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The Synthetic Antiviral Drug Arbidol Inhibits Globally Prevalent Pathogenic Viruses

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ABSTRACT

Arbidol (ARB) is a synthetic antiviral originally developed to combat influenza viruses. ARB is currently used clinically in several countries but not in North America. We have previously shown that ARB inhibits *in vitro* hepatitis C virus (HCV) by blocking HCV entry and replication. In this report, we expand the list of viruses that are inhibited by ARB and demonstrate that ARB suppresses *in vitro* infection of mammalian cells with Ebola virus (EBOV), Tacaribe arenavirus, and human herpesvirus 8 (HHV-8). We also confirm suppression of hepatitis B virus and poliovirus by ARB. ARB inhibited EBOV Zaire Kikwit infection when added before or at the same time as virus infection and was less effective when added 24 h after EBOV infection. Experiments with recombinant vesicular stomatitis virus (VSV) expressing the EBOV Zaire glycoprotein showed that infection was inhibited by ARB at early stages, most likely at the level of viral entry into host cells. ARB inhibited HHV-8 replication to a similar degree as cidofovir. Our data broaden the spectrum of antiviral efficacy of ARB to include globally prevalent viruses that cause significant morbidity and mortality.

IMPORTANCE

There are many globally prevalent viruses for which there are no licensed vaccines or antiviral medicines. Some of these viruses, such as Ebola virus or members of the arenavirus family, rapidly cause severe hemorrhagic diseases that can be fatal. Other viruses, such as hepatitis B virus or human herpesvirus 8 (HHV-8), establish persistent infections that cause chronic illnesses, including cancer. Thus, finding an affordable, effective, and safe drug that blocks many viruses remains an unmet medical need. The antiviral drug arbidol (ARB), already in clinical use in several countries as an anti-influenza treatment, has been previously shown to suppress the growth of many viruses. In this report, we expand the list of viruses that are blocked by ARB in a laboratory setting to include Ebola virus, Tacaribe arenavirus, and HHV-8, and we propose ARB as a broad-spectrum antiviral drug that may be useful against hemorrhagic viruses.

The past several decades have witnessed significant advances in the control of globally prevalent viral infections, with hepatitis C virus (HCV) as the most recent example (1). Nonetheless, even with successful vaccines and therapies for some of these pathogens, viral mutation, drug resistance, and viral reemergence pose problems for global control and eradication efforts. Even more distressing is the recent outbreak of the filovirus Ebola virus (EBOV), affecting multiple countries in West Africa (2) and including two imported into cases the United States (with one death) and two locally acquired infections in U.S. health care workers. Other than supportive care and hydration therapy, there currently exist no treatments, licensed vaccines, or antiviral drugs for acute EBOV infection. Moreover, there are no prophylactic treatments that could reduce spread during an outbreak and protect health care workers who treat an EBOV-infected patient.

There are other gaps in the armamentarium against global viral infections. For example, arenaviruses represent a family of ambisense RNA viruses capable of causing fatal hemorrhagic diseases, such as Lassa fever (3). Tacaribe arenavirus can also cause febrile illness (4). There exist few effective therapies for human herpesvirus 8 (HHV-8), the causative agent of Kaposi's sarcoma (5). Poliovirus still shows clusters of reemergence in several countries, including Pakistan, Afghanistan, and Nigeria (6). Viral hepatitis, caused by chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, affects over 350 million people worldwide and is the fifth leading cause of cancer, killing nearly 1 million people annually (7).

There is a growing appreciation that arbidol (ARB) {ethyl 6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-(phenylsulfanylmethyl)indole-3-carboxylate;hydrate; hydrochloride} has broad-spectrum antiviral activity. ARB was developed in Russia to combat influenza virus (8) and has been in clinical use in Russia and China for decades (9). Since then, ARB has been shown to inhibit the replication of multiple virus

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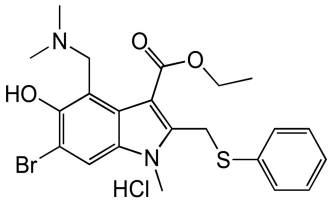

FIG 1 Structure of ARB, ethyl 6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-(phenylsulfanylmethyl)indole-3-carboxylate;hydrate; hydrochloride (PubChem compound 131410).

families that exert clinical impacts globally. ARB inhibits members of the families *Orthomyxoviridae*, *Paramyxoviridae*, and *Picornaviridae* (10), *Bunyaviridae*, *Rhabdoviridae*, *Reoviridae*, *Togaviridae*, and *Hepadnaviridae* (11), and *Hepaciviridae* (12). In this report, we demonstrate for the first time the antiviral potential of ARB against Ebola virus (EBOV), arenaviruses (Tacaribe virus), and herpesviruses (HHV-8). We also confirm ARB antiviral activity against poliovirus and HBV.

MATERIALS AND METHODS

Starting material. ARB (Fig. 1) was synthesized commercially and provided by Gary Rohrabaugh of Good Earth Medicine, LLC. The University of Washington Mass Spectrometry Center evaluated our stocks of ARB by nuclear magnetic resonance spectroscopy and mass spectrometry and confirmed that they are >99% pure and identical to the original 1970 formulation (data not shown). ARB was dissolved in dimethyl sulfoxide (DMSO) for all *in vitro* tests except for testing against EBOV, where ARB was solubilized in ethanol.

Cells and viruses. For *in vitro* testing of HHV-8, Tacaribe virus, poliovirus, and HBV, the University of Washington utilized the nonclinical and preclinical services program offered by the National Institute of Allergy and Infectious Diseases (NIAID). For HHV-8, BCBL-1 cells were induced to undergo lytic replication by the addition of 100 ng/ml phorbol 12-myristate 13-acetate (Promega, Madison, WI). One hour later, activated cells were seeded in 96-well plates containing 5-fold dilutions of ARB. The replicated DNA was then isolated and quantified by quantitative PCR (qPCR) using methods reported previously (13). HBV (strain ayw) replication was tested in HepG2 2.2.1 cells, which constitutively replicate the HBV genome (14). HBV replication was quantified using PCR as described previously (15). Tacaribe virus (strain TRVL11573) and poliovirus type 3 (strain WM-3) were tested in Vero cells by viral cytopathic effect inhibition assays using neutral red uptake as an indicator of cell viability (16, 17). ARB was added 10 min prior to infection with Tacaribe virus or poliovirus at a multiplicity of infection (MOI) of 0.001.

For EBOV infection, HepG2 cells were seeded at 2×10^5 cells per well in 12-well plates the day prior to infection. Cells were then infected with EBOV strain Zaire Kikwit at an MOI of 0.5 for 1 h at 37°C. The inoculum was removed, and the cells were washed with phosphate-buffered saline (PBS) four times. ARB was dissolved in 96% ethanol to create a 10-mg/ml stock, and ultrapure water was then added to make a final stock of 1 mg/ml. Working dilutions were made in complete minimal essential medium (MEM). ARB treatments included adding ARB 24 h before infection, at the same time as viral adsorption, 1 h after virus infection, and 24 h postinfection. Medium containing the indicated concentrations of ARB was always added after the appropriate pretreatment and virus adsorption period. Supernatants were harvested at 96 h postinfection and diluted in most instances at least 100-fold prior to titration on Vero E6 cells. This dilution step reduced the concentration of carryover ARB to less than 0.1 μg/ml, which does not inhibit EBOV. Thus, the resultant titer data are reflective of ARB suppression of EBOV replication in HepG2 cells as opposed to carryover ARB suppression of EBOV in Vero E6 cells during viral titer determination.

For experiments with vesicular stomatitis virus (VSV), Vero cells, seeded at 200,000 cells per well, were treated with vehicle or 10 μM ARB for 1 h before infection, at the time of infection, or 1 h after infection. Cells were infected with wild-type VSV or recombinant VSV expressing the Zaire Ebola virus glycoprotein (VSV-EbGP) at an MOI of 0.001. After an infection of 1 h, cells were washed 4 times to remove unbound virus, and medium with vehicle or 10 μM ARB was added back to cells. Twenty-four hours after infection, cell culture supernatants were harvested, and virus titers were determined by standard plaque assay on Vero cells.

Cytotoxicity of ARB on HepG2 cells was evaluated by measuring cellular ATP levels with a commercial kit (ATPlite assay; PerkinElmer).

Data analysis. The effective concentration of compound that suppresses viral replication by 50% (EC_{50}) and concentration of compound that causes 50% cytotoxicity (CC_{50}) were calculated with PRISM 4.0 (GraphPad, USA) using a built-in 4-parametric sigmoid function with variable slope. The ratio of antiviral EC_{50} to CC_{50} was used to calculate a selectivity index (SI) (CC_{50}/EC_{50}). *t* tests were performed to compare differences between controls and doses of ARB.

RESULTS

ARB inhibits the early stages of Ebola virus infection. The CC_{50} for ARB (Fig. 1) in HepG2 cells was 24.36 ± 0.55 μM (Fig. 2A). HepG2 cells were pretreated for 24 h with 0 to 18.8 μM ARB before infection with wild-type EBOV. ARB caused significant dose-dependent inhibition of EBOV at all tested doses ($P < 0.0001$; $EC_{50} = 2.7$ μM) (Fig. 2A), with a selectivity index (SI) of 9 (Table 1). At the highest dose of 18.8 μM ARB, more than a 4-log inhibition of EBOV was observed. ARB appeared to protect, in a dose-dependent manner, HepG2 cells from EBOV-induced cytopathic effects (Fig. 2B). Specifically, increasing doses of ARB reduced the number of rounded cells and increased the overall cell density in EBOV-infected cultures.

We have shown that ARB blocks HCV internalization into cells by slowing clathrin-mediated endocytosis and inhibiting fusion of HCV membranes with cellular membranes (18–21). Since EBOV also enters cells via an endocytotic process (22), we performed time-of-addition experiments. ARB was added to cells 24 h before, at the same time as, 1 h after, or 24 h after virus adsorption. ARB caused dose-dependent inhibition of EBOV when the compound was added to cells 24 h before or at the same time as ($EC_{50} = 4.9$ μM) or 1 h after ($EC_{50} = 4.9$ μM) virus infection (Fig. 3A). Compared to control treated cells, ARB caused significant suppression of EBOV infection at all tested doses when added before, during, or immediately after infection ($P < 0.03$). In contrast, when ARB was added at 24 h postinfection, low doses of ARB (i.e., 2.35 and 4.7 μM) failed to inhibit EBOV. However, higher doses (i.e., 9.4 and 18.8 μM) of ARB still showed significant suppression (0.26- and 0.53-fold log suppression, respectively; $P < 0.002$), although the level of suppression was far lower than when ARB was added before or during infection. These data suggest that ARB optimally inhibits EBOV infection when added before or during the early stages of infection and that ARB has some efficacy against established infection.

To further explore whether ARB targets an early stage in the EBOV life cycle, we used recombinant VSV expressing the EBOV glycoprotein (GP) from the Zaire isolate. ARB inhibited virus replication when the drug was added to cells 1 h before, at the same time as, and 1 h after infection (Fig. 3B). Note, however, that maximal suppression of infection occurred when ARB was added prior to virus infection, and the efficacy of ARB declined as treatment was delayed. ARB did not inhibit replication of wild-type

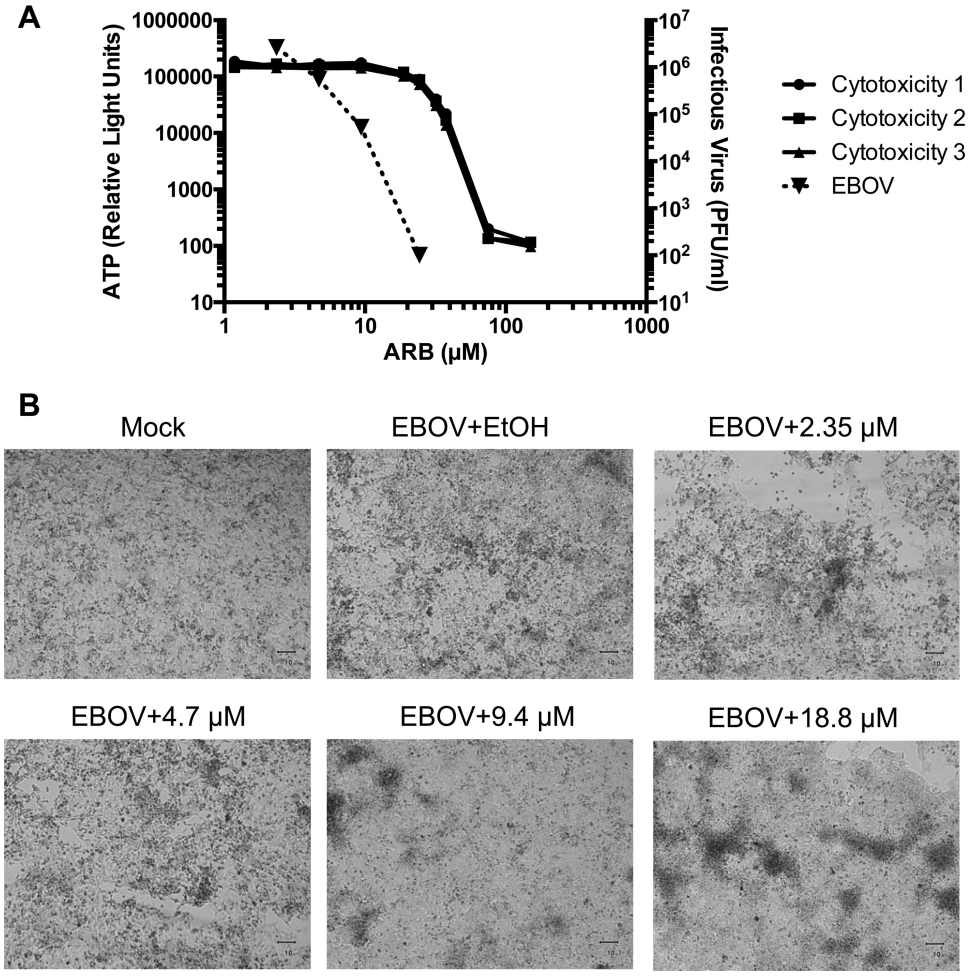

FIG 2 ARB inhibits EBOV. (A) Cytotoxicity and anti-EBOV profile of ARB on HepG2 cells. Left *y* axis, triplicate cultures of HepG2 cells were incubated with 0, 1.18, 2.35, 4.7, 9.4, 18.8, 24.4, 32, 37.6, 75.2, and 150.4 μM ARB prior to measurement of cellular ATP levels 3 days later. The experiment was performed three independent times, and the replicates are designated cytotoxicity 1, cytotoxicity 2, and cytotoxicity 3. Right *y* axis, ARB was incubated, in quadruplicate, with HepG2 cells for 24 h prior to infection with EBOV. Virus was adsorbed for 1 h (in the presence of ARB), followed by four cell washings with PBS and addition of medium with 0, 2.35, 4.7, 9.4, and 18.8 μM ARB. At 96 h postinfection, culture supernatants were harvested and diluted in fresh medium, and titers were determined on Vero E6 cells. Error bars are mostly contained within the symbols and represent standard error of the mean. (B) Light micrographs of the mock- and EBOV-infected cells, as well as cells infected with EBOV and treated with the indicated doses of ARB. Images were captured at 120 h postinfection.

VSV in this system, suggesting that the effect of ARB was directed against virus entry mediated by the EBOV GP. Collectively, these data suggest that ARB targets an early stage of the EBOV life cycle, most likely the entry step.

ARB inhibits other globally prevalent viruses. ARB was tested by NIAID's *in vitro* antiviral testing program. Table 1 shows that poliovirus, HHV-8, Tacaribe virus, and HBV were inhibited by ARB, with selectivity indexes (SIs) of 7.7, 6.2, >11, and >37, respectively. The primary hits against poliovirus, HHV-8, Tacaribe, and HBV were confirmed in secondary testing by NIAID. Finally, ARB inhibited HHV-8 replication to a similar degree as cidofovir (CDV), with the only difference being slightly increased cytotoxicity of ARB relative to CDV at the highest dose (60 μM) (Fig. 4).

TABLE 1 ARB inhibits multiple viruses[a]

Virus	Family	EC_{50} (μM)	CC_{50} (μM)	SI_{50} (μM)
Ebola virus Zaire	*Filoviridae*	2.7	24.4	9
Poliovirus type 3	*Picornaviridae*	4.1 ± 3.2	28.6 ± 9.6	7.7
HHV-8	*Herpesviridae*	1.6	>60	>37
Tacaribe virus	*Arenaviridae*	5.8 ± 3.0	31.0 ± 3.9	6.2
HBV	*Hepadnaviridae*	17.9	>188	>11

[a] EC_{50}, effective concentration of ARB that leads to 50% suppression of virus infection; CC_{50}, concentration of ARB that causes 50% cytotoxicity; SI_{50}, selectivity index, which is the ratio of CC_{50} to EC_{50}. Errors are standard deviations.

DISCUSSION

We show here that ARB inhibits multiple viruses of global medical significance, including EBOV. In support of our findings, suppression of EBOV by ARB was recently described, in supplemental data, in a large drug screen (23).

Mechanistically, we have previously shown that ARB inhibits HCV entry by blocking viral fusion (20, 21, 24) and that ARB also impairs clathrin-mediated endocytosis (18). Since the viruses inhibited by ARB in this study may use clathrin to gain entry into cells (25–31), a possible unifying mode of action might be via

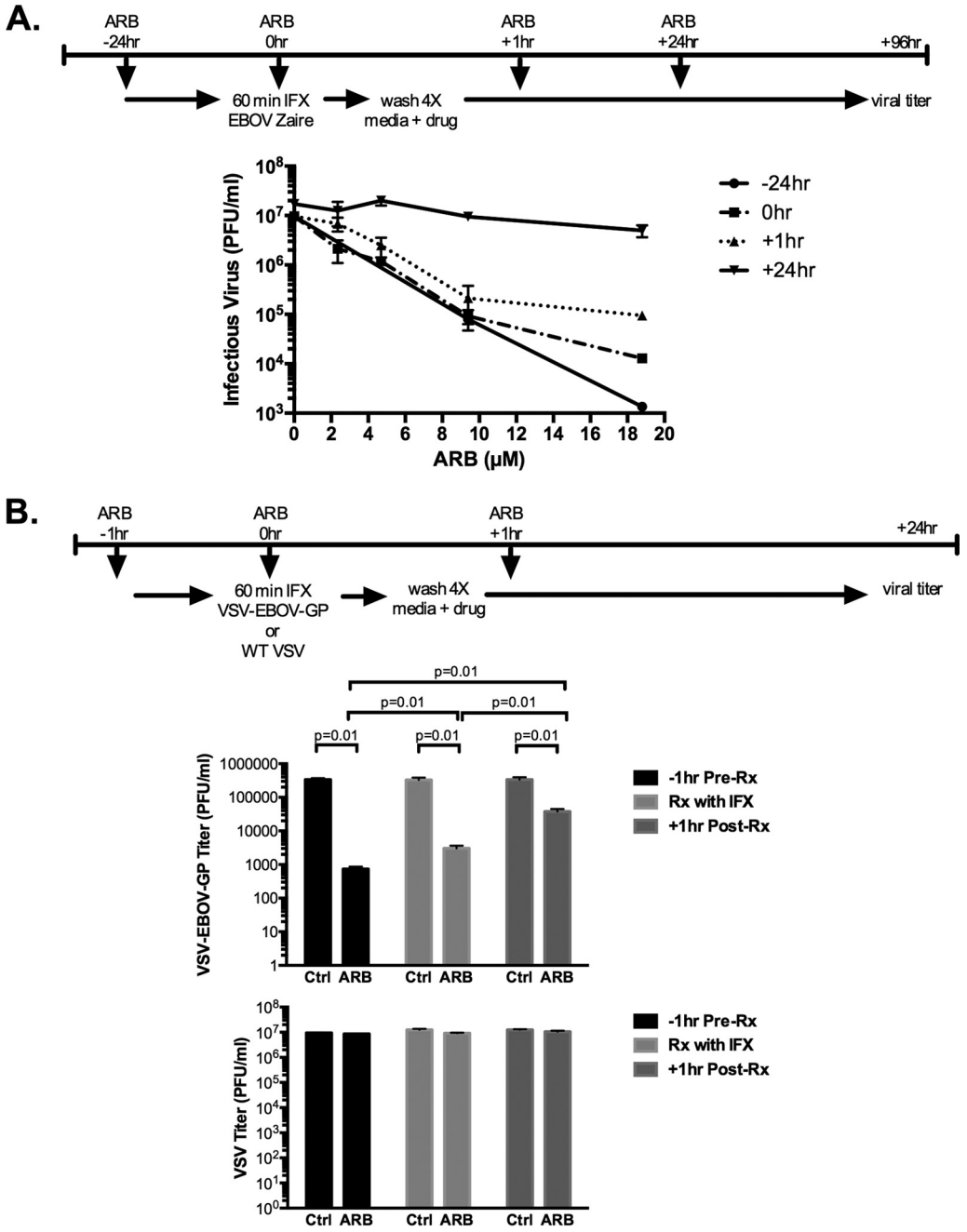

FIG 3 ARB inhibits an early stage of the EBOV life cycle. (A) HepG2 cells were treated with 0, 2.35, 4.7, 9.4, and 18.8 μM ARB for 24 h before infection (−24 h), at the same time as infection (0 h), 1 h after infection (+1 h), or 24 h after infection (+24 h). Cells were infected with EBOV Zaire at an MOI of 0.5 for 1 h and washed, and fresh medium containing ARB was added back to cultures. Infectious virus in culture supernatants harvested at 96 h postinfection was determined by viral plaque assay on Vero E6 cells. Error bars represent the standard error of the mean for triplicate cultures. (B) Vero cells were pretreated for 1 h before infection (−1 h) or at the same time as infection (0 h) with VSV pseudoviruses expressing EBOV Zaire glycoprotein or wild-type (WT) VSV. After an infection of 1 h, cells were washed 4 times to remove unbound virus, and medium with vehicle or 10 μM ARB was added back to cells. ARB was also added to cells 1 h after infection (+1 h). Supernatants were harvested at 24 h postinfection, and titers were determined on naive Vero cells. Values represent mean viral titers, and error bars represent the standard error of the mean for triplicate cultures.

blockade of virus entry. Time-of-addition experiments support this mode of action of ARB against EBOV. However, since EBOV and HHV-8 also enter cells by macropinocytosis (32–36), it will be important to discriminate actions of ARB on clathrin versus the macropinocytic routes of entry. In this regard, the other herpesviruses not inhibited by ARB, including herpes simplex virus 1 (HSV-1) (37), HSV-2 and human cytomegalovirus (HCMV) (38), and Epstein-Barr virus (EBV) (39), all use clathrin-independent pathways to gain entry into cells. Finally, the possible membrane-associating actions of ARB (20, 21) need to be considered as potential mechanisms for suppression of virus replication. In fact, since ARB is an indole-based hydrophobic molecule (Fig. 1), it displays a

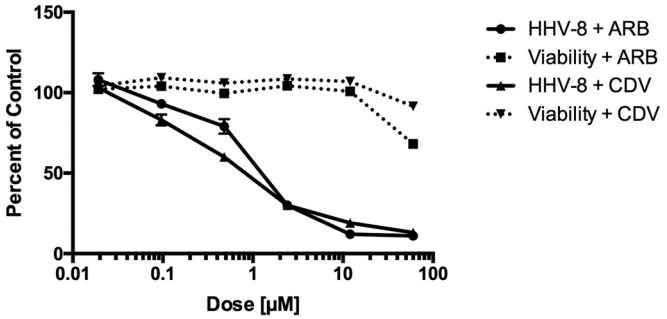

FIG 4 ARB inhibits HHV-8 replication to a similar extent as cidofovir. Human BCBL-1 cells, which are infected with HHV-8 (50), were treated with ARB or CDV, HHV-8 DNA was quantified by reverse transcription-PCR (RT-PCR), and cell viability was measured by Cell Titer Glow assay (Promega) 7 days later. Error bars represent the standard error of the mean for triplicate cultures.

capacity for dual binding to both lipid membrane interfaces and aromatic protein residues (21). The physico-chemical properties of ARB may enable it to form supramolecular conformations through aromatic stacking interactions with selective amino acid residues of proteins (phenylalanine, tyrosine, and tryptophan). As such, ARB may impair many steps in the life cycle of viruses, including virus binding to cells, fusion of viral and cellular membranes during virus entry (20, 21, 24, 40–42), clathrin-mediated endocytosis (18), virus replication on intracellular membranes such as membranous webs (19, 43), and virus assembly and egress from cells.

Why did ARB display clear antiviral activity against HHV-8 yet not inhibit other herpesviruses? Contrary to our findings, HSV-1 infection was previously shown to be inhibited by ARB and derivatives of the molecule (44). That study used human keratinocytes and a very low MOI (0.0001), which might account for the discordance with our study, which used a higher MOI (0.01). As described above, since HSV-1 uses clathrin-independent pathways, it may not be accessible to ARB's antiviral mode of action. It is also possible that the assay system might influence results. The HHV-8 assay derives from induction of lytic replication from latently infected BCBL-1 cells, while the HSV-1 assay deploys live virus in an acute-infection setting. Further studies are required to sort out these possibilities.

We confirmed a prior study showing that ARB inhibits *in vitro* replication of HBV (45). We further tested ARB in homozygous transgenic HBV mice (46). ARB was prepared and given to mice by daily oral gavage of 50 or 100 mg/kg/day of ARB for 14 days. Unfortunately, ARB did not inhibit HBV replication in this mouse model (data not shown). Pharmacokinetic studies were not performed due to budget limitations, so additional studies are required before unequivocal conclusions can be made.

ARB suppresses virus infection in the low micromolar range, which is higher than the value for directly acting antiviral (DAA) agents that target viral proteins or enzymes. We suspect that this is likely because ARB is a cell-targeting antiviral, based on multiple studies suggesting that ARB blocks virus entry by incorporating into cellular membranes and modifying their physico-chemical properties (reviewed in reference 12). Moreover, host-targeting compounds often function in higher concentration ranges than DAA compounds (47, 48). Finally, derivatives of ARB with improved bioactivity have been synthesized (44, 45), suggesting that it may be possible to lower the dose of ARB and improve the selectivity index.

ARB inhibited members of both *Filoviridae* and *Arenaviridae*, which are known to cause lethal hemorrhagic fevers in humans. It is noteworthy that ARB has been used clinically for decades in other countries, with minimal side effects and a good pharmacokinetic profile (9, 12, 49). Given that ARB seems to be most efficacious when administered prior to or at the same time as virus infection, ARB might be considered for prophylactic use in hemorrhagic fever outbreaks, which could limit the deadly spread of filoviruses and arenaviruses. Prophylactic use of ARB might also limit the risk of infection for health care workers when they treat infected subjects during outbreaks, and following repatriation of infected subjects. As a therapeutic antiviral drug, ARB could potentially act as a roadblock against spread of the virus to uninfected target cells. Additional studies at different multiplicities of infection may reveal the therapeutic potential of ARB for treatment of acute EBOV infection.

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