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Article

Novel Antiviral Activities of Obatoclax, Emetine, Niclosamide, Brequinar, and Homoharringtonine

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Abstract: Viruses are the major causes of acute and chronic infectious diseases in the world. According to the World Health Organization, there is an urgent need for better control of viral diseases. Re-purposing existing antiviral agents from one viral disease to another could play a pivotal role in this process. Here we identified novel activities of obatoclax and emetine against herpes simplex virus type 2 (HSV-2), human immunodeficiency virus 1 (HIV-1), echovirus 1 (EV1), human metapneumovirus (HMPV) and Rift Valley fever virus (RVFV) in cell cultures. Moreover, we demonstrated novel activities of emetine against influenza A virus (FluAV), niclosamide against HSV-2, brequinar against HIV-1, and homoharringtonine against EV1. Our findings may expand the spectrum of indications of these safe-in-man agents and reinforce the arsenal of available antiviral therapeutics pending the results of further *in vivo* tests.

Keywords: virus; broad-spectrum antiviral; antiviral agent; drug target; systems biology

1. Introduction

Every year, emerging and re-emerging viruses, such as Ebola virus (EBOV), Marburg virus (MARV), and Rift Valley fever virus (RVFV), surface from natural reservoirs and kill people [1, 2]. In addition, influenza A (FluAV), human immunodeficiency (HIV-1), herpes simplex (HSV), and other viruses regularly infect human population and represent substantial public health and economic burden [3, 4]. The World Health Organization (WHO) and United Nations (UN) has called for better control of viral diseases (https://www.who.int/blueprint/priority-diseases/en/; https://sustainabledevelopment.un.org/). Developing novel virus-specific vaccines and antiviral drugs can be time-consuming and costly [5, 6]. In order to overcome these time and cost issues, academic institutions and pharmaceutical companies focused on repositioning of existing antivirals from one viral disease to another, taking into account that the most viruses utilize the same host factors and pathways to replicate inside a cell [6-12].

Broad-spectrum antivirals (BSAs) are small-molecules that inhibit a wide range of human viruses. We have recently reviewed approved, investigational and experimental antiviral compounds and identified 108 BSAs, whose pharmacokinetics (PK) and toxicity had been studied *in vivo* and in clinical trials [13]. We tested 40 of these BSAs against HMPV, hepatitis C virus (HCV), cytomegalovirus (CMV), and hepatitis B virus (HBV). We demonstrated novel antiviral effects of azacytidine, itraconazole, lopinavir, nitazoxanide, and oritavancin against HMPV, as well as cidofovir, dibucaine, azithromycin, gefitinib, minocycline, oritavancin, and pirlindole against HCV [14]. We also tested 55 BSAs, including these 40, against FluAV, RVFV, EV1, ZIKV, CHIKV, RRV, HIV-1 and HSV-1. We identified novel activities for dalbavancin against EV1, ezetimibe against HIV-1 and ZIKV, azacitidine, cyclosporine, minocycline, oritavancin and ritonavir against RVFV [15].

Here, we evaluated the efficacy of 43 BSAs, which do not overlap with 55 agents we tested recently. We identified novel *in vitro* activities of obatoclax and emetine against HSV-2, HIV-1, EV1, HMPV and RVFV. Moreover, we demonstrated novel antiviral effects of emetine against FluAV, niclosamide against HSV-2, brequinar against HIV-1, and homoharringtonine against EV1. Thus, we expanded spectrum of activities of these BSAs in cell cultures.

2. Materials and Methods

2.1. Compounds

Forty-three compounds used in this study, their suppliers and catalogue numbers are summarized in Table S1. To obtain 10 mM stock solutions compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) or milli-Q water. The solutions were stored at −80 °C until use.

2.2. Cells

Madin–Darby canine kidney (MDCK) and African green monkey kidney epithelial cells (Vero-E6) cells were grown in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Paisley, Scotland) supplemented with 100 U/mL penicillin and 100 μg/ml streptomycin mixture (Pen/Strep; Lonza, Cologne, Germany), 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS; Lonza, Cologne, Germany). Human telomerase reverse transcriptase-immortalized retinal pigment epithelial (RPE) cells were grown in DMEM-F12 medium supplemented with Pen/Strep, 2 mM L-glutamine, 10% FBS, and 0.25% sodium bicarbonate (Sigma-Aldrich, St. Louis, USA). ACH-2 cells, which possesses a single integrated copy of the provirus HIV-1 strain LAI (NIH AIDS Reagent Program), were grown in RPMI-1640 medium supplemented with 10% FBS and Pen/Strep. TZM-bl cells were grown in DMEM supplemented with 10% FBS and PenStrep. Human lung adenocarcinoma epithelial A549 cells were cultured in DMEM medium containing 10% FBS and PenStrep. A549-NPro cells, which stably express BVDV NPro protein, which inhibits IFN production, were cultured in DMEM containing 10% FBS, Pen/Strep, and 10 μg/ml puromycin. All cell lines were grown in humidified incubator at 37°C in the presence of 5% CO2.

2.3. Viruses

All the experiments with viruses were performed in compliance with the guidelines of the national authorities using appropriate biosafety laboratories under appropriate ethical and safety approvals. GFP-expressing influenza A/PR/8-NS116-GFP strain (FluAV-GFP) was amplified in a monolayer of MDCK cells in DMEM containing Pen/Strep, 0.2% bovine serum albumin, 2 mM L-glutamine, and 1 μg/mL L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (TPCK)-trypsin (Sigma-Aldrich, St. Louis, USA). HMPV NL/1/00 strain, encoding green fluorescent protein (HMPV-GFP), RVFV encoding the far-red fluorescent protein instead of NSs protein (RVFV-RFP) and wild-type HSV-2 strain were amplified in a monolayer of Vero-E6 cells in the DMEM medium containing Pen/Strep, 0.2% bovine serum albumin, 2 mM L-glutamine, and 1 μg/mL TPCK-trypsin. EV1 was amplified in a monolayer of A549 cells in the DMEM media containing Pen/Strep, 0.2% bovine serum albumin, and 2 mM L-glutamine.

For production of HIV-1, 6×10^6 ACH-2 cells were seeded in 10 mL medium. Virus production was induced by the addition of 100 nM phorbol-12-myristate-13-acetate. The cells were incubated for 48 h. The HIV-1 containing medium was collected. HIV-1 concentration was estimated by measuring concentration of HIV-1 p24 in the medium using anti-p24-ELISA, which was developed in-house. Recombinant purified p24 protein was used as reference. The virus stocks were stored at -80 °C.

2.4. Microscopy

Approximately 4×10^4 RPE cells were seeded per well in 96-well plates. The cells were grown for 24 h in DMEM-F12 medium supplemented with 10% FBS, and Pen/Strep. The medium was replaced with DMEM-F12 medium containing 0.2% bovine serum albumin, 2 mM L-glutamine, and 1 µg/ml TPSK-trypsin. The compounds were added to the cells in 3-fold dilutions at seven different concentrations starting from 10 or 30 µM. No compounds were added to the control wells. RPE cells were infected with HSV-2, FluAV-GFP, HMPV-GFP or RVFV-RFP viruses at multiplicity of infections (moi) of 0.1, 0.5, 0.1 and 1, respectively. RVFV-mediated RFP expression was visualized after 24 h, whereas HMPV- and FluAV-mediated GFP expression was recorded after 72 h using fluorescent microscopy (Zeiss Observer Z1, Zaventem, Belgium). HSV-2-infected RPE cells were imaged after 72 h in phase-contrast mode.

2.5. Cell viability and toxicity assays

RPE cells were treated as described above and infected with HSV-2, EV1, FluAV-GFP, HMPV-GFP or RVFV-RFP viruses at multiplicity of infections (moi) of 0.1, 0.1, 0.5, 0.1 and 1, respectively. After 48 h of infection, the medium was removed from the cells. Viability of mock- and virus-infected cells were measured using Cell Titer Glow assay (CTG; Promega, Madison, USA). The luminescence/fluorescence were read with a PHERAstar FS plate reader (BMG Labtech, Ortenberg, Germany).

For testing compound toxicity and efficacy against HIV-1, approximately 4×104 TZM-bl cells were seeded in each well of a 96-well plate. TZM-bl cells express firefly luciferase under control of HIV-1 LTR promoter allowing quantitation of the viral infection (tat-protein expression by integrated HIV-1 provirus) using firefly luciferase assay. The cells were grown for 24 h in cell growth medium. Compounds were added to the cells in three-fold dilutions at seven different concentrations starting from 30 µM. No compounds were added to the control wells. The cells were infected with HIV-1 (corresponding to 300 ng/ml of HIV-1 p24) or mock. At 48 hpi, the media was removed from the cells, the cells were lysed, and firefly luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and PHERAstar FS plate reader. In a parallel experiment, Cell Tox Green reagent (CTxG; Promega, Madison, WI, USA) was added to the cells and fluorescence was measured with a plate reader.

The half-maximal cytotoxic concentrations (CC_{50}) for each compound were calculated based on viability/death curves obtained on mock-infected cells after non-linear regression analysis with a variable slope using GraphPad Prism software version 7.0a. The half-maximal effective concentrations (EC_{50}) were calculated based on the analysis of reporter protein expression or viability/death of infected cells. The relative effectiveness of the drug was quantified as the selectivity index ($SI = CC_{50}/EC_{50}$).

2.6. Drug combination experiment

RPE cells were treated with combinations of increasing concentrations of obatoclax and emetine. The cells were infected with FluAV-GFP at moi 0.5. After 24h GFP fluorescence was recorded using fluorescent microscopy. In a parallel experiment, viability of infected cells was measured using the CTG assay. To test if the drug combinations acts synergistically, the observed responses were compared with expected combination responses calculated by means of zero interaction potency (ZIP) model [16].

2.6. Virus titration

For testing production of HSV-2 and EV1 viruses in compound-treated and non-treated RPE cells, the media from the cells were serially diluted in serum-free growth media containing 0.2% bovine serum albumin and applied to a monolayer of A549-NPro cells in 12 well/plates. After one hour, cells were overlaid with growth medium containing 1% carboxymethyl cellulose and 1% FBS and incubated for 72 hours. The cells were fixed and stained with crystal violet dye. The plaques were calculated in each well. The titers were expressed as plaque-forming units per ml (PFU/ml).

The presence of HMPV-GFP, RVFV-RFP and FluAV-GFP in the media of non- or drug-treated cells was evaluated on fresh RPE cells in 96 well-plates. The media was serially-diluted and reporter protein expression was visualized after 24-72 h using fluorescence microscopy.

3. Results

3.1. Forty-three BSAs target 52 viruses

We obtained 43 BSAs from commercial sources. Altogether, these BSAs inhibit replication of 52 viruses belonging to (–) single-stranded (ss)RNA, (+)ssRNA, ssRNA-reverse transcriptase (RT), ssDNA, double-stranded (ds)DNA, or dsDNA-RT virus groups (Fig. 1, Tables S2 and S3).

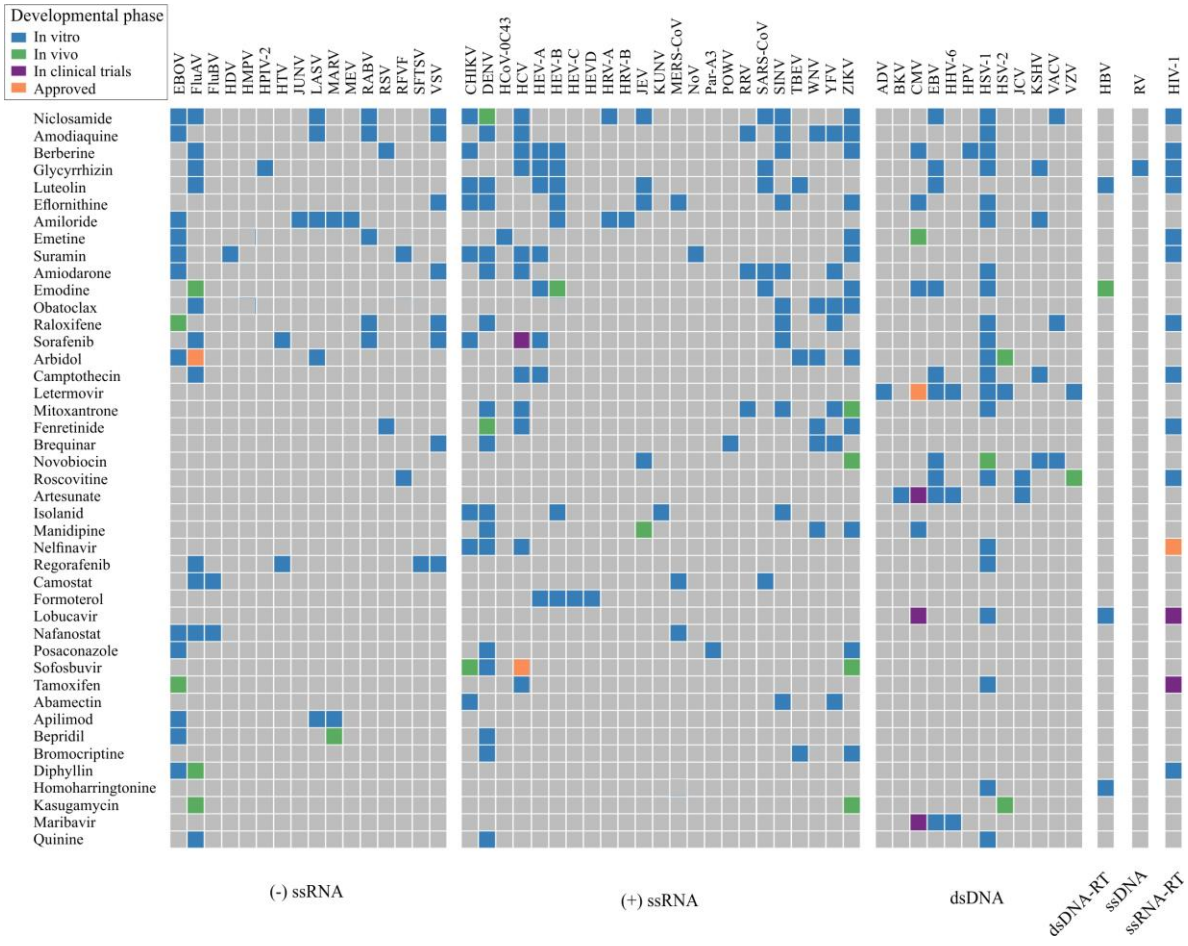

Figure 1. Forty-three broad-spectrum antiviral agents (BSAs) and viruses they inhibit. Viruses are clustered by virus groups. BSAs are ranged from the highest to lowest number of targeted viruses. Blue shading indicates that antiviral activity has been reported for the compound *in vitro*, green – *in vivo*, whereas, violate and orange shading indicate that the antiviral agent is in clinical trial or approved. Gray shading indicates that antiviral activity has not been either reported or studied. Abbreviations: ds, double-stranded; RT, reverse transcriptase; ss, single-stranded.

3.2. Obatoclax, emetine and niclosamide inhibit HSV-1 replication in RPE cells

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We tested 43 BSAs against wild-type HSV-2 in RPE cells. Seven different concentrations of the compounds were added to virus or mock-infected cells. Cell viability was monitored by microscopy and CTG assay. After the initial screening, we identified four compounds (obatoclax, emetine, niclosamide and ganciclovir), which rescued cells from HSV-2 mediated death at none-cytotoxic concentrations (Fig. 2A). To determine efficiency and toxicity of novel HSV-2 inhibitors, we measured the viability of mock- and virus-infected cells after 72 h using CTG assay (Fig. 2B). The SI for obatoclax was 40 (CC_{50}=3.3 µM, EC_{50}=0.1 µM), SI for emetine was 110 (CC_{50}=3.3 µM, EC_{50}=0.03 µM), and SI for niclosamide was 3 (CC_{50}=1.1 µM, EC_{50}=0.4 µM). We titrated HSV-2 produced from drug-treated and non-treated cells in A540-Npro cells (Fig. 2C). The experiment revealed that 0.12 µM obatoclax, 0.04 µM emetine and 0.37 µM niclosamide lowered production of HSV-2 in RPE cells, confirming novel anti-HSV-2 activities of the three compounds.

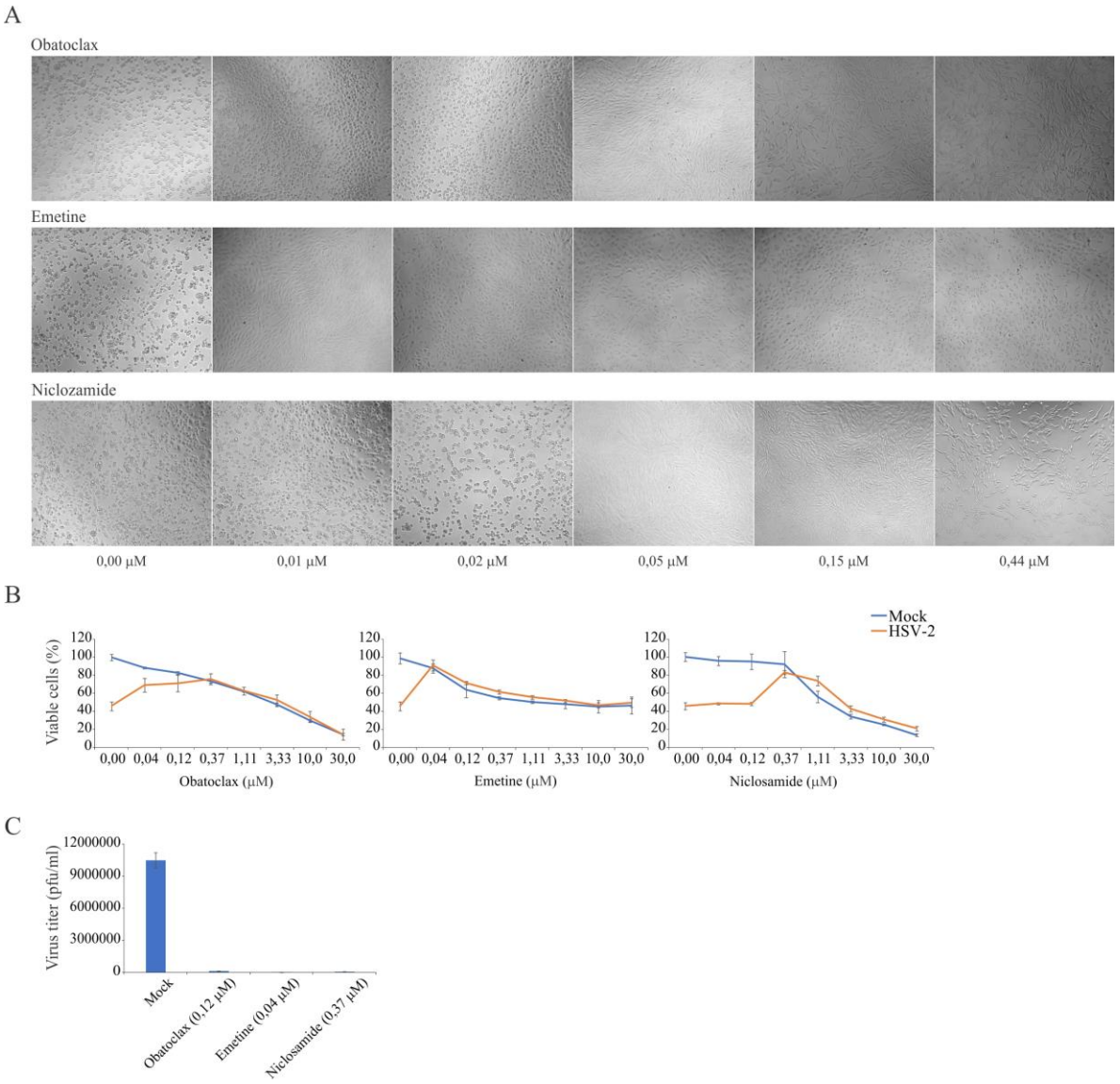

Figure 2. Effect of obatoclax, emetine and niclosamide on production of HSV-2 and viability of mock- and virus infected RPE cells. (A) RPE cells were treated with increasing concentrations of a compound and infected with HSV-2 (moi, 0.1). Cells were imaged after 72 h in phase-contrast mode. (B) RPE cells were treated with increasing concentrations of a compound and infected with mock- or HSV-2 (moi, 0.1). The viability of the cells was determined 72 hpi with the CTG assay. Mean ± standard deviation (SD), n = 3 (experimental replicates). (C) HSV-2 production in infected drug- or DMSO-treated RPE cells was measured by plaque assay using A549-NPro cells (Mean±SD, n=3).

3.3. Obatoclax, emetine and homoharringtonin inhibit EV1 replication in RPE cells

Similarly, we examined 43 BSAs against wild-type EV1 in RPE cells. We monitored the viability of mock- and virus-infected cells by microscopy and CTG assay. After the initial screening, we identified three compounds (obatoclax, emetine, and homoharringtonine), which rescued cells from virus-mediated death at none-cytotoxic concentrations. To determine efficiency and toxicity of novel EV1 inhibitors, we measured viability of mock- and virus-infected cells after 48 h using CTG assay (Fig. 3A). The SI for obatoclax was 250 (CC_{50}=30 μM, EC_{50}=0.12 μM), SI for emetine was >300 (CC_{50}>30 μM, EC_{50}=0.1 μM), and SI for homoharringtonine was >3000 (CC_{50}>30 μM, EC_{50}=0.01 μM). We titrated EV1 produced from drug-treated and non-treated cells in A540-Npro cells (Fig. 3B). The experiment revealed that obatoclax, emetine and homoharringtonine lowered production of EV1, confirming novel anti-EV1 activities of the three compounds.

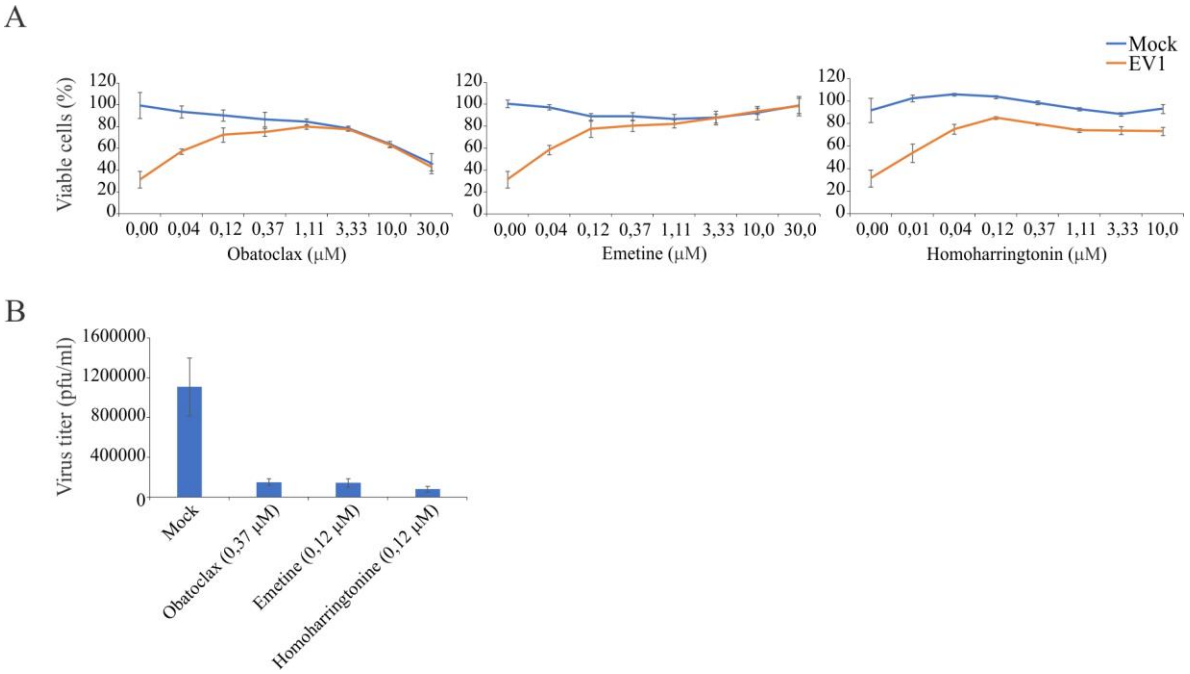

Figure 3. Effect of obatoclax, emetine and homoharringtonine on production of EV1 and viability of mock- and virus infected RPE cells. (A) RPE cells were treated with increasing concentrations of a compound and infected with EV1 (moi, 0.1). The viability of the cells was determined 48 hpi with the CTG assay. (Mean±SD, n=3). (C) EV1 production in infected, drug- or DMSO-treated RPE cells was measured by plaque assay using A549-NPro cells (Mean±SD, n=3).

3.4. Brequinar and suramin inhibit HIV-1-mediated luciferase expression in TZM-bl cells

We also examined toxicity and antiviral activity of 43 BSAs against HIV-1 mediated firefly luciferase expression which is integrated into the genome of TZM-bl cells under HIV-1 LTR promoter. Our primary screen identified, and validation experiment confirmed, anti-HIV-1 activity of brequinar and suramin (Fig. 4A). The SI for brequinar was >750 (CC_{50}>30 μM, EC_{50}=0.04 μM), and SI for suramin was >350 (CC_{50}>30 μM, EC_{50}=0.08 μM). CTxG assay showed that mock- and HIV-infected cells remained viable at selected range of drug concentrations (Fig. 4B). Brequinar is a novel inhibitor, whereas suramin is known anti-HIV-1 agent [17].

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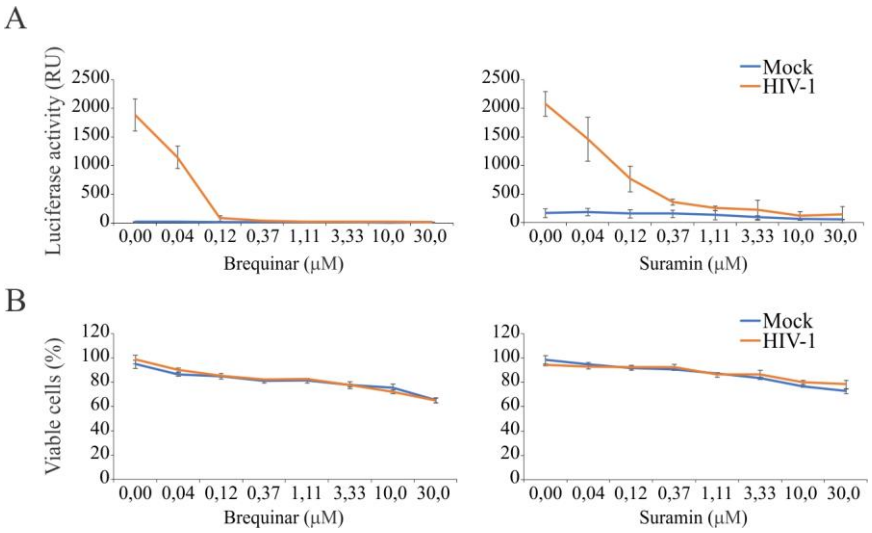

Figure 4. Effect of brequinar and suramin on HIV-1-mediated luciferase expression and viability of mock- and virus-infected TZM-bl cells. (A) TZM-bl cells were treated with increasing concentrations of a compound and infected with mock or HIV-1 (300 ng/ml of HIV-1 p24). After 24 hpi, the medium was removed from the cells, cells were lysed, and firefly luciferase activity was measured using the Luciferase Assay System (Mean±SD, n=3). (B) In a parallel experiment, CTxG reagent was added to the cells and the fluorescence was measured. Percentage of viable cells was calculated. Mean ± standard deviation (SD), n = 3 (experimental replicates).

3.5. Obatoclax and emetine inhibit RVFV-mediated RFP expression in RPE cells

In addition, we examined antiviral activity and toxicity of 43 BSAs against RFP-expressing RVFV in RPE cells. Both fluorescent microscopy and CTG assays showed that obatoclax and emetine inhibited RVFV-mediated RFP expression at non-cytotoxic concentrations (Fig. 5A-C). The SI for obatoclax was >300 (CC_{50}>30 μM, EC_{50}=0.1 μM), and SI for emetine was >600 (CC_{50}>30 μM, EC_{50}=0.05 μM). Both compounds are novel inhibitors of RVFV infection.

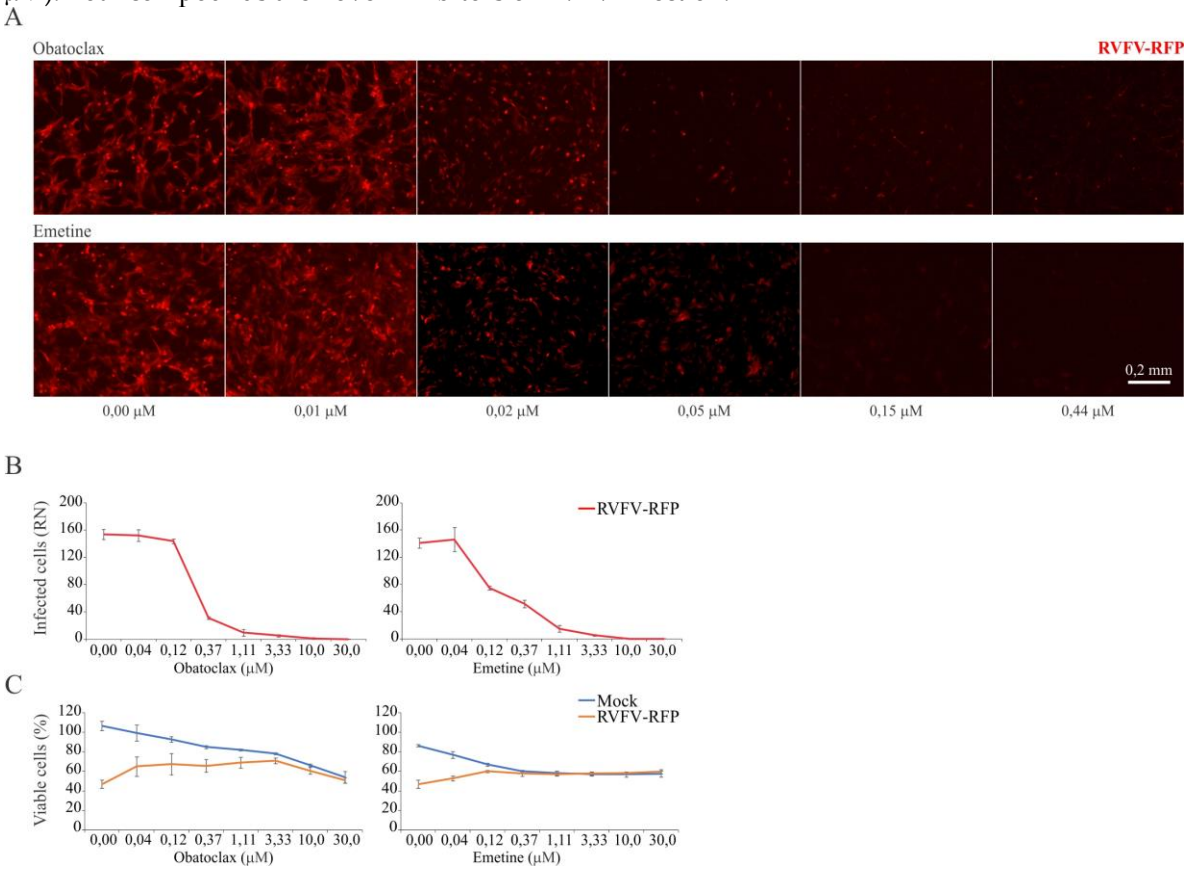

Figure 5. Effect of obatoclax and emetine on RVFV-mediated RFP expression and on viability of mock- and virus-infected RPE cells. (A-B) RPE cells were treated with increasing concentrations of a compound and infected with RVFV-RFP (moi, 1). After 24 h, the cells were imaged and fluorescence intensity was quantified (Mean±SD, n=3). (C) Cell viability was determined using the CTG assay (Mean±SD, n=3).

3.6. Obatoclax and emetine inhibit HMPV-mediated GFP expression in RPE cells

Next, we tested 43 BSAs against GFP-expressing HMPV. HMPV-mediated GFP expression and cell viability were measured after 72 h. After the initial screening, we identified 2 compounds, which lowered GFP-expression without detectable cytotoxicity. We repeated the experiment with these compounds. The experiment confirmed novel anti-HMPV activities of obatoclax and emetine (Figure 6A-C). The SI for obatoclax was 8 (CC_{50}=0.8 μM, EC_{50}=0.1 μM), and SI for emetine was 20 (CC_{50}=2 μM, EC_{50}=0.1 μM).

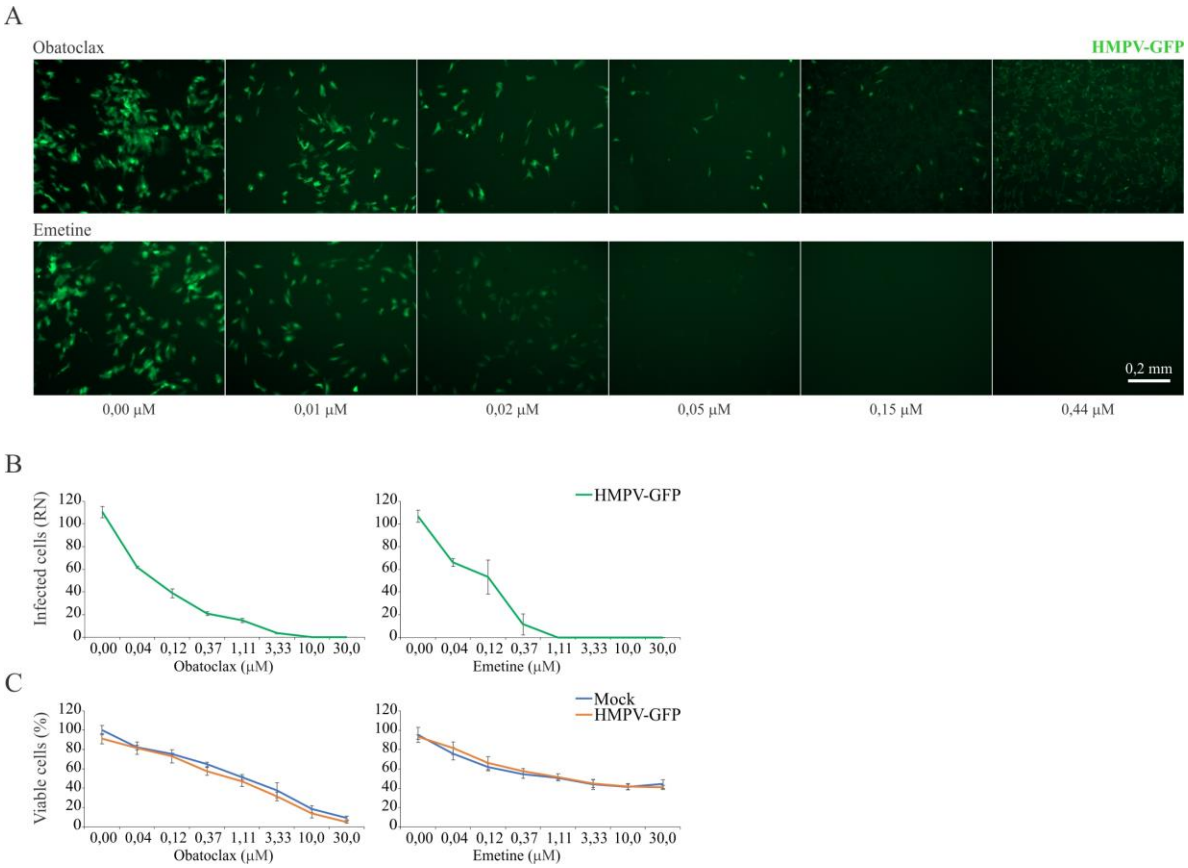

Figure 6. Effect of obatoclax and emetine on HMPV-mediated GFP expression and on viability of mock- and virus-infected RPE cells. (A-B) RPE cells were treated with increasing concentrations of a compound and infected with HMPV-GFP (moi, 0.1). After 72 h, the cells were imaged and fluorescence intensity was quantified (Mean±SD, n=3). (C) Cell viability was determined with the CTG assay (Mean±SD, n=3).

3.7. Obatoclax and emetine inhibit FluAV-mediated GFP expression in RPE cells

Next, we tested 43 BSAs against GFP-expressing FluAV in RPE cells. Both compounds at non-cytotoxic concentrations inhibited FluAV mediated GFP expression at 24 hpi (Fig. 7A-C). The SI for obatoclax was 31 (CC_{50}=3.1 μM, EC_{50}=0.1 μM) and SI for emetine was >250 (CC_{50}>10 μM, EC_{50}=0.04 μM). Thus, we identified novel anti-influenza activity of emetine and confirmed anti-FluAV action of obatoclax [18].

To test if the obatoclax-emetine combinations acts synergistically against FluAV mediated GFP expression and rescue infected cells from death, the observed responses were compared with expected combination responses. The deviations in observed and expected responses showed no

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synergistic effect (Fig. 7D,E). This result indicates that obatoclax and emetine target distinct cellular pathways involved in virus replication cycle.

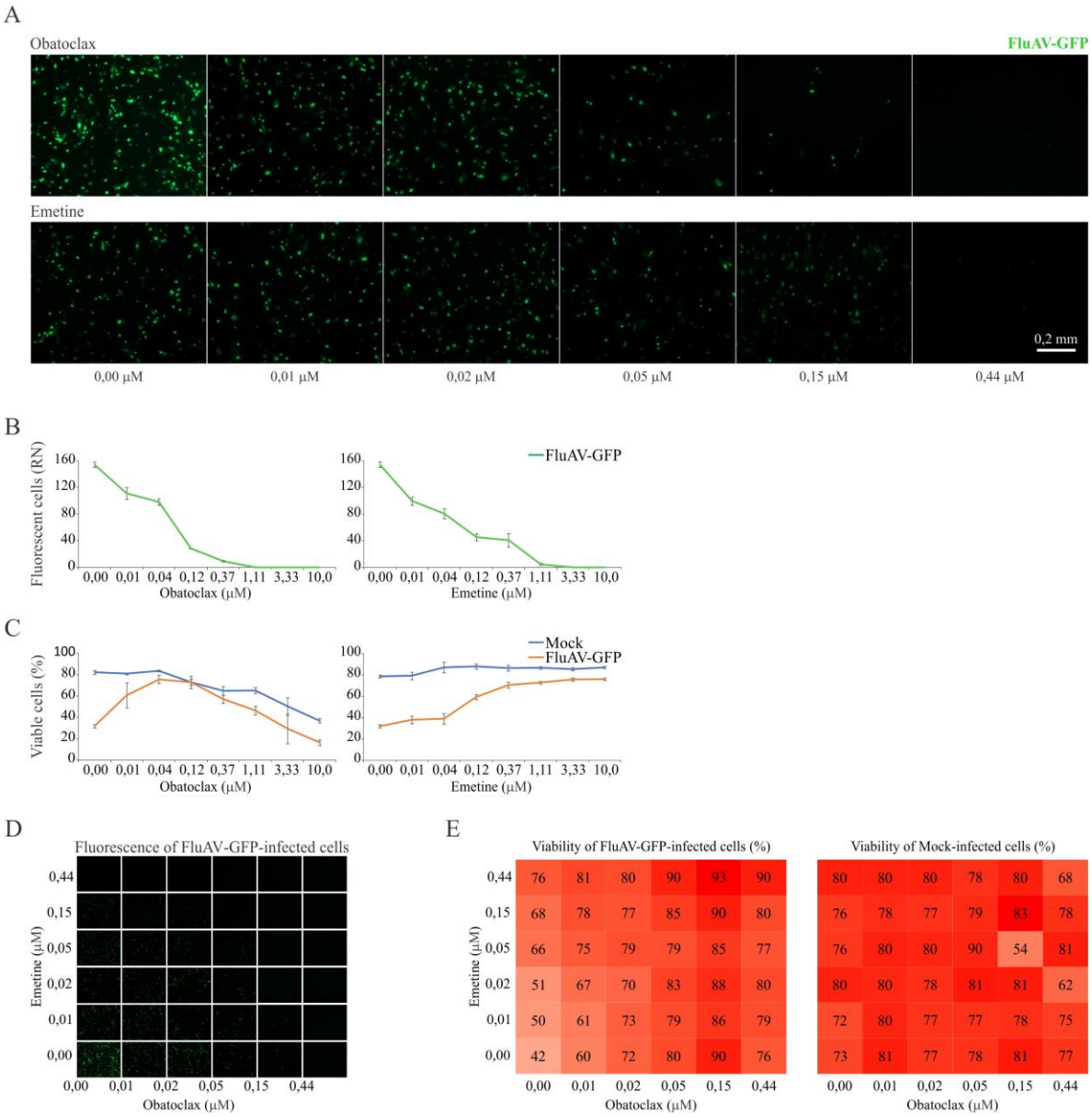

Figure 7. Effect of obatoclax, emetine and their combinations on FluAV-mediated GFP expression and viability of mock- and virus-infected RPE cells. (A-B) RPE cells were treated with increasing concentrations of a compound and infected with FluAV-GFP (moi, 0.5). After 24 h, the cells were imaged and fluorescence intensity was quantified (Mean±SD, n=3). (C) Cell viability was determined with the CTG assay (Mean±SD, n=3). (D-E) The interaction landscapes of emetine-obatoclax drug combination on FluA-mediated GFP expression and viability of FluAV- and mock-infected RPE cells, as measured with CTG assay.

4. Discussion

Here, we tested 43 BSAs against (-)ssRNA, (+)ssRNA, RT-ssRNA and dsDNA viruses and identified novel activities for 5 agents (Fig. 8). We identified novel activities of niclosamide against HSV-2, brequinar against HIV-1, homoharringtonine against EV1, obatoclax against HSV-2, HIV-1, EV1, HMPV and RVFV and emetine against HSV-2, HIV-1, EV1, HMPV, RVF and FluAV. We also confirmed antiviral activities of obatoclax against FluAV and suramin against HIV-1. Thus, existing BSAs could be re-positioned to other viral infections. Our results also pointed out that evasion mechanism observed in one virus could be relevant for other viruses.

Figure 8. The interaction network between 52 viruses and 43 BSAs. Yellow spheres represent antiviral agents. Blue spheres represent viruses. The diameter of spheres corresponds to the number of interactions between the viruses and the drugs. Novel interactions between BSAs and viruses are shown in red and known - in grey.

Obatoclax was originally developed as an anticancer agent. Several Phase II clinical trials were completed that investigated use of obatoclax in the treatment of leukemia, lymphoma, myelofibrosis, and mastocytosis. In addition, its antiviral activity was reported against FluAV, ZIKV, WNV, YFV, SINV, JUNV, LASV, and LCMV in vitro [18-21]. It was shown that obatoclax inhibited viral endocytic uptake by targeting cellular Mcl-1 protein [18]. Given that obatoclax also inhibits RVFV, EV1, HMPV and HSV-2 it could be pursued as a potential BSA candidate.

Emetine is an anti-protozoal drug. It is also used to induce vomiting. In addition, it possesses antiviral effects against ZIKV, EBOV, RABV, CMV, HCoV-OC43 and HIV-1 [22-26]. It was proposed that emetine can directly inhibit viral polymerases, though it may have some other targets as well [27]. Given that emetine also inhibits FluAV, RVFV, EV1, HMPV and HSV-2, it may represent a promising BSA candidate.

Niclosamide is an orally bioavailable anthelmintic drug and potential antineoplastic agent. In addition, it inhibits the broadest range of viruses, including HSV-2, in vitro and, in some cases, *in vivo* [28-37]. It was shown that niclosamide induces endosomal neutralization and prevents virus entry into host cells. This supports further development of niclosamide as BSA agent.

Homoharringtonine is an anticancer drug which is indicated for treatment of chronic myeloid leukemia. It also possesses antiviral activities against HBV, MERS-CoV, HSV-1 and VZV [38-41]. Homoharringtonine binds to the 80S ribosome and inhibits viral protein synthesis by interfering with chain elongation [39]. Given that homoharringtonine also inhibits EV1, it may represent a promising BSA candidate.

Brequinar is an investigational anticancer agent (phase I/II). Brequinar attenuates replication of DENV, WNV, YFV, LASV, JUNV, LCMV, VSV, HIV-1, and POWV (NCT03760666) [19, 42]. It inhibits dihydroorotate dehydrogenase, thereby blocking de novo pyrimidine biosynthesis, which is essential

for transcription and replication of viral RNA. Given that brequinar also inhibits HIV-1, it may represent a promising BSA candidate.

Altogether, we expanded the spectrum of antiviral actions of niclosamide, brequinar, homoharringtonine, obatoclax and emetine. Importantly, PK and safety studies have been performed on these compounds in laboratory animals and humans. This information could be used to initiate efficacy studies *in vivo* and in clinics, saving time and resources of drug development process. The most effective and tolerable BSAs or their combinations will have a global impact improving the preparedness and protection of the general population from emerging and re-emerging viral threats, rapid management of drug-resistant strains, as well as for first-line treatment or for prophylaxis of viral co-infections.

5. Conclusions

Repurposing existing antiviral agents from one viral disease to another could have a pivotal role in the battle against emerging and re-emerging viral diseases. New activities of existing BSAs can be discovered in cell cultures, confirmed *in vivo*, and studied in clinical trials against other viruses. These studies may expand a spectrum of BSAs' indications and lead to novel options for treatment of viral disease.

Supplementary Materials: The following are available online. Table S1: Compounds, their suppliers and catalogue numbers, Table S2: Broad-spectrum antivirals used in the study, Table S3: Compound information, Table S3: Human viruses and associated diseases.

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