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Letter

Coadministration of ribavirin and arenaviral entry inhibitor LHF-535 enhances antiviral benefit against authentic lassa virus

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Dear Editor,

Lassa virus (LASV) is the causative agent of the acute viral hemorrhagic Lassa fever (LF), which is classified into *Mammarenavirus* within the *Arenaviridae* family, with a single-stranded, negative-sense, bi-segmented RNA genome. Due to its high pathogenicity and lethality, LASV is considered as a priority threat to public health, with an estimated cases of 300,000 infections and 5000 deaths annually. LASV was first isolated and described as a clinical entity in 1969 in Lassa, Nigeria (Garry, 2023). LASV isolates of different geographic and host origins are highly diverse in genomic sequences and phylogenetically classified into up to seven lineages, with each lineage predominately localized in specific countries. Although the research on LF has been carried out for decades since the pathogen first characterized, there is no approved antiviral drugs or vaccines for clinical use against LASV to date (Grant et al., 2023). One possible reason that hindered the development of countermeasures is that the preclinical studies on authentic LASV are restricted in high bio-containment biosafety level 4 (BSL-4) facilities. In this letter, we describe isolation, and characterization of the LASV from the clinical samples. And we applied a coadministration assay of antiviral drugs for LASV by using a clinically isolated *Mammarenavirus lassaense* strain in the BSL-4 facility, aiming to investigate new therapeutic strategies for LASV infection.

The LASV positive specimen (Ran et al., 2024; Li and Luo, 2025) was centrifuged at 15,000×*g* for 10 minutes in BSL-4 laboratory; the supernatant was then diluted 10 times in minimal essential medium and inoculated onto monolayers of African green monkey kidney (Vero E6) cells. The cells were cultured at 37 °C in 5 % carbon dioxide and observed daily to monitor for cytopathic effect.

As suggested by the results of IFAs, LASV was isolated and amplified after third passages on Vero E6 cells (Fig. 1A). Viral titers were quantified via plaque assay. The second-generation LASV isolate yielded 1.0×10^5 PFU/mL, while the third-generation isolate reached 1.0×10^6 PFU/mL. The TEM analysis showed that LASV is a particle with a diameter of approximately 100 nm although the precise structure of spikes was not obviously shown on the virus surface (Fig. 1B). The immature LASV particles without envelope were mainly located in cytoplasmic vesicles of Vero E6 cells (Fig. 1C). On the third passage a total number of non-redundant 9,444,636 reads were obtained by RNAseq from the third passage supernatant. The sequence comparison analysis showed that 153,402 reads were closely related to the LASV, which had never been reported in China. The full-length genomic sequence of a new LASV strain, which contains two single-stranded negative-sense RNA segments (L and S). The L segment is 7.2 kb in length, and the S segment is 3.4 kb in length. Phylogenetic analysis revealed that the L and S segments of this LASV isolate cluster with strains from lineage IV, indicating that this strain belongs to lineage IV (Supplementary Fig. S1). The complete genome sequence of the LASV strain (Lassa_HX strain) has been deposited in Science Data Bank under the CSTR identifier: 31253.11.sciencedb.22517 (https://www.scidb.cn/s/veQvMr).

Although approved antiviral agents against LASV infections are still lacking, the nucleoside analog ribavirin is currently the primary treatment available for LF (Eberhardt et al., 2019; Groger et al., 2023). As an inhibitor of viral RNA-dependent RNA polymerase (RdRp), ribavirin can inhibit viral genome replication and upregulate host IFN type I responses (Feld and Hoofnagle, 2005). Although commonly used, ribavirin is considered only effective when administered in the early course of disease and cause significant side effects in clinical trials (Cheng et al., 2022;

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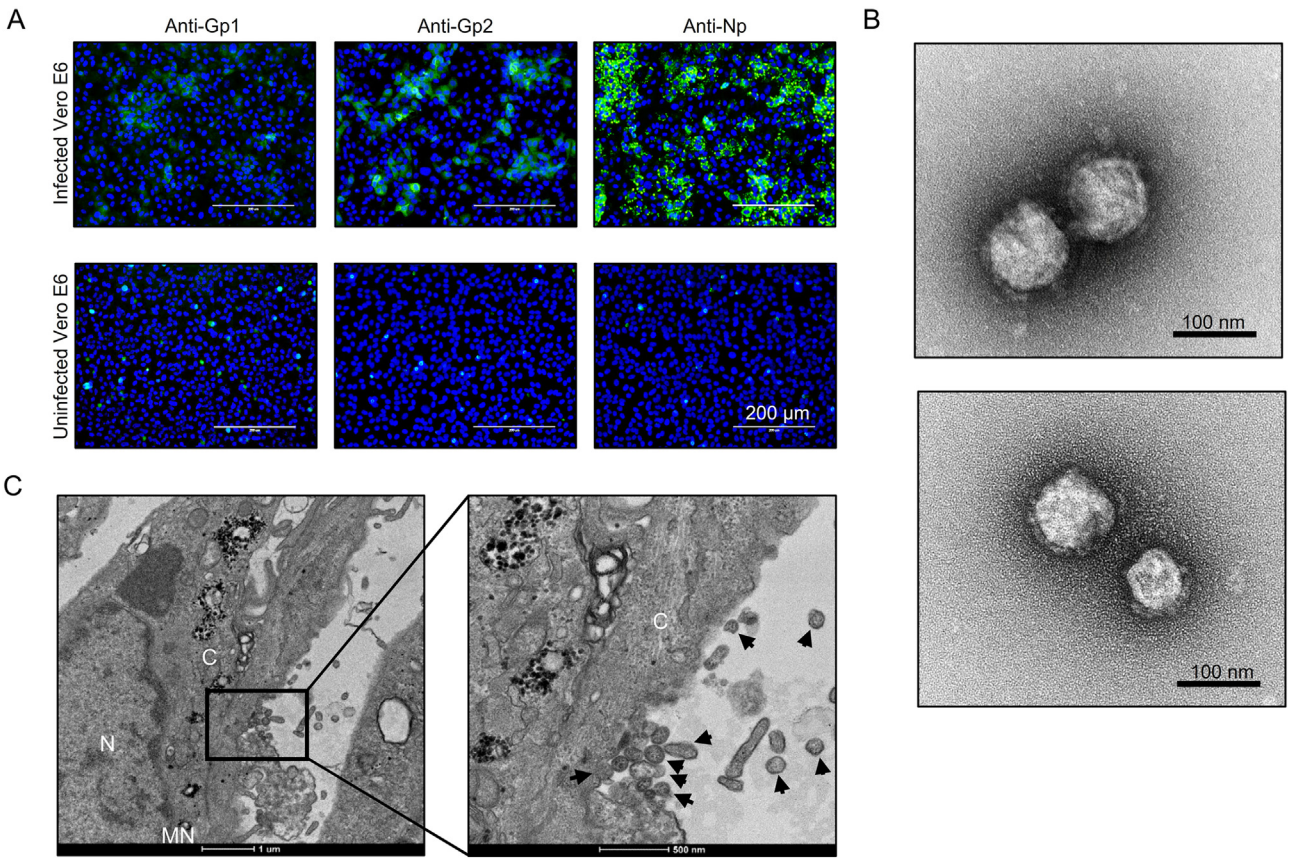

Fig. 1. Isolation, characterization, and analysis of lassa virus (LASV). **A** Immunofluorescence assays to identify LASV isolation in Vero E6 cells. Cells were immunestained and green fluorescence indicates the cells infected by LASV. **B** TEM analysis of LASV particles purified from supernatants harvested from Vero E6 cells infected with LASV at 4 days post-infection. Clarified supernatants were subjected to ultracentrifugation, and fractions containing viral particles were harvested for negative staining. **C** Viral particles in the ultrathin sections were imaged using electron microscopy at 100 kV. The TEM image revealing the localization of LASV particles within the cytoplasm and extracellular of infected Vero E6 cells. The enlarged area on the right highlights clusters of virus particles (indicated by arrows). Abbreviations: N, nucleus; C, cytoplasm; NM, nuclear membrane.

Grant et al., 2023). Interestingly, the entry step of LASV appears to be an effective target for the development of antiviral inhibitors. LHF-535, an analog of the viral entry inhibitor ST-193, which displays an inhibitory activity against the viral envelope glycoproteins from all LASV lineages (Madu et al., 2018), was perceived as a leading antiviral candidate for LF treatment (Amberg et al., 2022; Garry, 2023).

For the development of pharmacotherapy, the combination of drugs targeting different steps in viral life cycle may provide a promising treatment for LF. As Fig. 2 shows, the combined effect of antiviral reagents ribavirin and LHF-535 against LASV were evaluated *in vitro* by using the immunofluorescence assay. After infecting Vero E6 cells with LASV, the numbers of IFA-positive cells in the presence of antiviral reagents were compared with those in the vehicle control group at 48 hours post-infection. The results suggested that both ribavirin and LHF-535 inhibit LASV infection in Vero E6 cells (Fig. 2A). Moreover, the combination of ribavirin and LHF-535 showed a higher inhibitory effect compared to the use of either drug alone (Fig. 2A). According to the analysis by the MacSynergy II, the combinations of LHF-535 with ribavirin had significant synergistic antiviral effects with the volume of 377.57 nM^2%, which mechanistically reflects their complementary inhibition of viral entry and RNA-dependent RNA polymerase activity (Fig. 2B).

The combination of the RdRp inhibitor ribavirin and the viral entry inhibitor LHF-535 appears synergistic, with drug combination assay showing enhanced protection against LASV infection. With synergistic interactions, the combination of antiviral drugs may achieve stronger therapeutic effects. Our findings establish a relationship between viral RdRp and entry inhibitors, highlighting the clinical significance of ribavirin-based combinations in LASV infection treatment.

In a clinical case of pharmacotherapy, a combination of ribavirin and another RdRp inhibitor favipiravir was proved a survival improvement on patients with LF (Raabe et al., 2017), indicating effective antiviral inhibition on LASV replication. The coadministration of drugs targeting different processes in LASV lifecycle are seldom reported. The fusion inhibitor LHF-535 and the nucleoside analog favipiravir have shown better promise in animal models of LF than administered the drugs individually (Westover et al., 2024), evidencing the benefits of the drug combination. The data presented in this study expand the possible pharmaceutical compositions in preventing LASV infection, providing promising progress to address the need for new therapeutics of LF.

Even tremendous efforts have been made in antiviral drug development, the emergence of drug resistance in pathogenic virus hampers the effectiveness of antiviral agents and brings challenges to the treatment of infectious viral diseases. In LASV, generation of the resistance to entry inhibitors in mutants were also reported (Wang et al., 2018; Zhang et al., 2019; Liu et al., 2021). Upon LASV cell entry, the binding of LASV envelope glycoprotein complex (GPC) to the cell surface receptor α-dystroglycan (α-DG) is the major process mediates the entry of the virus. As reports showed, the alternation of single amino acids within GPC complex modulates sensitivity of LASV on entry inhibitors (Madu et al., 2018; Zhang et al., 2024), especially the amino acids in and near the predicted transmembrane domain of the GP2 subunit (Larson et al., 2008). Building on the data from our previous screen of adaptive mutants, the F446L mutation was proved to be a barrier to the antiviral activity of

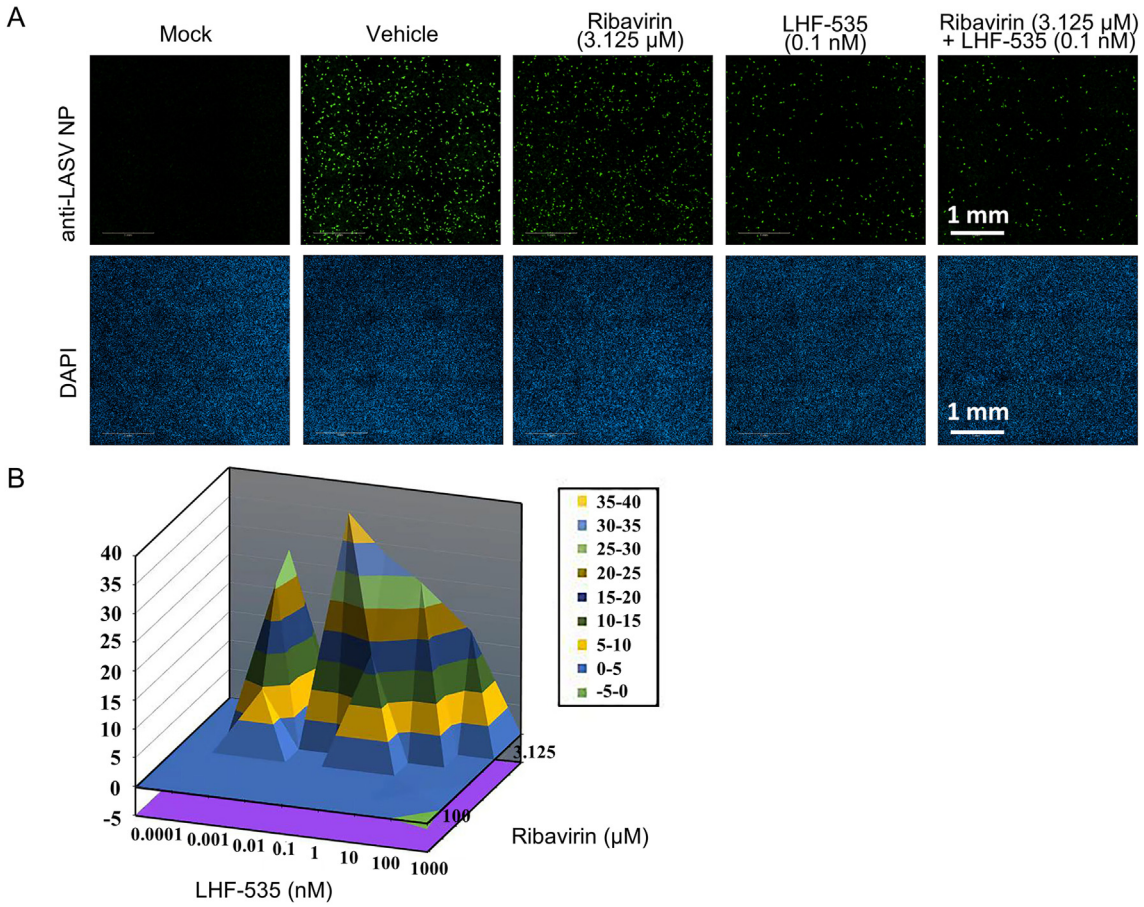

Fig. 2. Synergistic effect of ribavirin and LHF-535. **A** Effects of ribavirin and LHF-535 on inhibiting the LASV infection. Vero E6 cells were preincubated with compounds or vehicle at 37 °C for 1 h, followed by incubation with LASV (MOI, 0.175) at 4 °C for 1 h. Cells were stained with an anti-LASV NP antibody (green), and the nuclei were stained with DAPI (blue). Scale bars, 1 mm. **B** Differential surface plots at 95 % confidence level (CI) were calculated and generated using MacSynergy II for the drug-drug interactions for evaluating combinations of ribavirin with LHF-535 targeting LASV. MacSynergy II defines nM^2% values as follows: strong synergy: >100 nM^2%; slight synergy: 50–100 nM^2%; additive effect: −50 to 50 nM^2%; antagonism: <−50 nM^2%.

bergamottin and compound 57 (Zhang et al., 2019; Liu et al., 2021). Besides, the V434I mutation on GP2 attenuates the effect of LHF-535 on LASV inhibition, according to the divergent drug sensitivity of the LP strain carrying this mutant (Madu et al., 2018). In addition, the replacement of amino acid T40 located in the stable-signal peptide (SSP) subunit endowed LASV resistance to the entry inhibitor lacidipine (Wang et al., 2018). Moreover, ribavirin has recently been characterized as an RNA mutagen, inducing mutagenesis in intracellular viral genomes to inhibit LASV infection (Hu et al., 2024). In our results, the great inhibition of LASV on the application of drug combinations provides new therapeutic strategies to combat the pathogen, and provides several alternatives to prevent the emergence of antimicrobial resistance. In future studies, we would employ long-term serial passaging of LASV under escalating ribavirin/LHF-535 concentrations to systematically characterize resistance pathways and identify potential compensatory mutations.

In summary, we isolated a strain of LASV. Moreover, we described the viral characteristics of the isolation and evaluated the co-administration efficacy of nucleoside analog and entry inhibitor against the LASV infection, demonstrating enhanced antiviral activity.

FOOTNOTES

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