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Synergistic drug combination effectively blocks Ebola virus infection

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ABSTRACT

Although a group of FDA-approved drugs were previously identified with activity against Ebola virus (EBOV), most of them are not clinically useful because their human blood concentrations are not high enough to inhibit EBOV infection. We screened 795 unique three-drug combinations in an EBOV entry assay. Two sets of three-drug combinations, toremifene-mefloquine-posaconazole and toremifene-clarithromycin-posaconazole, were identified that effectively blocked EBOV entry and were further validated for inhibition of live EBOV infection. The individual drug concentrations in the combinations were reduced to clinically relevant levels. We identified mechanisms of action of these drugs: functional inhibitions of Niemann–Pick C1, acid sphingomyelinase, and lysosomal calcium release. Our findings identify the drug combinations with potential to treat EBOV infection.

1. Introduction

The EBOV entry pathway has been extensively studied (Jae and Brummelkamp, 2015; Moller-Tank and Maury, 2015), although a few components involved at the early and the late stages are still unknown. Macropinocytosis and endocytosis have been implicated in EBOV entry and intracellular trafficking. Involvements of cathepsin B/L (Chandran et al., 2005), Niemann Pick C1 protein (NPC1) (Carette et al., 2011; Cote et al., 2011), acid sphingomyelinase (ASM) (Miller et al., 2012), and two-pore channel (TPC) (Sakurai et al., 2015) in EBOV entry have been reported. Cathepsin B/L proteolytically hydrolyzes EBOV glycoprotein (GP) to trigger subsequent downstream infection events and cathepsin inhibition blocks EBOV replication. The hydrolyzed EBOV GP binds to NPC1 activating lysosome escape and virus genome injection to the cytoplasm where viral replication occurs. Fibroblasts derived from patients deficient in NPC1 and NPC1 knock-out mice showed resistance to EBOV infection (Herbert et al., 2015). More recently, tetrandrine, a small molecule inhibitor of TPCs, showed a moderate protection in the EBOV mouse model (Sakurai et al., 2015).

Our previous drug repurposing screen identified 53 FDA approved drugs that blocked Ebola virus-like particle (VLP) entry but the concentrations required for most of them were greater than the maximum blood concentrations of these drugs in human (Kouznetsova et al., 2014). In pharmacokinetic studies, the

Abbreviations: EBOV, Ebola virus; NPC1, Niemann Pick C1 protein; IC_{50}, half-maximum inhibitory concentrations.

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maximum drug concentration in human blood or plasma is the peak drug concentration detected after the drug is administrated in an *in vivo* experiment. Weak potency and insufficient plasma concentration are the common issues encountered for the active compounds found from drug repurposing screens (Sun et al., 2016). In this study, we report a targeted drug combination approach and identification of unique three-drug combinations that synergistically block EBOV infection. The reduced individual drug concentrations in the combinations are reachable in human plasma. Further, we validated their inhibitions of live virus infection in cells and identified their mechanisms of inhibition. These drug combinations may have a potential for the treatment of EBOV infection.

2. Materials and methods

2.1. Materials

Ebola VLPs containing a beta-lactamase-fused VP40 protein (EBOV BlaVP40) and GP were produced in Dr. García-Sastre's lab, as previously described (Tscherne et al., 2010). LiveBLAzer FRET−B/G Loading Kit with CCF2-AM were purchased from Life Technologies (Carlsbad, CA, USA); eGFP-EBOV was produced as previously described (Johansen et al., 2013). Constructs of RFP organelle marker were purchased from Thermo Fisher Scientific. An ATP content cell viability assay kit was purchased from Promega (Madison, WI, USA). Polystyrene plates (384-well and 1536-well black, clear bottom, sterile, tissue culture treated) were purchased from Greiner Bio-One (Monroe, NC, USA). The compounds were purchased from Sigma (St. Louis, MO, USA) at the highest available purity.

2.2. Ebola VLP beta-lactamase assays for combination HTS in 1536-well plate

The Ebola VLP assay was performed as described previously (Kouznetsova et al., 2014). Briefly, the first drug and the second drug were premixed and 0.8 μL of this mixture was then transferred into assay plate containing Hela cells; 23 nL of the third drug was transferred into the assay plate. The cells were treated with 1 μL/well VLP. The CCF2-AM beta-lactamase substrate was added and fluorescence intensities were measured using an Envision plate reader.

2.3. Ebola live virus assays

Vero E6 cells were plated in the 96-well plate (black with optical bottom). Briefly, serial dilutions of 7 drugs (diluted in DMEM 2% FBS starting at 46 μM) and combinations, and DMSO as control, were added to the wells, and incubated for 1 h at 37 °C with 5% CO_2. The cells were infected with EBOV/Mayinga-eGFP at a MOI of 0.1 TCID50/cell. The assay was run in triplicate. The fluorescence was read 72 h after infection using a BioTek Synergy HT.

2.4. NAADP stimulated calcium release

Fibroblasts (GM05659) were seeded on 96-well plates (3000 cells/well). The cells were loaded with Nuc. Blue live staining dye (Invitrogen) and incubated for 15 min. The cells were washed twice before loading with Cal-520-AM (AAT-Bioquest, CA) as previously described (Xu et al., 2012b). The cells were treated with each drug at 37 °C for 2 h. Once mounted on the microscope, the reaction of cells to NAADP-AM (10 μM) or vehicle DMSO was monitored by capturing images every 1 s for a total of 180 s. Cal-520 fluorescence was then measured per cell using INCell Analyzer 2200 and analyzed by INCell Analyzer workstation software.

2.5. Co-localization assay

U2OS cells were seeded on 96-well plates (3000 cells/100 μL/well). Medium was removed after 24 h. The cells were transfected with 0.5–1.5 μL/well of RFP-plasma membrane, RFP-early endosomes, RFP-late endosomes, and RFP-lysosomes and incubated for 24 h. The cells were washed with medium, treated with 50 μL drug for 1 h at 37 °C, infected with 50 μL eGFP-EBOV, and then incubated at 37 °C for 4 h. The medium was removed and the cells were stained with nuclear dye for 30 min. The fluorescence was then measured using IN Cell Analyzer 2200.

2.6. Cathepsin B/L assay

Cathepsin L (Novus Biologicals, Littleton, CO) was reconstituted with reaction buffer. 5 ng of cathpsin L was added into each well in 384-well plate. Indicated drugs were added into cathpsin L and premixed for 29 min. 200 μM cathepsin L substrate Ac-FR-AFC (Abcam) was added to initiate the reaction. Fluorescence was measured using Tecan Infinite M1000 Pro (Ex = 400 ± 20, Em = 505 ± 20). Cathepsin L inhibitor was used as a positive control. The cathepsin B (Abcam) was assayed in the same manner as cathepsin L, but with the cathepsin B substrate Ac-RR-AFC (Abcam) in the presence of cathepsin B inhibitor Z-FA-FMK.

2.7. Functional assays for NPC1 (Amplex-red), acid sphingomyelinase (ASM), filipin staining and lysotracker-red staining

Amplex-red cholesterol assay, filipin assay and lysotracker-red assay were performed as previously described (Xu et al., 2012b). ASM assay was performed previously described (Xu et al., 2012a).

2.8. Data analysis and statistics

The primary screen data and curve fitting were analyzed using software developed internally at the NIH Chemical Genomics Center (NCGC) (Wang et al., 2010). We constructed a heat map of triple drug combinations where rows enumerate drug 1, columns enumerate drugs 2, 3 and heat map elements are ratios defined as AC_{50} (drug 1 alone)/AC_{50} (drugs 1 + 2 + 3). The AC_{50} values were computed by first normalizing the channel 0 signal to positive control and then correcting for background effects. Half maximal inhibitory concentration (IC_{50}) values and 95% CI of compound confirmation data were calculated using Prism software (GraphPad Software, Inc. San Diego, CA). Results in the figures are expressed as a mean of triplicates ± SEM. Differences in anti-EBOV activity between 3-drug treatment and single drug treatments were evaluated using the student's *t*-test (two-tailed) to compare two groups and to calculate P values. In the figures, a p-value < 0.001 indicates a significant difference between experimental groups.

3. Results

3.1. Screening of synergistic drug combinations to reduce individual drug concentrations needed to inhibit EBOV infection

To overcome the weak activities of previously identified FDA-approved drugs against EBOV, we applied a targeted drug combination approach that selects individual drugs based on different mechanisms of action or therapeutic indications. We hypothesize that a combination therapy of three-drugs with different mechanisms of action will be able to block EBOV infection with lower individual drug concentrations due to the synergistic effect.

To increase the screening throughput for large numbers of