave 40% overall survival (p = 0.016). (b) Repeat of amiodarone (90 mg/kg, ip, twice daily) gave no increase in survival.

Table 3. Summary of in Vivo Efficacy Results Summary for Additional Compounds Tested

compound	dose concentration (mg/kg)	route of treatment	regimen	% mouse survival (treatment group)	% mouse survival (control group)
amodiaquine	60	ip	BID	0	0
clomiphene	60	ip	BID	10	0
clomiphene	21	po	SID	0	0
prochlorperazine	10	ip	SID	10	20
benztropine	13	po	SID	10	0
benztropine	5	ip	BID	10	0
chlorotetracycline	200	po	SID	0	0
chlorotetracycline	50	ip	BID	0	0

DOI: 10.1021/acsinfecdis.5b00030
ACS Infect. Dis. 2015, 1, 317–326

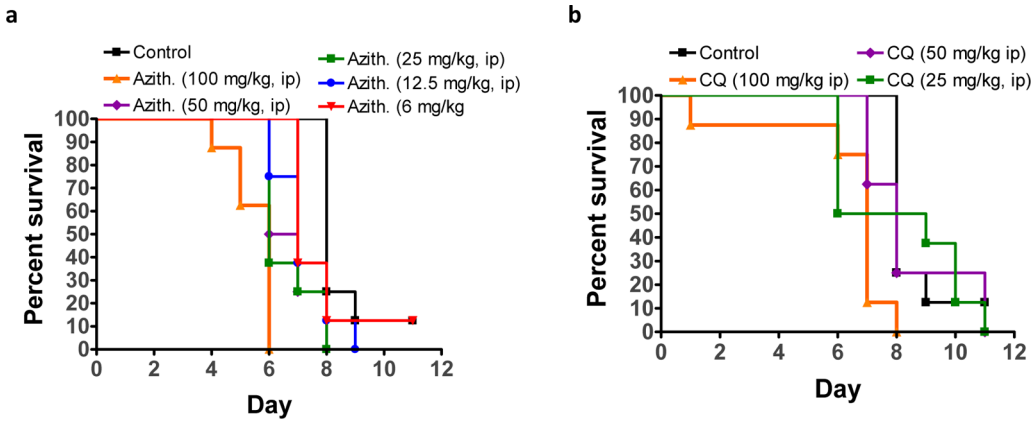


Figure 5. Kaplan–Meier survival curves for efficacy studies with in vitro EBOV inhibitors in guinea pigs. (a) Azithromycin (6–100 mg/kg, ip, once daily) gave no increase in survival with these dosing regimens. (b) CQ (25–100 mg/kg, ip, once daily) gave no increase in survival with these dosing regimens.

Azithromycin was first tested in a mouse study with 100 mg/kg dosing twice daily by ip administration (Figure 2a). This dosing regimen gave 60% overall survival with 20% survival in the vehicle group (p = 0.02). Due to the low significance and relatively high survival rate in the control group, this study was repeated identically. In this second independent evaluation, the azithromycin-treated group had 30% survival, whereas the vehicle group had 0% survival, and there was no statistical significance between the two groups (p = 0.06) (Figure 2b). A third independent evaluation using the same dosing regimen resulted in only 10% survival (Figure 2c). Despite the initially promising results, these studies collectively suggest that azithromycin is not reproducibly effective with this dosing regimen. A once-daily oral dosing regimen at 210 mg/kg (the body-surface area scaled human dose equivalent) was subsequently tested to evaluate whether oral dosing might produce a more reproducible effect, but this dosing regimen also failed to increase survival or time to death (Figure 2d).

CQ was tested in three separate efficacy studies, with significant increased survival in the studies that used ip dosing at 90 mg/kg (Figure 3a,b), but not in the study with po dosing at 40 mg/kg (Figure 3c). The first ip study gave 70% overall survival (n = 10 mice/group), but the vehicle control in this particular study resulted in 30% survival, which is greater than the more typical 0–10% survival for the control group and reduced the significance of the CQ efficacy. The study was subsequently repeated using the same dosing and produced 80% survival with 0% survival in the vehicle control group as previously reported.[2] This result prompted us to test CQ with po route at a dose of 40 mg/kg, which is the equivalent to the approved human dose scaled to mice using a body-surface area scaling factor. This dosing regimen did not increase the overall survival or the time-to-death relative to the controls.

The antiarrhythmia drug amiodarone gave a modest, but significant, increase in survival when given at 90 mg/kg with ip dosing (Figure 4a). This efficacy study was then repeated, but gave no increase in survival or time to death in the second study (Figure 4b).

Five other drugs that were in vitro hits (amodiaquine, prochlorperazine, benztropine, chlortetracycline, and clomiphene) were also evaluated in the mouse model of EBOV infection. These compounds were tested via ip or po dosing, but they did not show any efficacy at the selected doses (Table 3).

These efficacy results demonstrate some important limitations with our in vivo efficacy screening paradigm. In an effort to maximize throughput, compounds were tested at only a single dose level using a nonoptimized dosing frequency and administration route. Initially, ip administration was chosen to maximize drug exposure and as the most convenient dosing modality for operating within an ABSL4 environment. Later, it was decided that because oral dosing is the ultimate route of administration, it would be more efficient to dose orally for initial efficacy screening in the mouse model. The challenge with oral dosing is that without a complete pharmacokinetic analysis for each compound, it is difficult to choose a dose level and dosing regimen that maximized drug exposure without undesired toxicities. The variability between our results with ip and po dosing for CQ demonstrates the importance of optimizing dosing prior to efficacy testing. For the development of anti-EBOV drugs, for which the efficacy testing must be performed within an ABSL4 environment, it is particularly important to rigorously optimize dosing prior to efficacy testing, so that critical development decisions can be made about compounds from data generated with optimal dosing using a clinically relevant route of administration.

The variability observed with the in vivo efficacy testing for azithromycin, CQ, and amiodarone also highlights the importance of repeated testing with this EBOV efficacy model. The model of EBOV infection has many known limitations, such as its inherent resistance to wild-type EBOV strains and highly effective type I interferon response to infection, but it also has demonstrated utility as a screening tool for identifying potential therapeutics.[8,9] The difficulty and expense of performing EBOV efficacy studies often limit the size and numbers of studies, but independent replicates are important for confirming any measured anti-EBOV in vivo efficacy.

In Vivo Efficacy Screening in Guinea Pigs. The initial demonstration of anti-EBOV efficacy in vivo in mice led us to further evaluate CQ and azithromycin in a guinea pig EBOV infection model. Guinea pigs have frequently been used as a model for EBOV infections, and they exhibit pathology that better mimics some of the hallmarks of EBOV infection in nonhuman primates and humans.

Azithromycin was tested in the guinea pig model at doses ranging from 6 to 100 mg/kg given once daily by ip administration (Figure 5a). None of the tested dose levels

DOI: 10.1021/acsinfecdis.5b00030
ACS Infect. Dis. 2015, 1, 317–326

gave increase in survival or delayed time to death. The three highest dose levels (25, 50, and 100 mg/kg) appear to exhibit toxicity, with animals dying primarily at day 6, whereas in the vehicle controls, the animals did not succumb to infection until day 8. The lower dose groups were not significantly different from the vehicle group, indicating a lack of efficacy in this model.

CQ was also tested in a guinea pig model of EBOV infection at doses ranging from 25 to 100 mg/kg given once a day with ip dosing (Figure 5b). Similarly to azithromycin, there was no increased survival or time to death at any of the tested doses, and also there was an indication of compound-related toxicity in all three dose groups. This result led us to subsequently perform a pharmacokinetic study of CQ in male Hartley guinea pigs at 7.5, 15, 30, and 45 mg/kg single doses with ip administration (Supporting Information Figure S1 and Table S3). These results indicated that at doses at which there were no adverse effects (<25 mg/kg) in the guinea pigs, there was much lower drug serum concentrations and overall exposures relative to those in the mouse. Together, these results indicate that the unanticipated higher toxicity of CQ in the guinea pig makes it unlikely to be a useful model for evaluating its anti-EBOV activity.

EBOV Inhibitors for Further Consideration. The results of our drug-repurposing efforts have identified several EBOV inhibitors that merit further consideration based on their in vitro and in vivo activities as well as other pharmacological considerations. There are many advantages associated with drug-repurposing efforts, particularly the wealth of existing data for each drug, but one of the limitations is that the compounds cannot be modified to improve specific aspects of their pharmacological activities, as one would do in a typical drug discovery program. With this key difference in the development paradigm, it is particularly important that promising candidates are evaluated as thoroughly as possible and candidates with fundamental limitations are eliminated early. With this in mind, several of the EBOV inhibitors identified warrant additional studies for potential repurposing as EBOV antivirals.

Azithromycin has many desirable features as an anti-EBOV repurposing candidate such as good in vitro potency, low toxicity, and high dosage forms. The lack of reproducibility in the mouse model as well as the lack of efficacy in the guinea pig model raises concerns about the ability to repurpose this drug as an EBOV antiviral. Oral dosing with azithromycin also did not show any efficacy, but on the basis of the high volume of distribution of this drug (31 L/kg in humans), it may require administration through continuous infusion or the performance of several loading doses prior to viral challenge to maximize any effect.

The antimalarial drug CQ has demonstrated the most promising initial activities and has reasonable pharmacological properties for repurposing as an EBOV antiviral. CQ is the only compound that has given reproducible efficacy in the mouse model, despite its modest potency. There was no efficacy observed in the guinea pig model of EBOV infection, but a pharmacokinetic analysis showed that this is likely due to an inability to achieve sufficient drug concentration in a guinea pig due to the higher toxicity of CQ to guinea pigs. There is a clear mechanistic hypothesis for the antiviral activity of CQ based on its in vitro ability to inhibit viral entry in EBOV as well as for other enveloped viruses.[2,10,11] This mechanistic hypothesis is somewhat confounded by the immunological activities of CQ that may also play a role in its in vivo activity.[12,13] CQ is administered orally as an antimalarial in high doses (1 g followed by 500 mg) and has high bioavailability (>75%) and a long half-life (3−6 days in humans). PK analysis showed that the efficacious dosing regimen produced a steady-state serum concentration of ∼2500 ng/mL.[2] Scaling of this dose to humans by body surface area gives an equivalent dose of 7 mg/kg or around a 500 mg dose, which is similar to the dose used for antimalarial therapy. A phase I clinical trial that administered 1500 mg of CQ over 3 days gave serum concentrations of ∼1100 ng/mL with a terminal elimination half-life of 13.2 days.[4] Therefore, CQ given to humans in the approved dosage or just slightly higher should produce serum concentrations similar to those shown to be efficacious in the mouse study. The enormous volume of distribution for CQ (>100 L/kg) is another factor that must be taken into account because this greatly affects the available blood concentration of drug and the time required to reach steady-state concentrations. Continuous infusions of drug might be a preferable manner to deliver CQ despite its excellent oral bioavailability.[14]

The antimalarial drugs hydroxychloroquine and amodiaquine are both structurally and mechanistically related to CQ, with their own potential advantages/disadvantages. Hydroxychloroquine is primarily used as a treatment for rheumatoid arthritis (RA) and routinely administered as a chronic treatment with daily dosing. The human pharmacokinetics are very similar to that of CQ, but it is typically given at slightly lower doses.[15] Amodiaquine was found to be slightly less potent than CQ and in humans is rapidly metabolized to a desethyl derivative that is believed to be responsible for most of its antimalarial activity. The lack of efficacy of amodiaquine in the mouse model could be due to the lack of anti-EBOV activity of the desethyl metabolite, because the parent drug is present only at very low concentrations. Because the entire class of 4-aminoquinoline antimalarial drugs have demonstrated anti-EBOV activity, including a newer drug still in clinical testing called AQ-13,[2,4,16] additional tests to identify additional EBOV repurposing candidates from this series are warranted.

The antiarrhythmia drug amiodarone gave a small, but significant, amount of increased survival in one efficacy study, but did not reproduce this effect subsequently. The in vitro activities of amiodarone suggest that it is slightly more potent than CQ and also appear to inhibit viral entry. Inhibition of viral entry for hepatitis C virus has also been demonstrated for amiodarone.[17] There are several known molecular targets for amiodarone, including the β-andrenergic receptor as well as potassium channels, so it is unclear how these activities contribute to the EBOV antiviral effects.[18] The dosing and pharmacokinetics of amiodarone are reasonably good for potential anti-infective repurposing, but there are also numerous side effects and toxicities associated with this drug. Procainamide is a related antiarrhythmia drug with a similar, but slightly less potent, activity. The higher bioavailability and serum concentrations of procainamide could lead to great in vivo efficacy compared to amiodarone.

The anthelmintic drug niclosamide is a salicylanilide drug used for the treatment of tapeworm infections. This drug was one of the most potent EBOV inhibitors identified (EC_{50} = 1.5 μM), but has not yet been tested in an animal efficacy model. This compound is also being investigated for its anticancer activity[19,20] and has reported antiviral activity against SARS-CoV.[21] Niclosamide can be given in large doses (2 g human dose; LD_{50} in rats is >5000 mg/kg), but has limited bioavailability (∼10%). This dosing does produce low micro-

DOI: 10.1021/acsinfecdis.5b00030
ACS Infect. Dis. 2015, 1, 317−326

molar serum concentrations in animals and humans after a single dose. Intravenous dosing can produce serum concentrations of around 25 μM, making this drug a good candidate for further evaluation.[19]

Many of the other classes of hits identified have important limitations that are likely to prevent their repurposing as EBOV antivirals. The phenothiazine/thioxanthene antipsychotics, antihistamines, and SSRIs are all used therapeutically at very low doses and generally have strong central nervous system effects at higher doses. Given the micromolar level anti-EBOV potencies of these compounds, the inability to dose these compounds at high doses is likely to preclude their development as EBOV antivirals. The SERM clomiphene has been reported to have in vivo efficacy using a higher, but less frequent, dosing regimen (60 mg/kg, ip, every-other-day dosing),[1] but was ineffective when we dosed it at 40 mg/kg ip with twice daily dosing. The difference in observed efficacy reinforces the importance of optimizing dosing and repeating the EBOV mouse efficacy studies. The approved doses for clomiphene and other SERMs is typically low (50 mg daily) and produces serum concentrations (~12 ng/mL) 2 orders of magnitude below the EC_{50} values.[22] Intravenous dosing can produce concentrations that are much higher in humans,[23] but the large difference between the approved dosages and the doses that have demonstrated efficacy increases the risk associated with repurposing compounds from this class. Chlortetracycline was viewed as a promising hit due to its high dosage form and tolerability, but its low potency and lack of efficacy at 200 mg/kg po daily dosing is not promising. Vincristine was not further pursued due to its general toxicity and low approved dosage. The calcium channel block bepridil is no longer marketed in the United States due to potential for cardiotoxicity, but its potency and pharmacokinetics warrant additional evaluation even though there are a number of risks associated with this compound.

Conclusion. The repurposing of FDA-approved therapeutics for new indications against potential pandemic or bioterrorism threats represents a rapid and cost-effective approach for meeting these unmedical needs. The overall workflow of a drug-repurposing effort is similar to typical drug discovery effort, but there are fundamental differences that result from the discrete nature of the screening set and inability to use medicinal chemistry to modify compound properties.

Although repurposing screens are performed on a smaller number of compounds with more limited chemical diversity, the inherent bias toward bioactivity in this set can yield numerous hits in a primary screen. The evaluation of hits as members of different pharmacological classes, rather than just as individual compounds, is useful for prioritizing the top compounds from distinct structural and mechanistic families of compounds. The prioritized compounds can be evaluated in terms of potencies and toxicities as well pharmacokinetic and toxicity data in animals and humans for each compound. The wealth of data available for existing drugs can be used to make more informed development decisions at an early stage. Despite the wealth of data available for most drugs, it can still be critical to optimize the dosing through pharmacokinetic analysis to ensure that candidates are administered appropriately for the new target indication.

The results of our efforts have identified a set of antimalarials as candidates for the treatment or prophylaxis of EBOV infection, as well as potentially an anthelmintic and antibiotic. In all of these cases, the existing drugs are approved for use at high doses with serum concentrations close to their antiviral potencies. The ability to repurpose these drugs for EBOV infection will depend on their efficacy in additional animal models with optimized dosing regimens for the new indication.

METHODS

Chemicals and Materials. All chemicals were purchased from Sigma-Aldrich and were verified to be >95% purity by HPLC analysis.

Animal Use and Care. Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other federal statutes and regulations relating to animals and experiments involving animals. The IACUC committee approving this protocol is the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) IACUC. The facility where this research was conducted, USAMRIID, is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International, and adheres to principles stated in the eighth edition of the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 2011.

Virus Strains. Ebola Zaire was propagated at USAMRIID (Fort Detrick, MD, USA) under BSL-4 conditions. Viral stocks were made by propagation in Vero cells using viral maintenance media (serum-free MEM supplemented with L-glutamine, penicillin G, streptomycin, TPCK trypsin, and BSA) and titered using standard plaque assays.

Mammalian Cells and Media. Vero cells (CCL-81), Vero 76 cells (CRL-1587), MDCK cells (CCL-34), HEK 293T (CRL-11268), and HeLa cells (CCL-2) were obtained from ATCC (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT, USA) or Eagle's minimum essential medium (MEM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ATCC). The 293 FT cells were procured from Invitrogen and maintained in DMEM.

EBOV Replication Assays. Vero 76 cells were seeded in 96-well high-content imaging plates at 80−90% confluency. Cells were pretreated with either DMSO (negative control), bafilomycin A1 (positive control), or test compound (10 or 50 μM final concentration) for 1 h at 37 °C. The cells were infected with EBOV-eGFP (1:5 MOI)[24] and incubated at 37 °C with 5% CO_2 for 48 h. The supernatant was removed, and cells were fixed with 10% formalin for 72 h before being washed with phosphate-buffered saline (PBS). The EBOV-eGFP infected cells were stained with nuclear Hoechst dye 33342 (1 μg/mL diluted in PBS) and CellMask Deep Red cytoplasmic/nuclear stain (5 μg/mL diluted in PBS). High content image acquisition was performed using an Opera QEHS confocal imaging reader. Images were processed and analyzed using Acapella and Definiens image analysis packages to determine the number of eGFP-positive (Ebola replication-positive) cells and the total number of cells remaining in each well as an in-well control of cell toxicity. EC_{50} values were derived from plotting the eGFP-positive cells as a function of compound concentration across a range of eight concentrations.

Pseudotype EBOV Virus Entry Assay. HEK 293T cells were grown in DMEM supplemented with 10% FCS. One day before drug treatment, cells encoding Renilla luciferase (marker of cell viability) were plated into 96-well white-walled tissue

DOI: 10.1021/acsinfecdis.5b00030
ACS Infect. Dis. 2015, 1, 317−326

culture plates to allow attachment. Cell density was adjusted to ~80% confluence on the day of drug exposure. Cells were then pretreated for 1 h with compounds at concentrations from 0.5 to 50 μM in 2-fold serial dilutions. After 1 h, the media containing the compound were replaced with fresh media containing compound and envelope glycoprotein-pseudotyped vesicular stomatitis virus (VSV)-encoding firefly luciferase. Pseudotyped virus construction was performed as described earlier, using glycoprotein genes derived from EBOV.[25] After 9 h, cells were washed in fresh media and incubated for an additional 10 h. These time periods were chosen for two reasons: (1) to provide sufficient delay in firefly luciferase expression to permit easy detection of any effect of each compound on virus infection and (2) to limit cytotoxicity by reducing the time that cells were exposed to the compound. At the end of the incubation period, the medium was removed, and firefly and Renilla luciferase activities were measured by the Dual-Glo Luciferase Assay System using a Veritas 96-well plate luminescence reader (Turner Instruments, Sunnyvale, CA, USA). Data were analyzed to determine percent inhibition compared with inhibition for the positive control and in-well cytotoxicity (Renilla luciferase measurement compared to the no-drug control). Bafilomycin A1 was used as a positive control, and DMSO-only wells were used as negative controls. EC_{50} values were determined from plotting the percent inhibition as a function of compound concentration and fitting the results to a four-parameter logistical function in PRISM.

Cell Toxicity Screening. Cells were plated in 96-well plates (~5×10^4 cells/well) and incubated overnight in appropriate cell culture media. Stock solutions of test compounds were added to cells at concentrations from 0.5 to 50 μM concentrations with a final DMSO concentration of 0.5% for 24 h. At the end of this incubation period, cell viability was measured using a modified MTT assay Cell Counting Kit-8 (Aldrich). CC_{50} values were derived from plotting the calculated percent viability as a function of compound concentration and fitting the results to a four-parameter logistical function in GraphPad Prism.

In Vivo Efficacy Testing. Selected in vitro EBOV inhibitors were tested in an EBOV mouse infection model for their ability to increase survival. The efficacy screening dose levels were chosen on the basis of a maximum tolerated dose study with uninfected Balb/c mice dosed across a range of three dose levels (body mass-scaled human dose, body surface area (BSA)-scaled human dose, and 10 times the BSA-scaled human dose). The highest dose level that gave no adverse effects in mice was selected for the efficacy studies. Prior to use, delivery vehicle (10% DMSO, 18% Cremaphor, 72% water) was added with vortexing to produce a uniform solution or suspension. Suspended compounds were stored at 4 °C between doses, warmed to room temperature, and vortexed prior to use. Each compound was tested in 10 Balb/c female mice, administered once or twice daily starting on day of infection (day 0) and continuing for an additional 7 days. Route of treatment was ip or oral (po), and infection was via ip route (1000 pfu, mouse-adapted EBOV). For each study, a single control group of 10 animals was used. Animals were monitored post challenge for up to 14 days or until death (or severe morbidity and euthanasia criteria were achieved), whichever occurred first. Clinical observations were made and recorded daily. These included weight loss (total for challenge group, time phased), morbidity (number of mice showing morbidity, type of morbidity, time-phased), and time to death for all mice (within 12 h window). The Kaplan−Meier survival curves were analyzed using a log rank test with the control and treatment groups and analyzed in GraphPad Prism.

The drugs CQ and azithromycin were also evaluated in Hartley guinea pigs that were infected via the ip route with 1000 pfu of guinea pig adapted Ebola Mayinga. Treatment was initiated on day 0 via ip route and given once daily for 7 days. Guinea pigs were monitored for survival and weights for 14 days or until death.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00030.

Additional information on preliminary anti-EBOV screening hits (Table S1) and pharmacokinetic studies of CQ dosed in Hartley guinea pigs (Figure S1 and Table S2) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*(P.B.M.) Phone: (650) 859-2253. Fax: (650) 859-3153. E-mail: peter.madrid@sri.com.

Present Addresses

$^{\Delta}$(A.K.) PharmEco, Moscow, Russian Federation.
$^{\Pi}$(R.D.) Texas Biomedical Research Institute, San Antonio, TX, USA.
$^{\Theta}$(L.G.) Rhumbline Consultants, Woodbridge, VA, USA.

Author Contributions

$^{\|}$R.G.P and T.K.W. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by internal grants of SRI International and the Defense Threat Reduction Agency (DTRA) TMTI Grant HDTRA1-07-C-0083. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Jay Wells, Sean Vantongeren, and Kelly Stuthman for execution of the in vivo studies and other project team members, Rae Lyn Burke, Tiffany R. Keepers, Lalitha V. Iyer, Ricardo Carrion Jr., and Jean L. Patterson, for their valuable contributions to the overall DTRA drug-repurposing project. Opinions, interpretations, conclusions, and recommendations stated within the paper are those of the authors and are not necessarily endorsed by the U.S. Army nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

■ REFERENCES

(1) Johansen, L. M., Brannan, J. M., Delos, S. E., Shoemaker, C. J., Stossel, A., Lear, C., Hoffstrom, B. G., DeWald, L. E., Schornberg, K. L., Scully, C., Lehár, J., Hensley, L. E., White, J. M., and Olinger, G. G. (2013) FDA-approved selective estrogen receptor modulators inhibit Ebola virus infection. *Sci. Transl. Med. 5*, 190ra79.

(2) Madrid, P. B., Chopra, S., Manger, I. D., Gilfillan, L., aKeepers, T. R., Shurtleff, A. C., Green, C. E., Iyer, L. V., Dilks, H. H., Davey, R. A., Kolokoltsov, A. A., Carrion, R., Jr., Patterson, J. L., Bavari, S., Panchal, R. G., Warren, T. K., Wells, J. B., Moos, W. H., Burke, R. L., and

DOI: 10.1021/acsinfecdis.5b00030
ACS Infect. Dis. 2015, 1, 317−326

Tanga, M. J. (2013) A systematic screen of FDA-approved drugs for inhibitors of biological threat agents. *PLoS One 8*, No. e60579.
(3) Gehring, G., Rohrmann, K., Atenchong, N., Mittler, E., Becker, S., Dahlmann, F., Pohlmann, S., Vondran, F. W., David, S., Manns, M. P., Ciesek, S., and von Hahn, T. (2014) The clinically approved drugs amiodarone, dronedarone and verapamil inhibit filovirus cell entry. *J. Antimicrob. Chemother. 69*, 2123.
(4) Mzayek, F., Deng, H., Mather, F. J., Wasilevich, E. C., Liu, H., Hadi, C. M., Chansolme, D. H., Murphy, H. A., Melek, B. H., Tenaglia, A. N., Mushatt, D. M., Dreisbach, A. W., Lertora, J. J., and Krogstad, D. J. (2007) Randomized dose-ranging controlled trial of AQ-13, a candidate antimalarial, and chloroquine in healthy volunteers. *PLoS Clin. Trials 2*, No. e6.
(5) Tett, S. E., Cutler, D. J., Day, R. O., and Brown, K. F. (1989) Bioavailability of hydroxychloroquine tablets in healthy volunteers. *Br. J. Clin. Pharmacol. 27*, 771.
(6) Churchill, F. C., Patchen, L. C., Campbell, C. C., Schwartz, I. K., Nguyen-Dinh, P., and Dickinson, C. M. (1985) Amodiaquine as a prodrug: importance of metabolite(s) in the antimalarial effect of amodiaquine in humans. *Life Sci. 36*, 53.
(7) Bray, M., Davis, K., Geisbert, T., Schmaljohn, C., and Huggins, J. (1999) A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. *J. Infect. Dis. 179* (Suppl. 1), S248.
(8) Bray, M. (2001) The role of the type I interferon response in the resistance of mice to filovirus infection. *J. Gen. Virol. 82*, 1365.
(9) Gowen, B. B., and Holbrook, M. R. (2008) Animal models of highly pathogenic RNA viral infections: hemorrhagic fever viruses. *Antiviral Res. 78*, 79.
(10) Rolain, J. M., Colson, P., and Raoult, D. (2007) Recycling of chloroquine and its hydroxyl analogue to face bacterial, fungal and viral infections in the 21st century. *Int. J. Antimicrob. Agents 30*, 297.
(11) Savarino, A., Di Trani, L., Donatelli, I., Cauda, R., and Cassone, A. (2006) New insights into the antiviral effects of chloroquine. *Lancet Infect. Dis. 6*, 67.
(12) Murray, S. M., Down, C. M., Boulware, D. R., Stauffer, W. M., Cavert, W. P., Schacker, T. W., Brenchley, J. M., and Douek, D. C. (2010) Reduction of immune activation with chloroquine therapy during chronic HIV infection. *J. Virol. 84*, 12082.
(13) Thome, R., Lopes, S. C., Costa, F. T., and Verinaud, L. (2013) Chloroquine: modes of action of an undervalued drug. *Immunol. Lett. 153*, 50.
(14) Krishna, S., and White, N. J. (1996) Pharmacokinetics of quinine, chloroquine and amodiaquine. Clinical implications. *Clin. Pharmacokinet. 30*, 263.
(15) Lim, H.-S., Im, J.-S., Cho, J.-Y., Bae, K.-S., Klein, T. A., Yeom, J.-S., Kim, T.-S., Choi, J.-S., Jang, I.-J., and Park, J.-W. (2009) Pharmacokinetics of hydroxychloroquine and its clinical implications in chemoprophylaxis against malaria caused by *Plasmodium vivax*. *Antimicrob. Agents Chemother. 53*, 1468.
(16) Ramanathan-Girish, S., Catz, P., Creek, M. R., Wu, B., Thomas, D., Krogstad, D. J., De, D., Mirsalis, J. C., and Green, C. E. (2004) Pharmacokinetics of the antimalarial drug, AQ-13, in rats and cynomolgus macaques. *Int. J. Toxicol. 23*, 179.
(17) Cheng, Y. L., Lan, K. H., Lee, W. P., Tseng, S. H., Hung, L. R., Lin, H. C., Lee, F. Y., Lee, S. D., and Lan, K. H. (2013) Amiodarone inhibits the entry and assembly steps of hepatitis C virus life cycle. *Clin. Sci. 125*, 439.
(18) Brunton, L.; Chabner, B.; Knollman, B. *Goodman and Gilman's The Pharmacological Basis of Therapeutics*; McGraw-Hill Professional: Blacklick, OH, USA, 2011.
(19) Khanim, F. L., Merrick, B. A., Giles, H. V., Jankute, M., Jackson, J. B., Giles, L. J., Birtwistle, J., Bunce, C. M., and Drayson, M. T. (2011) Redeployment-based drug screening identifies the anti-helminthic niclosamide as anti-myeloma therapy that also reduces free light chain production. *Blood Cancer J. 1*, e39.
(20) Osada, T., Chen, M., Yang, X. Y., Spasojevic, I., Vandeusen, J. B., Hsu, D., Clary, B. M., Clay, T. M., Chen, W., Morse, M. A., and Lyerly, H. K. (2011) Antihelminth compound niclosamide downregulates Wnt signaling and elicits antitumor responses in tumors with activating APC mutations. *Cancer Res. 71*, 4172.
(21) Chang, Y. W., Yeh, T. K., Lin, K. T., Chen, W. C., Yao, H. T., and Lan, S. J. (2006) Pharmacokinetics of anti-SARS-CoV agent niclosamide and its analogs in rats. *J. Food Drug Anal. 14*, 329.
(22) Mikkelson, T. J., Kroboth, P. D., Cameron, W. J., Dittert, L. W., Chungi, V., and Manberg, P. J. (1986) Single-dose pharmacokinetics of clomiphene citrate in normal volunteers. *Fertil. Steril. 46*, 392.
(23) Szutu, M., Morgan, D. J., McLeish, M., Phillipou, G., Blackman, G. L., Cox, L. W., and Dollman, W. (1989) Pharmacokinetics of intravenous clomiphene isomers. *Br. J. Clin. Pharmacol. 27*, 639.
(24) Panchal, R. G., Kota, K. P., Spurgers, K. B., Ruthel, G., Tran, J. P., Boltz, R. C., and Bavari, S. (2010) Development of high-content imaging assays for lethal viral pathogens. *J. Biomol. Screening 15*, 755.
(25) Saeed, M. F., Kolokoltsov, A. A., and Davey, R. A. (2006) Novel, rapid assay for measuring entry of diverse enveloped viruses, including HIV and rabies. *J. Virol. Methods 135*, 143.

DOI: 10.1021/acsinfecdis.5b00030
ACS Infect. Dis. 2015, 1, 317−326