Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Antiviral Research 160 (2018) 175–182

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Novel amodiaquine derivatives potently inhibit Ebola virus infection

Yasuteru Sakurai[a,b], Norikazu Sakakibara[c,1], Masaaki Toyama[d], Masanori Baba[d], Robert A. Davey[a,e,*]

[a] Department of Virology and Immunology, Texas Biomedical Research Institute, San Antonio, TX, USA
[b] National Research Center for the Control and Prevention of Infectious Diseases, Nagasaki University, Nagasaki, Japan
[c] Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, Sanuki, Japan
[d] Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Kagoshima University, Kagoshima, Japan
[e] National Emerging Infectious Diseases Laboratories, Boston University, Boston, MA, USA

A B S T R A C T

Ebola virus disease is a severe disease caused by highly pathogenic *Ebolaviruses*. Although it shows a high mortality rate in humans, currently there is no licensed therapeutic. During the recent epidemic in West Africa, it was demonstrated that administration of antimalarial medication containing amodiaquine significantly lowered mortality rate of patients infected with the virus. Here, in order to improve its antiviral activity, a series of amodiaquine derivatives were synthesized and tested for Ebola virus infection. We found that multiple compounds were more potent than amodiaquine. The structure-activity relationship analysis revealed that the two independent parts, which are the alkyl chains extending from the aminomethyl group and a halogen bonded to the quinoline ring, were keys for enhancing antiviral potency without increasing toxicity. When these modifications were combined, the antiviral efficacy could be further improved with the selectivity indexes being over 10-times higher than amodiaquine. Mechanistic evaluation demonstrated that the potent derivatives blocked host cell entry of Ebola virus, like the parental amodiaquine. Taken together, our work identified novel potent amodiaquine derivatives, which will aid in further development of effective antiviral therapeutics.

1. Introduction

Ebola virus (EBOV) causes a severe disease with a high mortality rate in humans (Baseler et al., 2017). The disease progresses rapidly and can be easily transmitted through direct contact with patients and contaminated materials. Since being discovered in 1976, EBOV has caused sporadic outbreaks with increasing frequency and affected areas. The 2013–2016 epidemic in West Africa resulted in more than 28,000 infected cases and over 11,000 deaths with multiple cases found outside the endemic region (Lo et al., 2017). This has created a significant public health threat around the world. Although several antiviral candidates have been clinically tested, none have shown clear and significant benefits in patients (van Griensven et al., 2016; Sissoko et al., 2016; Dunning et al., 2016a,b; PREVAIL II Writing Group, 2016), emphasizing a need for further development of effective antiviral therapeutics.

One approach for therapeutic development is to repurpose existing drugs (Mercorelli et al., 2018). Indeed, several studies identified multiple approved drugs showing anti-EBOV activity in both in vitro and in vivo (Madrid et al., 2013; Johansen et al., 2013; Sakurai et al., 2015; Johansen et al., 2015; Madrid et al., 2015). Repurposing takes advantage of known drug kinetics, formulation knowledge and other chemical features while applying to a novel treatment purpose. A functional compound could potentially have a relatively simple track to the clinic (Strittmatter, 2014). Unfortunately, taking only this approach has produced few potent candidates for off-label use in the clinic because their effective antiviral dosages were much higher than those for clinical use (Bixler et al., 2017). This outcome is understandable given that most clinically approved small molecules experienced extensive structure-activity relationship (SAR) analyses, which focused on the specific disease indication. A more developed approach that takes advantage of the established synthesis chemistry, formulation and clinical knowledge is to evaluate the detailed SAR for the new indication using the initial hit as the starting point.

Recent screening efforts using clinically used small molecules identified 4-aminoquinoline antimalarial compounds as potent EBOV inhibitors (Madrid et al., 2013; Madrid et al., 2015; Ekins et al., 2015). One of them was amodiaquine, which has been clinically used as an oral antimalarial medication for more than 60 years. It is on the World Health Organization's List of Essential Medicines and widely available in Africa at a low cost (World Health Organization, 2017). After rapid absorption in humans, amodiaquine undergoes metabolism by

* Corresponding author. Department of Microbiology, Boston University School of Medicine and National Emerging Infectious Diseases Laboratories, Boston University, Boston, MA, USA.
E-mail address: radavey@bu.edu (R.A. Davey).
[1] Deceased.

https://doi.org/10.1016/j.antiviral.2018.10.025
Received 31 August 2018; Received in revised form 26 October 2018; Accepted 27 October 2018
Available online 03 November 2018
0166-3542/

cytochrome p450 2C8 (CYP2C8) enzyme to desethyl-amodiaquine, which has a long half-life of 9–18 days (Backman et al., 2016). Previous reports showed anti-EBOV activity of both amodiaquine and desethyl-amodiaquine in cell culture (Madrid et al., 2013; Zilbermintz et al., 2015), suggesting the potential long-lasting antiviral activity in humans. Although the detailed mechanism of action is not fully understood, virus entry into cells appears to be inhibited. Interestingly, in the 2013–2016 epidemic of EBOV, amodiaquine combined with an artemisinin derivative, was prescribed to some malaria patients in the endemic region. Later analysis revealed that among people infected with Ebola virus, those receiving amodiaquine showed significantly decreased case mortality (50% vs 65%) compared to those receiving only non-aminoquinoline-based antimalarial drugs such as artemisinin derivatives or no treatment (Gignoux et al., 2016). This suggests that amodiaquine may provide clinical benefit for Ebola virus disease patients, but will require a substantial improvement in potency before being useful.

In this study, we synthesized and mechanistically evaluated a series of existent and novel amodiaquine derivatives to define SAR for anti-EBOV activity, aiming to identify compounds with low toxicity and improved potency against EBOV infection.

2. Materials and Methods

2.1. Cells and reagents

Huh7 cells (gift from Dr. Stanley Lemon, University of North Carolina, NC) and Vero-E6 cells (CDC, Atlanta, GA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution. 293FT cells were purchased from Life Technologies and cultured in DMEM with 500 ng/ml of G418. For nuclear staining, Hoechst33342 dye was purchased from Life Technologies.

2.2. Chemical compounds

All the 4-aminoquinolines including amodiaquine were newly synthesized at Tokushima Bunri University. Synthetic methods are described in the patent application PCT/US18/27528. The purity of each compound was analyzed and the identity was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (data not shown). Compounds were dissolved to 20 mM in DMSO and stored at −80 °C until use.

2.3. EBOV-green fluorescent protein (GFP) cultivation and infection

A recombinant EBOV encoding a GFP reporter gene (EBOV-GFP) was provided by Dr. Heinz Feldmann (Rocky Mountain Laboratories, National Institute of Health, Hamilton, MT). The virus was cultivated on Vero-E6 cells by infection at a multiplicity of infection (MOI) of approximately 0.1. Culture supernatants were collected after 5 days, and clarified by centrifugation at 2000 × *g* for 15 min. The virus was concentrated by ultracentrifugation at 82,700 × *g* for 2 h pelleting through a 20% sucrose cushion and re-suspended in phosphate buffered saline (PBS). Virus titer was determined by serial dilution on Vero-E6 cells. After 24 h-incubation, the cells were fixed in 10% formalin for > 24 h, washed with PBS, stained with Hoechst 33342 dye and imaged by a Nikon Ti Eclipse inverted microscope with a 4 × lens. Counting of the cell nuclei and GFP-positive foci was performed using Cell Profiler image analysis software (Broad Institute, MIT, Boston, MA) and customized analysis pipeline (available on request). For infection assays, Huh7 cells were seeded in 384 well plates and incubated overnight at 37 °C. Cells were pretreated for at least 1 h with each compound diluted across a 2-fold serial dilution series. They were then challenged with EBOV-GFP at an MOI of 0.05 for 24 h, the time corresponding to approximately one round of replication and accumulation of sufficient GFP signal to be detected in infected cells. All cells were fixed and analyzed as described above. All experiments with replication competent Ebola virus were performed in a biosafety level 4 (BSL4) laboratory at Texas Biomedical Research Institute.

2.4. Cytotoxicity measurement

Drug cytotoxicity was measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) following the manufacturer's protocol. Huh7 cells were seeded in white walled 96 well plates (Corning, Lowell, MA) and incubated with each compound at 37 °C. After 24 h, the assay buffer was added to the culture plates and incubated for additional 10 min at room temperature. Luminescence was measured using a 96-well plate luminometer (Promega, Madison, WI).

2.5. Generation of recombinant VSV pseudotyped with virus glycoproteins

To evaluate compound inhibition of glycoprotein (GP) function in controlling virus entry into cells, GP-pseudotyped recombinant vesicular stomatitis viruses (VSV) were made with the G gene replaced by a luciferase reporter gene and the GP supplied exogenously (VSVΔG-VSV-G). VSVΔG pseudotyped with EBOV or Marburg virus GP (VSVΔG-EBOV-GP or VSVΔG-MARV-GP, respectively) were generated starting with VSVΔG-VSV-G. For VSVΔG-MARV-GP, the MARV GP was supplied by transfecting 293FT cells with 5 μg of plasmids pMARV Musoke GP and 10 μg of pβ-gal (a non-specific carrier plasmid) using the calcium phosphate method in 10 cm dishes. Twenty four hours after transfection, the cells were challenged with VSVΔG-VSV-G and left overnight. The supernatant was harvested 48 h after challenge and stored at −80 °C until use. For production of VSVΔG-EBOV-GP, cells were transfected with 1 μg of pEBOV Mayinga GP and 14 μg of pβ-gal plasmid as a carrier to 293FT cells. Twenty four hours after transfection, the cells were challenged with VSVΔG-MARV-GP overnight and washed twice with PBS. The latter virus was used instead of VSVΔG-VSV-G as the MARV GP is more labile than VSV-G and does not as readily carry over into the lower titer EBOV-GP bearing pseudotype. The supernatant was harvested 48 h after infection and stored at −80 °C until use. As a control for carry-over of the inoculating parent virus, cells were transfected with the plasmid expressing β-galactosidase alone and then challenged with the parent virus stock. The culture supernatant was collected and tested for the activity. Virus titers were determined by serial dilution on Huh7 cells with luciferase activity measured 16 h post-infection.

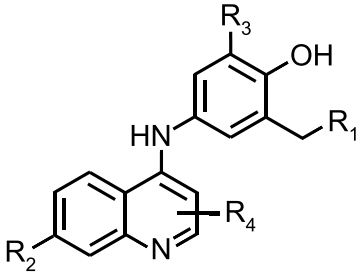
2.6. Infection assay with recombinant pseudotyped VSV

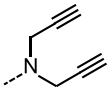
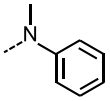
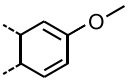
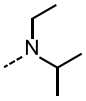
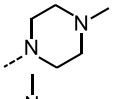
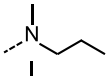
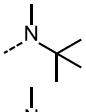
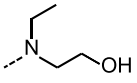
Huh7 cells were plated in white walled 96-well plates (Corning, Lowell, MA) and incubated with each compound in 2-fold serial dilutions. After 1 h, VSVΔG-EBOV-GP or VSVΔG-VSV-G was added. The cells were challenged with virus in the presence of compounds at 37 °C for 16 h, and the medium was replaced with luciferase assay buffer (20 mM Tricine-HCl, pH 7.5, 8 mM $MgSO_4$, 0.13 mM ethylenediaminetetraacetic acid (EDTA), 0.53 mM ATP, 33 mM dithiothreitol (DTT) 0.47 mM luciferin) containing 0.2% of Triton X-100 detergent. After the cells were incubated with the buffer for 10 min at room temperature, the luciferase activity was measured using a 96-well plate luminometer (Promega).

2.7. Minigenome assay

To evaluate the impact of small molecule treatment on EBOV genome replication/transcription steps, a plasmid-based minigenome assay was performed. p3E5E-Luc plasmid, encoding the EBOV minigenome containing a firefly luciferase reporter gene, was provided by Dr. Elke Muhlberger (Boston University, MA) and described previously (Muhlberger et al., 1999). Plasmids encoding the virus

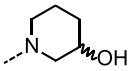
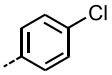
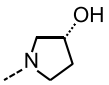
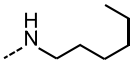
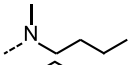
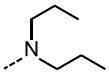
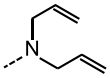
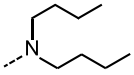
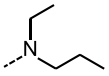
Table 1
The chemical structures of amodiaquine derivatives and antiviral activities against EBOV-GFP.



Compound #	R1	R2	R3	R4	IC_{50} (μM) for EBOV-GFP
Amodiaquine		Cl	H	H	2.13 ± 0.32
1		Cl	H	H	5.78 ± 1.49
2		CF3	H	H	5.87 ± 1.46
3		Cl	H	H	6.39 ± 0.93
4		Br	H	H	1.55 ± 0.14
5		F	H	H	2.78 ± 0.28
6		H	H		1.64 ± 0.22
7		Cl	H		0.73 ± 0.07
8		Cl	H	H	1.46 ± 0.14
9		Cl	H	H	1.21 ± 0.09
10		Cl	H	H	2.14 ± 0.21
11		Cl	H	H	1.46 ± 0.15
12		H	H	H	2.14 ± 0.18
13		Cl	H	H	1.68 ± 0.19
14		Cl	H	H	1.22 ± 0.11
15		Cl	H	H	1.28 ± 0.07
16		Cl	H	H	2.08 ± 0.22
17		Cl	H	H	1.77 ± 0.18

(*continued on next page*)

Table 1 (*continued*)

Compound #	R1	R2	R3	R4	IC_{50} (μM) for EBOV-GFP
18		I	H	H	0.64 ± 0.05
19		Cl	H	H	1.80 ± 0.28
20		Cl		H	1.31 ± 0.10
21		Cl	H	H	1.09 ± n.d.
22		Cl	H	H	1.73 ± 0.09
23		Cl	H	H	0.29 ± 0.03
24		Cl	H	H	6.03 ± 0.41
25		Cl	H	H	0.86 ± 0.08
26		Cl	H	H	0.94 ± 0.08
27		Cl	H	H	2.32 ± 0.32
28		Cl	H	H	0.72 ± 0.08
29		Cl	H	H	1.39 ± 0.13

ribonucleoprotein replication complex (Shtanko et al., 2018) were introduced into Huh7 cells plated in 24 well plates using TransIT LT1 transfection reagent and incubated overnight at 37 °C. Cells were transfected with 100 ng of p3E5E, 50 ng of pC-NP, 30 ng of pC-VP30, 50 ng of pC-VP35, 300 ng of pC-L, 100 ng of a plasmid encoding T7 polymerase and 100 ng of a plasmid encoding a renilla luciferase (to measure transfection efficiency). Twenty-four hours after transfection, cell culture medium was changed to fresh medium containing each compound and the cells were incubated at 37 °C for additional 24 h. Both firefly and renilla luciferase activities were measured using Dual-Luciferase® Reporter Assay System (Promega) according to the manufacture's instruction.

3. Results

3.1. Initial screening identifies 4-aminoquinolines with higher potency than amodiaquine

A series of 69 4-aminoquinolines structurally related to amodiaquine were synthesized (Table 1 and Table S1). At first, structural features were randomly modified and evaluated to identify those responsible for improved potency without increased toxicity. Each compound was tested for antiviral activity using replication competent EBOV encoding GFP as the infection reporter. Huh7 cells were chosen as target cells since they are derived from human liver cells, which is one major target of EBOV infection and pathology in vivo (Martines et al., 2015). Consistent with previous reports using other cell types, the parent compound, amodiaquine, inhibited EBOV infection, with a 50% inhibitory concentration (IC_{50}) in the micromolar range (2.13 μM, Fig. 1A). For the current work, the cut off for significant improvement in potency was set at an IC_{50} of 1.5 μM, which corresponded to amodiaquine's IC_{50} minus 2 × standard deviation (SD) of measurements. Fourteen among 69 tested compounds showed improved potency (Table 1, Fig. 1A). Those with the highest potency were compounds 7, 18, 23 and 28 with IC_{50}s of 0.73, 0.64, 0.29 and 0.72 μM, respectively.

Cytotoxicity was evaluated for the 14 potent compounds by measuring cell viability after 1-day incubation, as done for the infection assays. According to their 50% cytotoxic concentrations (CC_{50}s), the concentration corresponding to 50% cell cytotoxicity, a selectivity index (SI) for each compound was calculated by dividing the CC_{50} by the IC_{50}. Compared to amodiaquine with an SI of 37, compounds 18 and 28 yielded SIs of > 130 due to lowered cytotoxicity (Table 2). Compounds 11, 14, 25, 26 and 29 also gave SIs higher than amodiaquine. The derivatives with improved potency and low toxicity were chosen as lead compounds for further derivatization.

3.2. SARs of the initial derivative series

Our initial derivative screening revealed that improved anti-EBOV potency, in general, corresponded to modification of the alkyl chain extending from the aminomethyl group (R_1, Table 1), as seen in compounds 8, 9, 11, 14, 15, 21, 23, 25, 26, 28 and 29. Extension of this alkyl chain appeared to increase potency. However, the presence of triple covalent bonds (compound 1) or a benzene ring (compound 3) reduced antiviral activity. Amino groups as for compound 13 or hydroxyl groups such as compounds 17, 19 and 22 had no impact on potency.

The electronegativity of halogens bonded to position 7 of the quinoline ring (R_2) appeared to inversely correspond to potency. Fluorine (compound 5), with the highest electronegativity, produced a compound with lower potency than amodiaquine, which has a chlorine at

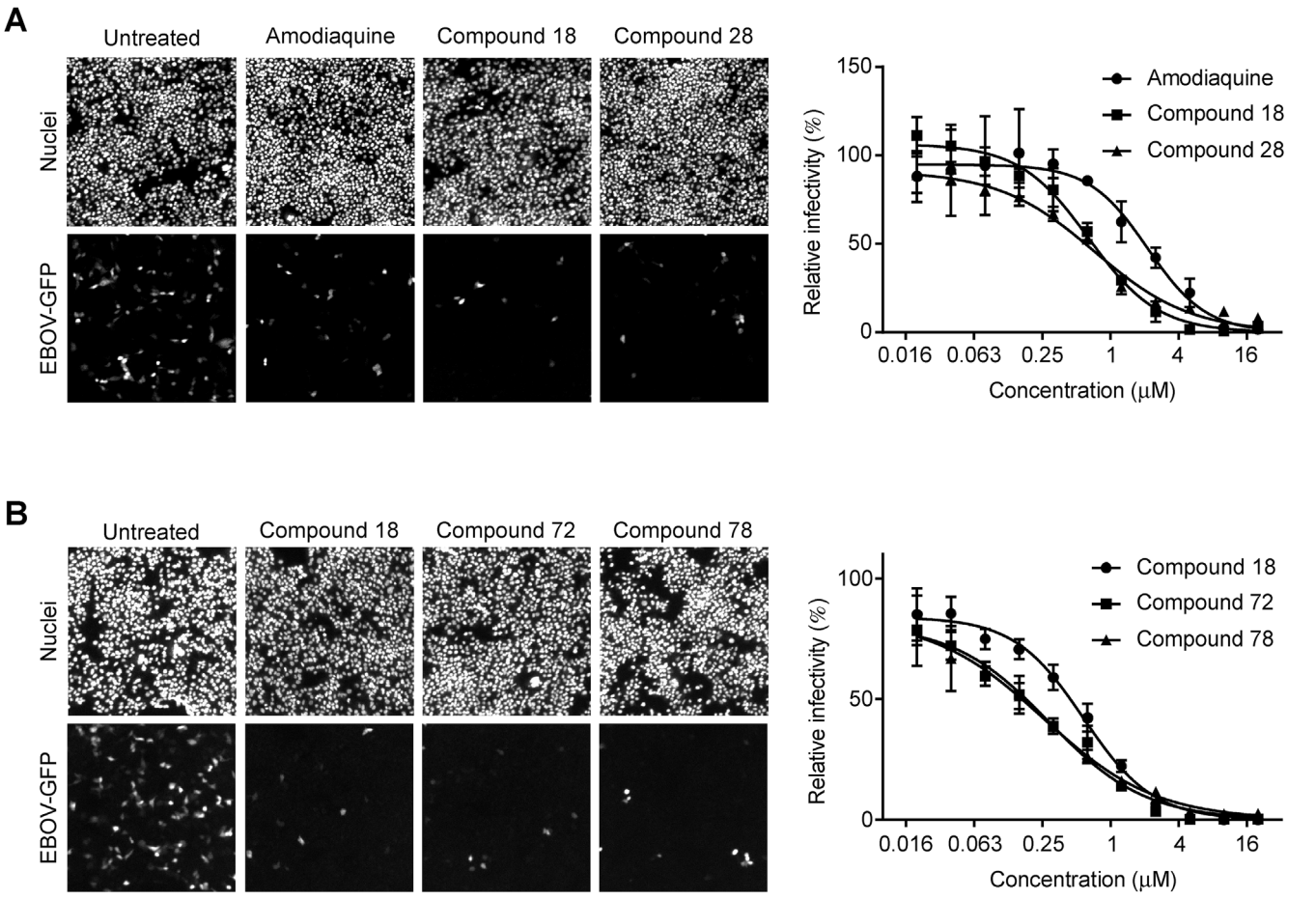


Fig. 1. Amodiaquine and its derivatives inhibit EBOV infection. Huh7 cells were challenged with recombinant EBOV encoding GFP (EBOV-GFP) in the presence of the indicated concentrations of amodiaquine, compound 18 or compound 28 (A) or compounds 18, compound 72 or compound 78 (B). After 24 h, cells were fixed, the nuclei were stained with Hoescht 33342 and images were captured by microscopy. The images of cells treated with each compound at 2.5 μM and untreated cells are shown (left panels). Infected cells expressing GFP and total cell numbers were counted to calculate the infectivity, which was normalized to those of untreated controls to obtain relative infectivity. They were plotted as a function of compound concentration to draw dose-response curves (right panels). All measurements were performed in at least triplicate and shown as mean ± SD. Similar results were obtained in replicate experiments.

Table 2
Selectivity indexes of potent amodiaquine derivatives.

Compound	IC_{50} (μM) for EBOV-GFP	CC_{50} (μM)	Selectivity index
Amodiaquine	2.13	78.95	37
7	0.73	14.75	20
8	1.46	39.18	27
9	1.21	26.09	22
11	1.46	60.5	41
14	1.22	> 100	> 82
15	1.28	41.77	33
18	0.64	> 100	> 156
20	1.31	19.48	15
21	1.09	33.16	30
23	0.29	5.18	18
25	0.86	65.91	77
26	0.94	> 100	> 106
28	0.72	> 100	> 139
29	1.39	> 100	> 72

this position. In contrast, bromine (compound 4) and iodine (compound 18) which have lowered electronegativity, improved potency by 1.4 and 3.3-fold, respectively (Table 1).

Phenol ring adducts (R_3), such as chlorobenzene, had a positive effect on antiviral activity. This was highlighted by 1.6-fold higher antiviral activity of compound 20 over amodiaquine (Table 1).

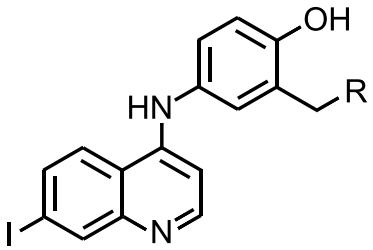
Compounds with a phenyl ring or benzene ring attached to the quinoline ring through position 3 (R_4), such as compound 7, which has a methoxy group, also showed higher potency (2.9-fold) against EBOV infection (Table 1). The results from these derivatives are consistent to that seen in quinacrine, which also has a phenyl group and is an anti-malarial agent that shows a significant anti- EBOV activity (Ekins et al., 2015).

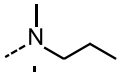
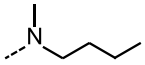
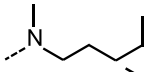
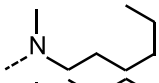
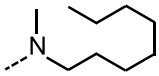
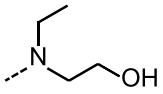
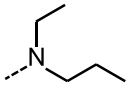
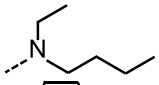
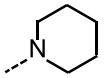
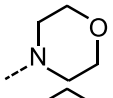
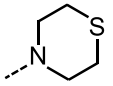
In contrast, compounds lacking the structure, in which an amino-quinoline and a phenyl ring or a benzene ring are bridged by an amino group, showed much lower potency than amodiaquine, as seen in compounds 34, 35 and 40 (Table S1), suggesting that the structural core is critical for the anti-EBOV activity. However, the amino group can be substituted from the original secondary amine to a tertiary amine because compound 43 showed similar potency to the parental amodiaquine (Table S1).

3.3. Combination of identified features improves potency against EBOV infection

SARs of the initial derivative set demonstrated that the alkyl chain moiety extending from the aminomethyl group and the halogen bonded to the quinoline ring were important features for improved potency against EBOV without increasing cytotoxicity. Importantly, each feature can be independently modified. Consequently, a second set of derivatives was synthesized with an iodine substitution at position 7 of the quinoline ring combined with a variety of alkyl chain lengths. All of

Table 3
The chemical structures of the 2nd series of amodiaquine derivatives and antiviral activities against EBOV-GFP.



Compound #	R	IC_{50} (μM) for EBOV-GFP	CC_{50} (μM)	Selectivity index
18		0.58 ± 0.05	> 100	> 172
70		0.69 ± 0.08	> 100	> 145
71		0.62 ± 0.06	> 100	> 161
72		0.29 ± 0.04	38.35	132
73		0.30 ± 0.04	32.34	108
74		0.43 ± 0.06	> 100	> 233
75		0.44 ± 0.04	66.13	150
76		0.37 ± 0.04	> 100	> 270
77		0.39 ± 0.02	> 100	> 256
78		0.26 ± 0.06	> 100	> 385
79		0.41 ± 0.05	> 100	> 244
80		0.36 ± 0.04	> 100	> 278
81		0.41 ± 0.04	60.36	147
82		0.66 ± 0.05	35.34	54
83		0.37 ± 0.07	> 100	> 270
84		1.59 ± 0.12	> 100	> 63
85		1.95 ± 0.22	> 100	> 51

the derivatives efficiently blocked EBOV infection with several of them showing more than 2-fold higher potency than compound 18, which has the same alkyl chain as amodiaquine (Fig. 1B). Consistent with the initial screening results, extension of the alkyl chain had positive effects on the antiviral activity with the most potent being compound 78, which had the longest chain length (Table 3). Cytotoxicity tests revealed that most of the compounds showed low toxicity with 74, 76, 77, 78, 79, 80 and 83 yielding SIs > 200, which are higher than those for the initial derivative sets. Therefore, they appeared to have higher potential as treatments for Ebola virus disease.

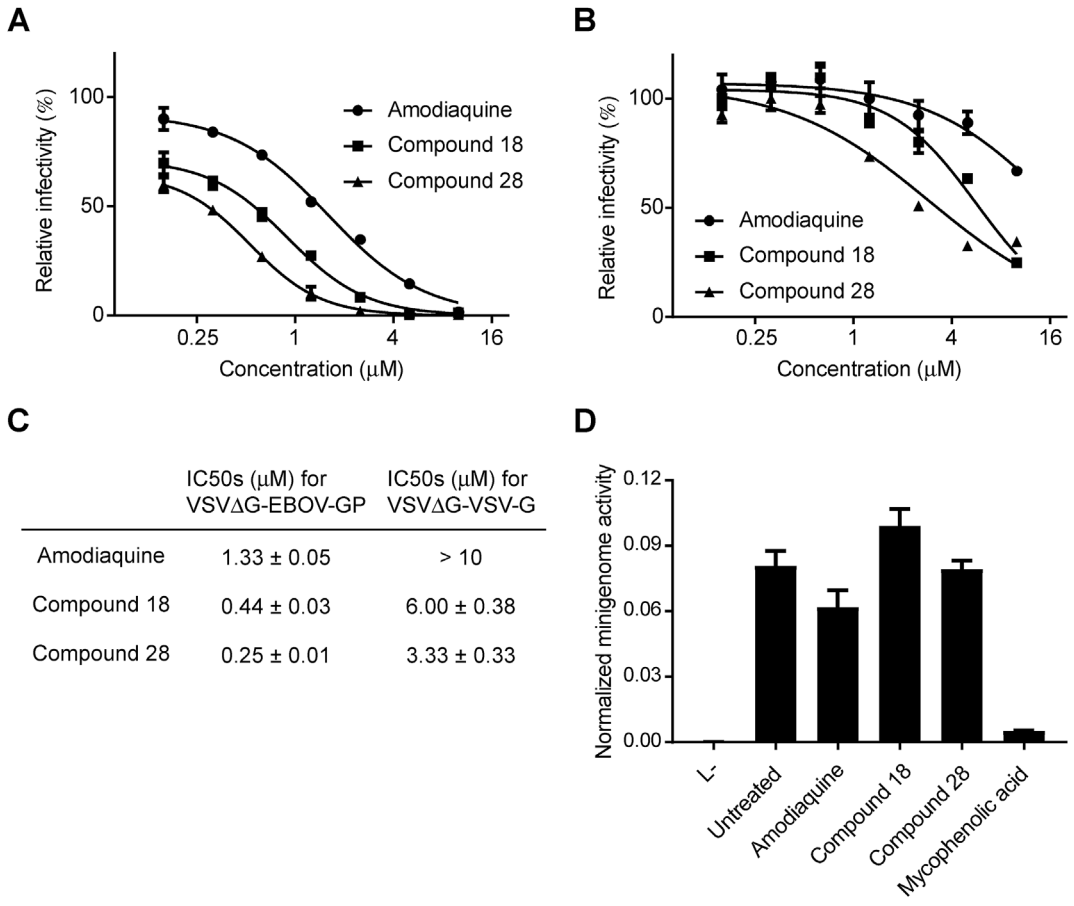
3.4. Potent amodiaquine derivatives block host cell entry of EBOV

In order to investigate how the potent compounds affected the EBOV replication cycle, each was tested for host cell entry and virus genome replication using pseudotyped virus and minigenome assays, respectively. As shown in Fig. 2A–C, infection by vesicular stomatitis virus (VSV) bearing EBOV glycoproteins (VSVΔG-EBOV-GP) was inhibited by the compounds over 10 times more potently than VSV bearing the native VSV glycoproteins (VSVΔG-VSV-G). The potency of the compounds against VSVΔG-EBOV-GP was similar (within 3-fold) to those against EBOV-GFP (Table 1, Fig. 2C). In contrast, the activity of EBOV minigenome was not significantly affected by the compounds, whereas mycophenolic acid, which is a known EBOV genome replication inhibitor, effectively blocked the signal from the minigenome (Fig. 2D) (Edwards et al., 2015). These results indicated that the derivatives specifically blocked host cell entry of EBOV. Moreover, both compounds 18 and 28 inhibited entry more efficiently than amodiaquine, which was consistent with the outcomes of EBOV-GFP infection (Fig. 1B). Compounds 18 and 28 are representative derivatives with one of the important structural features revealed in the initial screening. Therefore, these modifications improved antiviral potency of the compounds by specifically targeting host cell entry without gaining anti-replication effects.

3.5. Discussion

This work confirms that amodiaquine, a well-tolerated drug with a long history of use for treatment of malaria, also has anti-EBOV activity. Importantly, it was possible to modify this compound to improve its potency for an alternative use. A clear SAR was found for the anti-EBOV activity. Some of the modifications, which enhanced the antiviral effects, appeared to act independently of each other. The most important features were the length of the alkyl chain extending from the aminomethyl group and the electronegativity of the halogen bound to the quinoline ring. When each of these features were combined, further improvement in potency, toward the submicromolar range, was achieved. In addition to improving potency, many of these compounds were better tolerated in cultured cells than amodiaquine. Consequently, we identified multiple compounds showing much higher selectivity indexes than amodiaquine. These amodiaquine derivatives are now lead compounds for further medicinal chemistry development for use in the clinic.

Derivatization is one of the traditional and effective techniques for drug development. By modifying specific structural components, this approach can enhance the specific effects, reduce toxicity or improve pharmacokinetics of the parental drugs without diminishing their desirable features. Derivatization of artemisinin, an antimalarial drug, generated more effective drugs such as artesunate by improving the bioavailability (Balint, 2001). Brincidofovir, a promising experimental drug against some DNA viruses, is a derivative of an anti-herpes virus cidofovir with much higher activity against poxviruses and potential anti-EBOV activity as well as lower toxicity (Parker et al., 2008; Olson et al., 2014; McMullan et al., 2016). Interestingly, brincidofovir has a lengthened alkyl chain, which is the same structural feature as that of our potent derivatives. Similar to the parental drugs in these cases,



	IC50s (μM) for VSVΔG-EBOV-GP	IC50s (μM) for VSVΔG-VSV-G
Amodiaquine	1.33 ± 0.05	> 10
Compound 18	0.44 ± 0.03	6.00 ± 0.38
Compound 28	0.25 ± 0.01	3.33 ± 0.33

Fig. 2. Amodiaquine and its derivatives inhibit glycoprotein-dependent entry of EBOV. To address the effects of compounds on virus entry into the cell, pseudotyped viruses bearing the glycoprotein of (A) EBOV or (B) VSV and encoding a firefly luciferase reporter were used. Huh7 cells were treated with the indicated doses of each compound and challenged with either pseudotyped virus. Luciferase activities were measured and normalized to those of untreated controls (mean ± SD, n = 3). Each data set is representative of two independent experiments. (C) Comparison of activity of amodiaquine, compounds 18 or compound 28 for inhibition of pseudotyped virus infection. Calculated IC_{50} values are shown as mean ± SD from 2 independent experiments performed using triplicate samples. (D) To assess EBOV transcription and replication, Huh7 cells were transfected with a plasmid expressing EBOV minigenome RNA encoding a firefly luciferase reporter, plasmids expressing each component of the EBOV polymerase complex and a plasmid expressing a renilla luciferase reporter. Twenty-four hours later, cells were treated with DMSO (untreated), amodiaquine (10 μM), compound 18 (10 μM), compound 28 (10 μM) or mycophenolic acid (10 μM) as a positive control. Luciferase activities were measured after additional 24 h. Firefly luciferase activities normalized to renilla luciferase activities are shown (mean ± SD, n = 3). Each data set is representative of two independent experiments.

amodiaquine has a history as a medication in clinic and also showed a small but significant clinical benefit to patients infected with EBOV (Gignoux et al., 2016). Therefore, derivatization of amodiaquine appeared to be a reasonable process in order to discover promising therapeutics against Ebola virus disease. Our results demonstrated that this approach has a potential to work for this disease indication.

Our SAR analyses demonstrated that the alkyl chain extending from the aminomethyl group was one of the key modification target for increasing the anti-EBOV activity. Such modifications were reported to assists in drug accumulation in vacuolar compartments (Parhizgar and Tahghighi, 2017), suggesting that efficient and appropriate localization of the amodiaquine derivatives in infected cells is an important factor for its anti-EBOV activity. Indeed, amodiaquine and chloroquine in mammalian cells accumulate much less than in malaria parasites (Hawley et al., 1996), a desirable property as an anti-malarial but not useful for an antiviral. Consequently, the antiviral potency of these 4-aminoquinolines is much less than its reported anti-malarial activity, with an IC_{50} in the ten nanomolar range (O'Neill et al., 2003; Hocart et al., 2011). However, our study showed that extension of the alkyl chains of amodiaquine could improve the anti-EBOV potency by 7-fold, suggesting that the modification appeared to improve the efficacy of compound accumulation in host mammalian cells. In addition, the alkyl chain of amodiaquine is important for in vivo drug kinetics because it is a target of metabolism by CYP2C8, a member of the cytochrome P450 superfamily, and potentially affects the circulation half-lives of the compounds and their kinetics inside the cells (Backman et al., 2016). Although this metabolism may not be critical for amodiaquine because the major metabolite, desethyl-amodiaquine, still has a similar anti-EBOV activity, metabolic forms of our derivatives and their antiviral potency are unknown and will need to be investigated. Moreover, as amodiaquine induces rare but serious liver injury, which was suggested to be caused by P450-mediated drug metabolism, these modifications may also affect toxicity of the derivatives in vivo and will need verification (Shimizu et al., 2009).

Our derivative screening also demonstrated that substitution of a halogen bonded to the quinoline ring could enhance the antiviral potency. Halogen substitution or insertion has been a commonly used technique for drug development (Hernandes et al., 2010). Such modification generally affects the interaction between the compound and the target by mechanisms such as steric effects and the formation of halogen bonds, which strength is proportional to electronegativity of the halogen. Interestingly, our SAR analysis revealed that the electronegativity of the halogens inversely corresponded to anti-EBOV potency, suggesting that the effect of the halogen substitutions seemed to depend on steric effects, in which larger halogens could efficiently occupy the space in the target. Although the inhibitory mechanism of

amodiaquine is unclear, our data suggest that the anti-EBOV effect of amodiaquine appears to have a molecular target, which is important for EBOV infection, or accumulates in a cell compartment required for EBOV to access. Moreover, our mechanistic assay indicated that the molecular target or the compartment, if any, is specific to EBOV entry step as VSV entry was comparatively resistant to the compound treatment. The halogen substitution must affect the physical interaction between an amodiaquine-derivative and such a target.

Amodiaquine was originally developed and has been widely used in the clinic officially since the 1970s for the treatment and prophylaxis of malaria. However, later studies revealed that it was active against a wide range of human pathogens, including multiple viruses such as flaviviruses, coronaviruses, alphaviruses, bunyaviruses and filoviruses (Boonyasuppayakorn et al., 2014; Baba et al., 2017; Han et al., 2018). Moreover, it inhibited the delivery of anthrax toxin and diphtheria toxins into cytoplasm as well as growth of the fungus, *Penicillium marneffei*, inside macrophages (Zilbermintz et al., 2015; Taramelli et al., 2001). Although using amodiaquine itself against these pathogens in the clinic is not practical due to the weak potency, it can be a lead candidate to create more potent compounds as exemplified by our study for Ebola virus. Moreover, multiple amodiaquine derivatives were synthesized previously and shown to be active against other pathogens (Boonyasuppayakorn et al., 2014; Baba et al., 2017; De et al., 1998). Besides the original medical purpose as an antimalarial, amodiaquine may now have another potential as a lead compound to develop therapeutic agents against a wide range of human pathogens.

Acknowledgements

N.S. has passed away during manuscript preparation and this paper is written in his memory and tribute to his devoted work to finding cures for human diseases. Since N.S. conducted the synthesis of all compounds, please contact the corresponding author (R.A.D.) or M.B. regarding the details of synthesis. The authors are grateful to the members of Davey laboratory (Texas Biomedical Research Institute) and Dr. Jiro Yasuda (Nagasaki University) for helpful discussions. The authors also thank to all those cited in Materials and Methods. Y.S., N.S., M.B. and R.A.D. are inventors on a patent entitled “NOVEL AMODIAQUINE ANALOGS AND METHODS OF USES THEREOF” (PCT/US18/27528). This work was supported by an R21 awarded to R.A.D (5R21AI115082-02).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.10.025.

References

Baba, M., et al., 2017. Establishment of an antiviral assay system and identification of severe fever with thrombocytopenia syndrome virus inhibitors. Antivir. Chem. Chemother. 25, 83–89.
Backman, J.T., Filppula, A.M., Niemi, M., Neuvonen, P.J., 2016. Role of cytochrome P450 2C8 in drug metabolism and interactions. Pharmacol. Rev. 68, 168–241.
Balint, G.A., 2001. Artemisinin and its derivatives: an important new class of antimalarial agents. Pharmacol. Ther. 90, 261–265.
Baseler, L., Chertow, D.S., Johnson, K.M., Feldmann, H., Morens, D.M., 2017. The pathogenesis of Ebola virus disease. Annu. Rev. Pathol. 12, 387–418.
Bixler, S.L., Duplantier, A.J., Bavari, S., 2017. Discovering drugs for the treatment of Ebola virus. Curr. Treat. Options Infect. Dis. 9, 299–317.
Boonyasuppayakorn, S., Reichert, E.D., Manzano, M., Nagarajan, K., Padmanabhan, R., 2014. Amodiaquine, an antimalarial drug, inhibits dengue virus type 2 replication and infectivity. Antivir. Res. 106, 125–134.
De, D., Krogstad, F.M., Byers, L.D., Krogstad, D.J., 1998. Structure-activity relationships for antiplasmodial activity among 7-substituted 4-aminoquinolines. J. Med. Chem. 41, 4918–4926.
Dunning, J., et al., 2016a. Experimental treatment of Ebola virus disease with TKM-130803: a single-arm phase 2 clinical trial. PLoS Med. 13, e1001997.
Dunning, J., et al., 2016b. Experimental treatment of Ebola virus disease with brincidofovir. PloS One 11, e0162199.
Edwards, M.R., et al., 2015. High-throughput minigenome system for identifying small-molecule inhibitors of Ebola virus replication. ACS Infect. Dis. 1, 380–387.
Ekins, S., et al., 2015. Machine learning models identify molecules active against the Ebola virus in vitro. F1000Research 4, 1091.
Gignoux, E., et al., 2016. Effect of artesunate-amodiaquine on mortality related to Ebola virus disease. N. Engl. J. Med. 374, 23–32.
Han, Y., Mesplede, T., Xu, H., Quan, Y., Wainberg, M.A., 2018. The antimalarial drug amodiaquine possesses anti-ZIKA virus activities. J. Med. Virol. 90, 796–802.
Hawley, S.R., Bray, P.G., Park, B.K., Ward, S.A., 1996. Amodiaquine accumulation in Plasmodium falciparum as a possible explanation for its superior antimalarial activity over chloroquine. Mol. Biochem. Parasitol. 80, 15–25.
Hernandes, M.Z., Cavalcanti, S.M., Moreira, D.R., de Azevedo Junior, W.F., Leite, A.C., 2010. Halogen atoms in the modern medicinal chemistry: hints for the drug design. Curr. Drug Targets 11, 303–314.
Hocart, S.J., et al., 2011. 4-aminoquinolines active against chloroquine-resistant Plasmodium falciparum: basis of antiparasite activity and quantitative structure-activity relationship analyses. Antimicrob. Agents Chemother. 55, 2233–2244.
Johansen, L.M., et al., 2013. FDA-approved selective estrogen receptor modulators inhibit Ebola virus infection. Sci. Transl. Med. 5 190ra179.
Johansen, L.M., et al., 2015. A screen of approved drugs and molecular probes identifies therapeutics with anti-Ebola virus activity. Sci. Transl. Med. 7 290ra289.
Lo, T.Q., Marston, B.J., Dahl, B.A., De Cock, K.M., 2017. Ebola: anatomy of an epidemic. Annu. Rev. Med. 68, 359–370.
Madrid, P.B., et al., 2013. A systematic screen of FDA-approved drugs for inhibitors of biological threat agents. PloS One 8, e60579.
Madrid, P.B., et al., 2015. Evaluation of Ebola virus inhibitors for drug repurposing. ACS Infect. Dis. 1, 317–326.
Martines, R.B., Ng, D.L., Greer, P.W., Rollin, P.E., Zaki, S.R., 2015. Tissue and cellular tropism, pathology and pathogenesis of Ebola and Marburg viruses. J. Pathol. 235, 153–174.
McMullan, L.K., et al., 2016. The lipid moiety of brincidofovir is required for in vitro antiviral activity against Ebola virus. Antivir. Res. 125, 71–78.
Mercorelli, B., Palu, G., Loregian, A., 2018. Drug repurposing for viral infectious diseases: how far are we? Trends Microbiol. 26, 865–876.
Muhlberger, E., Weik, M., Volchkov, V.E., Klenk, H.D., Becker, S., 1999. Comparison of the transcription and replication strategies of marburg virus and Ebola virus by using artificial replication systems. J. Virol. 73, 2333–2342.
O'Neill, P.M., et al., 2003. Isoquine and related amodiaquine analogues: a new generation of improved 4-aminoquinoline antimalarials. J. Med. Chem. 46, 4933–4945.
Olson, V.A., et al., 2014. In vitro efficacy of brincidofovir against variola virus. Antimicrob. Agents Chemother. 58, 5570–5571.
Parhizgar, A.R., Tahghighi, A., 2017. Introducing new antimalarial analogues of chloroquine and amodiaquine: a narrative review. Iran. J. Med. Sci. 42, 115–128.
Parker, S., et al., 2008. Efficacy of therapeutic intervention with an oral ether-lipid analogue of cidofovir (CMX001) in a lethal mousepox model. Antivir. Res. 77, 39–49.
PREVAIL II Writing Group, Multi-National PREVAIL II Study Team, Davey Jr., RT, Dodd, L, Proschan, MA, Neaton, J, Neuhaus Nordwall, J, Koopmeiners, JS, Beigel, J, Tierney, J, Lane, HC, Fauci, AS, Massaquoi, MBF, Shar, F, Malvy, D, 2016. A randomized, controlled trial of ZMapp for Ebola virus infection. N. Engl. J. Med. 375, 1448–1456.
Sakurai, Y., et al., 2015. Ebola virus. Two-pore channels control Ebola virus host cell entry and are drug targets for disease treatment. Science 347, 995–998.
Shimizu, S., et al., 2009. Metabolism-dependent hepatotoxicity of amodiaquine in glutathione-depleted mice. Arch. Toxicol. 83, 701–707.
Shtanko, O., et al., 2018. Retro-2 and its dihydroquinazolinone derivatives inhibit filovirus infection. Antivir. Res. 149, 154–163.
Sissoko, D., et al., 2016. Experimental treatment with favipiravir for Ebola virus disease (the JIKI trial): a historically controlled, single-arm proof-of-concept trial in guinea. PLoS Med. 13, e1001967.
Strittmatter, S.M., 2014. Overcoming drug development bottlenecks with repurposing: old drugs learn new tricks. Nat. Med. 20, 590–591.
Taramelli, D., et al., 2001. Inhibition of intramacrophage growth of Penicillium marneffei by 4-aminoquinolines. Antimicrob. Agents Chemother. 45, 1450–1455.
van Griensven, J., et al., 2016. Evaluation of convalescent plasma for Ebola virus disease in Guinea. N. Engl. J. Med. 374, 33–42.
World Health Organization, 2017. WHO model list of essential Medicines – 20th list. http://www.who.int/medicines/publications/essentialmedicines/en/.
Zilbermintz, L., et al., 2015. Identification of agents effective against multiple toxins and viruses by host-oriented cell targeting. Sci. Rep. 5, 13476.