Identification of the dietary supplement capsaicin as an inhibitor of Lassa virus entry

Ke Tang\textsuperscript{a,b,\dagger}, Xiaoyu Zhang\textsuperscript{a,b,\dagger}, Ying Guo\textsuperscript{a,b,*}

\textsuperscript{a}State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China
\textsuperscript{b}Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

Received 9 January 2020; received in revised form 19 February 2020; accepted 25 February 2020

Abstract The limited treatment options for the increasing occurrence of Lassa hemorrhagic fever in West Africa poses an urgent need for the discovery and development of novel therapeutics. Dietary supplements, especially natural products that are edible and safe for human use, are a good source of drug discovery with potential for uncovering novel applications. In this study, we tested 40 natural products of dietary supplements and identified capsaicin, a common dietary supplement abundant in chili peppers, as an inhibitor of Lassa virus (LASV) entry with EC\textsubscript{50} of 6.9–10.0 \textmu mol/L using an HIV based pseudovirus platform. Capsaicin inhibits the entry of five LASV strains but not against the Old World arenavirus lymphocytic choriomeningitis virus (LCMV), showing a preferential activity against LASV. Capsaicin inhibits LASV entry by blocking the pH dependent viral fusion through affecting the stable signal peptide (SSP)-GP2 transmembrane (GP2\textsubscript{TM}) region of the LASV surface glycoprotein. Mutational study revealed the key residues Ala25, Val431, Phe434 and Val435 in SSP-GP2\textsubscript{TM} region in capsaicin’s antiviral effect. This study for the first time reveals a direct acting antiviral effect of capsaicin against the hemorrhagic fever causing LASV, providing detailed interaction hot spots in the unique SSP-GP2\textsubscript{TM} interface of LASV glycoprotein that is crucial in fusion inhibition, and offering a new strategy in discovering and developing antivirals from natural products that are safe for human use.

\textcopyright 2020 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

\textsuperscript{*}Corresponding author. Tel: +86 10 63161716.
E-mail address: yingguo6@imm.ac.cn (Ying Guo).
\textsuperscript{\dagger}These authors made equal contributions to this work.
Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2020.02.014
2211-3835 \textcopyright 2020 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
1. Introduction

Lassa virus (LASV) is an Old World arenavirus that causes Lassa fever, a severe and fatal viral hemorrhagic fever in human. Endemic in West Africa, LASV causes an estimated 100,000 to 300,000 infections and 5000 deaths each year. In the years of 2018 and 2019, Nigeria witnessed two unprecedented Lassa fever outbreaks, resulting in 633 and 810 confirmed cases with case fatality rates of 27% and 20.6%, respectively. According to World Health Organization (WHO)'s brochure on pandemic and epidemic diseases, LASV is listed as one of the top priority pathogens, and urgent research and development for diagnostic tests, antivirals and vaccines are underway.

LASV is an enveloped single negative-strand RNA virus and the surface glycoprotein complex (GPC) is responsible for the entry of LASV into host cells. LASV GPC is a trimer of three non-covalently associated subunits stable signal peptide (SSP), GP1 and GP2. The 58 amino-acid-long SSP is a twice membrane-spanning stable signal peptide that is crucial in GPC mediated fusion. GP1 binds to α-dystroglycan (α-DG), the cell surface receptor of LASV, resulting in the virus–cell surface attachment followed by endocytosis. The fusion of LASV takes place in endosome, where LASV GP1 switches from α-DG to lysosome-associated membrane protein 1 (LAMP1) on the inner endosomal membrane. GP2 is a class I fusion protein that drives viral-cell membrane fusion under high proton environment in the late endosomes. Previous studies have shown that LASV GP2 exists in a non-covalent interaction with SSP, and that this interaction is indispensable in LASV GPC mediated membrane fusion. Moreover, this interaction between GP2 and SSP is conserved among arenaviruses, providing a unique target for arenavirus fusion inhibition.

There is currently no approved antivirals or vaccines against LASV. Clinical therapeutic treatment is limited to an off-label use of the broad-spectrum antiviral ribavirin. Although a few small molecule LASV entry inhibitors have been reported, LHF-535 is the only LASV entry inhibitor that is currently under clinical investigation. Natural products have long been recognized as a valuable source of drug discovery, while the natural product in dietary supplement is one category among millions of natural products with the property of high safety besides of their structural diversity and biological significance. In this study, we constructed a library of 40 natural products from dietary supplements and evaluated their anti-LASV entry activities. As a result, capsaicin, a well-known substance naturally abundant in chili peppers was identified as an inhibitor of LASV entry. As a predominant capsaicinoid, capsaicin is known as an agonist of the transient receptor potential vanilloid subtype 1 (TRPV1), or the capsaicin receptor resulting in the 'hot' and burning sensations associated with chili peppers as spice. This bioactivity was also applied clinically as an analgesic for the treatment of peripheral neuropathic pain, such as painful diabetic peripheral neuropathy, HIV-related neuropathic pain and postherpetic neuralgia. This study for the first time disclosed capsaicin’s activity against LASV entry, and it is the first report for this famous natural product as a direct acting antiviral.

2. Materials and methods

2.1. Cells and plasmids

HEK293T, A549, Vero and U-87MG cells were obtained from the China Infrastructure of Cell Line Resource (Beijing, China). Vero E6 cells were obtained from the American Type Culture Collection (ATCC). U-87 MG cells were cultured in MEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 µg/mL streptomycin and 100 IU/mL penicillin. All other cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% FBS, 100 µg/mL streptomycin and 100 IU/mL penicillin. Cells were maintained in a humidified atmosphere containing 5% CO2 at 37 °C.

The codon-optimized GP genes of LASV lineage I strain LP, lineage II strain 803213, lineage III strain GA391 and lineage IV strain Josiah and LCMV strain Armstrong 53b were described previously. The codon optimized GP gene of LASV lineage V strain AV (GenBank Accession No. AF246121.2) was synthesized and inserted into eukaryotic expression vector byTSINGKE Biotech (Beijing, China). Plasmids encoding chimeric LASV-GPs [i.e., LASV-GP-(SSP-GP2 TM) LCMV, LASV-GP-(SSP Gyb LCMV, LASV-GP-(SSP TM1) LCMV, LASV-GP-(SSP Glu LCMV) and LASV-GP-(SSP TM2) LCMV] and LASV-GPs with single residue mutations were constructed as described previously. The env-deficient HIV core plasmid (pNL4-3.Luc.R’−) and vesicular stomatitis virus glycoprotein plasmid (VSV-G) were described previously.

2.2. Compounds

The natural products were selected from an assembled compound library from dietary supplements (Cat. No. L6000, purity>95%, TargetMol, Boston, MA, USA; Cat. No. L1400, purity>95%, Selleck Chemicals, Houston, TX, USA). The capsaicin standard (purity>98%) was purchased from TargetMol (Cat. No. T1062). ST-193, F3406-2010, efavirenz and bafilomycin A1 were described previously. Doxorubicin hydrochloride was purchased from TargetMol (Cat. No. T1020). All compounds were dissolved in dimethyl sulfoxide (DMSO, Cat. No. 34943, Sigma–Aldrich, St. Louis, MO, USA) and stored at −20 °C until use. DMSO (0.1%, v/v) was used as a solvent control in all experiments.

2.3. Cell viability assay

HEK293T and A549 cells were seeded in 96-well plates as 8 × 103 cells/well on the day before the assay. The cells were treated with the tested compound and incubated for 48 h. The cell viability was evaluated by using CellTitere-Glo® Luminescent Cell Viability Assay (Cat. No.G7571, Promega, Madison, WI, USA). Cells treated with 0.1% DMSO (v/v) served as the solvent control, which is the indicator of 100% cell viability. Cells with medium served as a negative control. Cells treated with doxorubicin served as the positive control. Cell viability (%) and selectivity index (SI) were calculated as Eqs. (1) and (2):

\[
\text{Cell viability} (\%) = \frac{\text{Relative luminescence units (RLUs) of compound/RLUs of solvent control}}{100}
\]
SI = Half maximal cytotoxic concentration (CC_{50})/Half maximal effective concentration (EC_{50}) (2)

2.4. Pseudoviruses production

The pseudotyped-LASVs (lineages I–V) were generated as previously described. Briefly, the plasmids encoding LASV-GP and the HIV vector (pNL4.3.Luc–R’E’) were cotransfected into HEK293T cells by using jetPRIME transfection reagent (Polyplus-transfection, New York, NY, USA). Forty-eight hours post-transfection, the LASV-GP/HIV-luc were collected and filtered through a 0.45 μm pore size filter (Millipore, Burlington, MA, USA). The pseudoviruses were layered onto 20% (w/v) sucrose and centrifuged at 30,000×g (L-100XP Ultracentrifuge, Beckman Coulter Inc., Brea, CA, USA) for 2 h at 4 °C. After that, the LASV-GP/HIV-luc were re-suspended in PBS, quantified by the HIV-1 p24 ELISA kit (Cat. No. KIT11695, Sino Biological Inc., Beijing, China) and stored at −80 °C until use. Pseudoviruses bearing the GP of LASV (strain AV) or chimeric LASV-GPs were generated under the same experimental condition.

2.5. Pseudovirus infection assay and compound detection

Pseudovirus infectivity was measured by the Luciferase Assay System (Cat. No. E1501, Promega) as described previously. A549 cells were seeded into 24-well plates at a density of 4×10^4 cells/well the day before infection. The cells were incubated with reference compound or tested compounds 15 min prior to pseudoviron infection. Forty-eight hours post-infection, the cells were lysed, and luciferase activity was measured by a luciferase assay kit (Promega). All of these experiments were performed in triplicates.

2.6. Time-of-addition assay

This assay was performed as described previously. Briefly, A549 cells were incubated with LASV pseudovirions at 4 °C for 2 h for the attachment. The supernatant containing the unattached virions was removed, and fresh medium was replenished. The cells were incubated with reference compound or tested compounds 15 min prior to pseudoviron infection. Forty-eight hours post-infection, the cells were lysed, and luciferase activity was measured by the luciferase assay kit. Capsaicin (30 μmol/L), ST-193 (1 μmol/L) and bafilomycin A1 (3 nmol/L) were incubated with cells during LASV-GP/HIV attachment, post-attachment or throughout the entire process. The assay was performed in triplicates.

2.7. Virus binding assay

Capsaicin (300 μmol/L) was incubated with LASV-GP (strain Josiah)/HIV-luc or LCMV-GP/HIV-luc at 4 °C for 4 h. The pseudovirions were then layered onto 20% (w/v) sucrose and centrifuged at 300,000×g (L-100XP Ultracentrifuge, Beckman) for 2 h. The supernatant was removed, and the pseudovirions were resuspended in PBS. The harvested pseudovirions were incubated with A549 cells for 48 h. The infected cells were lysed, and luciferase activity was measured by the Luciferase Assay System. The luciferase activity of the DMSO (0.1%, v/v) solvent control was used as the 100% infectivity indicator. ST-193 (0.1 μmol/L), F3406-2010 (10 μmol/L) and bafilomycin A1 (0.1 μmol/L) were used as the reference compounds. The assay was performed in triplicates.

2.8. Cell–cell fusion assay

The assay was performed as described previously. Briefly, HEK293T cells were co-transfected with plasmids expressing LASV-GP (or LCMV-GP) and the enhanced green fluorescent protein (EGFP). The transfected cells were seeded into 48-well plates at a density of 1×10^5 cells/well. Twenty-four hours later, the medium was removed, and the cells were incubated with PBS (pH 4.7) for 20 min. Then the PBS was removed, and the fresh medium was added into the wells. The cells were incubated at 37 °C for 4 h, and syncytium formation was observed under a fluorescence microscope (IX71, Olympus Corp., Shinjuku, Tokyo, Japan). Capsaicin (50 μmol/L), bafilomycin A1 (10 nmol/L), ST-193 (0.1 μmol/L) or F3406-2010 (10 μmol/L) were added to the cells 4 h before low pH treatment, during low pH treatment or during the whole process, i.e., before and during low pH treatment.

2.9. Construction of chimeric LASV-GP and LCMV-GP plasmids

The genes of chimeric LCMV-GP [LCMV-GP-(SSP-GP2TM)LASV] and chimeric LASV-GP [LASV-GP-(SSP)LASV] were created by overlapping PCR. The SSP (Met1 to Thr58) and GP2TM (Leu428 to Gly58) open reading frame were amplified by PCR and then were both used to replace the SSP (Met1 to Gly58) and GP2TM (Leu434 to Leu455) region of LCMV-GP to get LCMV-GP-(SSP-GP2TM)LASV. The LCMV-GP SSP (Met1 to Gly58) open reading frame were amplified by PCR and then were used to replace the SSP region (Met1 to Thr58) of LASV-GP to get LASV-GP-(SSP)LASV. The chimeric gene sequences were inserted into pCMV3 vector and identified by DNA sequencing.

2.10. Statistical analysis

The mean values, standard deviation (SD), EC_{50}, and CC_{50} values were calculated by GraphPad Prism software. The statistical analysis in this study was performed by using Student’s t-test. The asterisks represent significant differences: *P < 0.05, **P < 0.01, and ***P < 0.001.

3. Results

3.1. Capsaicin is identified as a LASV specific entry inhibitor

Dietary supplements of potential health benefits could be categorized into vitamins, minerals, proteins and amino acids, body-building supplements, essential fatty acids, natural products, probiotics, etc. Among these categories, natural products, often building supplements, essential fatty acids, natural products, body-building supplements, etc. are the source of biologically active compounds with wide structural diversity. In this study, we evaluated 40 natural products from an assembled library of labeled dietary supplements for their inhibitory activity against LASV-GP/HIV-luc (strain Josiah) infection (Fig. 1A and Supporting Information Table S1). These compounds were first tested for cytotoxicity on HEK293T cells by CellTiter-Glo assay at a final concentration of 10 μmol/L, and those
resulting in less than 10% reduction of cell viability were selected for subsequent activity evaluations. The compounds were tested by VSV-G/HIV-luc pseudovirus infection to rule out compounds with any effect on VSV glycoprotein or HIV-1 replication, and compounds with less than 10% inhibitory activity on VSV-G/HIV-luc infection then proceed for evaluation against LASV-GP/HIV-luc infection. Compounds resulting in less than 50% infectivity of LASV-GP/HIV-luc were considered effective against LASV entry. Capsaicin was identified from this process with a specific inhibitory effect against LASV-GP/HIV-luc with an EC₅₀ of 6.9 μmol/L (Fig. 1B and C). We tested the anti-LASV activity of capsaicin on A549, Vero E6, Vero and U-87MG cells to validate its anti-LASV activity, and our results show that the anti-LASV activity of capsaicin is not cell type specific (Supporting Information Table S2).

LASV belongs to Old World arenaviruses and is phylogenetically close to the lymphocytic choriomeningitis virus (LCMV, 35,36). We tested capsaicin against LCMV-GP/HIV-luc infection and found that capsaicin had no significant effect on LCMV infection (Fig. 1C and Table 1). The LASV species demonstrates a high genetic diversity, which could be further divided into 5 or 6 lineages, consistent to their geographic distribution. In this study, in addition to the LASV strain Josiah (lineage IV) we used in the primary activity evaluation, we selected another four strains of LASV that are the genetic representative of each of their LASV lineages (Supporting Information Figs. S2 and S3), and tested capsaicin activity against the entry of these strains of LASV pseudotypes. Capsaicin showed a comparable activity towards 5 LASV strains with EC₅₀s of 6.5–15.9 μmol/L (Table 1 and Supporting Information Fig. S4).

Taken together, capsaicin specifically inhibit LASV rather than LCMV among Old World arenaviruses, while displaying a

### Table 1  
Effects of capsaicin on LASV-GP (lineages I–V) pseudotyped virus entry on A549 cells.

| Pseudovirus          | Capsaicin       | ST-193     | F3406-2010   |
|----------------------|-----------------|------------|-------------|
|                      | EC₅₀ (μmol/L)   | 95% CI     | EC₅₀ (μmol/L) | 95% CI     | EC₅₀ (μmol/L) | 95% CI     |
| LASV-GP/HIV-luc      |                 |            |             |
| Lineage I (strain LP)| 15.4            | 10.9–21.8  | 0.013       | 0.0048–0.037 | >10       | /           |
| Lineage II (strain 803213) | 15.9            | 10.0–25.1  | 0.0013      | 0.00058–0.0027 | >10       | /           |
| Lineage III (strain GA391) | 10.8            | 7.4–15.8   | 0.0013      | 0.00076–0.0022 | >10       | /           |
| Lineage IV (strain Josiah) | 8.4             | 6.9–10.2   | 0.0024      | 0.0016–0.0037 | >10       | /           |
| Lineage V (strain AV) | 6.5             | 5.4–7.8    | 0.00082     | 0.00055–0.0012 | >10       | /           |
| LCMV-GP/HIV-luc (strain Armstrong 53b) | >30             | /          | >10         | /          | 0.15       | 0.14–0.16   |

95% CI, 95% confidence intervals.
/ , not applicable.
Comparative effect on various strains within the LASV species, indicating that capsaicin is a specific inhibitor of LASV entry.

### 3.2. Capsaicin blocks LASV entry by inhibiting LASV-GP mediated fusion

Many studies have delineated the stepwise fashion of LASV entry into host cells. LASV attaches to the cell surface through interaction between virus GP1 and cellular receptor α-DG39,40. Following endocytosis and endosomal acidification, LASV GP1 detaches from α-DG and the GPC to bind LAMP18, meanwhile LASV GP2 undergoes conformational changes and triggers viral-endosomal membrane fusion9, releasing virus genome into the cytoplasm. In this study, we first identify the step in which capsaicin exerts its effect by a time-of-addition assay 32,33.

Capsaicin as well as reference compounds bafilomycin A1 and ST-193 were incubated with A549 cells as depicted (Fig. 2). As shown in Fig. 2A, capsaicin is effective in both the attachment and the post-attachment phase, displaying a similar inhibitory pattern to ST-193, a known arenavirus fusion inhibitor that targets GPC18, while the proton pump inhibitor bafilomycin A1 is only effective post-attachment, suggesting that capsaicin may interact directly with the LASV pseudovirions and that it may take effect at the viral fusion step.

We next used the established cell–cell fusion assay32,33 to confirm the effect of capsaicin on GPC mediated fusion. Capsaicin

![Figure 2](image)

**Figure 2** Capsaicin specifically inhibited low pH triggered LASV-GP mediated cell–cell fusion by binding directly to the virions. (A) Capsaicin affects the attachment and post-attachment, but not pre-attachment process of LASV entry. A549 cells were incubated with the test compounds or the same amount DMSO for 2 h at 37 °C (pre-attachment treatment) and then the supernatant was washed out. The LASV pseudovirions were added and incubated with the cells at 4 °C for 2 h (attachment). The supernatant which containing the unattached virions was removed and fresh medium was replenished. The cells were incubated at 37 °C for 48 h (post-attachment). The cells were lysed, and luciferase activity was measured by the luciferase assay kit. Capsaicin (30 μmol/L), ST-193 (1 μmol/L), bafilomycin A1 (3 nmol/L), or the same amount of DMSO was treated at pre-attachment, during the attachment, post-attachment or attachment + post-attachment. (B) and (C) Capsaicin inhibited low pH-triggered LASV-GP or LCMV-GP mediated cell–cell fusion. HEK293T cells were co-transfected with plasmids expressing LASV-GP (or LCMV-GP) and EGFP. The transfected cells were seeded into 48-well plates. Twenty-four hours later, the medium was removed, and the cells were incubated with PBS (pH 4.7) for 20 min. Then the low pH PBS was replaced with fresh medium and incubated for 4 h. Syncytium formation of the cells was observed via fluorescence microscopy. Capsaicin (50 μmol/L), bafilomycin A1 (10 nmol/L), ST-193 (0.1 μmol/L) or F3406-2010 (10 μmol/L) were added to the cells 4h before the low pH treatment, during low pH treatment or before + during low pH treatment. Scale bar, 100 μm. (D) Capsaicin blocks LASV entry by binding directly to LASV-GP/HIV-luc virions. LASV-GP/HIV-luc or LCMV-GP/HIV-luc was incubated with Capsaicin (300 μmol/L) at 4 °C for 4 h and then the supernatant was removed by ultracentrifugation. The pseudovirions were resuspended and used to infect A549 cells. Forty-eight hours post-infection, the cells were lysed, and luciferase activity was measured. The same amount of DMSO was used as the solvent control; ST-193 (0.1 μmol/L), F3406-2010 (10 μmol/L) and bafilomycin A1 (0.1 μmol/L) were used as the reference compounds. The luciferase activity of the solvent control was used as the 100% infectivity indicator. The data are represented as the mean ± SD (n = 3). Statistical significances between treatment group and DMSO group were calculated by Student’s t-test using GraphPad Prism software, with asterisks representing significant differences: *P < 0.05, **P < 0.01, and ***P < 0.001.
as well as reference compounds ST-193, F3406-2010, and bafilomycin A1 were added to cells expressing GPC and EGFP at specific time of low pH triggering as indicated in Fig. 2B and C, and cell—cell fusion was observed 4 h after low pH treatment as indicated by green fluorescence. The results showed that capsaicin inhibited LASV-GP mediated fusion, but it did not affect LCMV-GP mediated fusion, suggesting that capsaicin specifically targets the LASV fusion process.

A virus binding assay was also employed to explore whether there is an interaction between capsaicin and LASV virions. Capsaicin, along with reference compounds ST-193, F3406-2010 and bafilomycin A1 were incubated with LASV-GP/HIV-luc or LCMV-GP/HIV-luc. The compound-treated pseudoviruses were collected through ultracentrifugation to exclude un-bounded compound as well as supernatants. The harvested viruses were then used to infect A549 cells. The results showed that LASV-GP/HIV-luc treated with capsaicin had a low infectivity, similar to that of ST-193, indicating that capsaicin interacts directly with LASV-GP (Fig. 2D).

### 3.3. Capsaicin targets the SSP-GP2 interface of LASV glycoprotein

The arenavirus stable signal peptide (SSP) is a unique functional glycoprotein that is essential to the fusion process. Compounds that interact with this complex directly will lead to a failure of the viral fusion. In this study, we investigated whether the specific inhibition of LASV-GP mediated fusion by capsaicin was associated with this interaction between SSP and GP2. To identify the region with which capsaicin interacted, we established two chimeric constructs where one is LASV-GP with its SSP and GP2 substituted by LCMV SSP and GP2, termed LASV-GP-(SSP-GP2TM)LCMV, and another is LCMV-GP with its SSP and GP2 substituted by the respective LASV SSP and GP2, termed LCMV-GP-(SSP-GP2TM)LASV (Table 2 and Fig. 3A). We tested the activity of capsaicin against the above chimeric GPC packed pseudoviruses, along with reference compounds ST-193 and F3406-2010. As shown in Table 2 and Supporting Information Fig. S5, the LASV fusion inhibitor ST-193 lost its activity against LASV-GP-(SSP-GP2TM)LCMV/HIV-luc while the LCMV specific inhibitor F3406-2010 was effective against LASV-GP-(SSP-GP2TM)LCMV/HIV-luc, which are consistent to their mode of action. Capsaicin showed a comparable activity against LCMV-GP-(SSP-GP2TM)LASV/HIV-luc and LCMV wild type (strain Josiah LCMV pseudotype), while it completely lost its activity against LASV-GP-(SSP-GP2TM)LASV/HIV-luc, indicating that capsaicin indeed targets the LASV SSP-GP2TM interface of GPC.

### 3.4. The N-terminal transmembrane region of SSP is a crucial region for the anti-LASV activity of capsaicin

SSP is a 58-residue long signal peptide that spans the membrane twice, with two cytoplasmic regions, two hydrophobic putative transmembrane regions and one ectodomain. To further evaluate the specific regions in SSP-GP2TM and determine how they affect capsaicin activity, we divided LASV SSP into four segments (Table 3 and Fig. 3B) and replaced each segment with its LCMV SSP counterpart. Capsaicin was tested against infections of pseudoviruses bearing these chimeric LASV-GPs (Table 3 and Supporting Information Fig. S6) with ST-193 and F3406-2010 as references. Capsaicin showed a similar inhibitory activity on LASV-GP-(SSP)CMV/HCVM/HIV-luc, LASV-GP-(SSP)CMV/HCVM/HIV-luc and LASV-GP-(SSP)CMV/HIV-luc compared with wild type (strain Josiah LCMV pseudotype), while it lost its activity on LASV-GP-(SSP)CMV/HIV-luc as well as LCMV-GP-(SSP)CMV/HIV-luc (Table 3), indicating that SSPTM1 is a key module in the anti-LASV activity of capsaicin.

Amino acid sequence alignment in the SSPTM1 region showed eight distinct residues between LASV and LCMV (Table 4 and Fig. 3C). To identify which residues affect the anti-LASV activity of capsaicin, we generated eight LASV-GP constructs, each bearing one of these single residues mutated to its LCMV counterpart (Supporting Information Fig. S7). We tested the activity of capsaicin against these LASV pseudoviruses with single residue mutations in SSPTM1 and found that capsaicin completely lost its activity on A25V mutant virus, and a slightly activity loss on S27I mutant virus with 2-fold EC50 increase, while displaying comparable activity on other single residue mutants (Table 4).

Taken together, these results indicated that residue Ala25 in SSPTM1 is associated with the anti-LASV activity of capsaicin, and implied an interaction between the SSPTM1 and GP2 possibly mediated by the residue.

### 3.5. Val431, Phe434 and Val435 in the transmembrane region of GP2 involve in capsaicin activity

SSP is known to interact non-covalently with the fusion subunit GP2, participates in GPC mediated fusion and functions by stabilizing GP2 structure. After identifying SSPTM1 as an important region in the SSP-GP2 interface, we next sought to identify residues in the respective GP2TM region that might associate with capsaicin activity. We inspected the amino acid sequences of LASV and LCMV in the GP2TM region and they revealed 6 distinct residues (Table 5 and Fig. 3D). We established 6 LASV pseudoviruses bearing these mutant LASV-GPs (Fig. S7), and tested capsaicin activity against their infection (Table 5). The

---

**Table 2** Effects of capsaicin on LASV-GP-(SSP-GP2TM)LCMV and LCMV-GP-(SSP-GP2TM)LASV mediated viral entry on A549 cells.

| Pseudovirus                  | Capsaicin | ST-193 | F3406-2010 |
|-----------------------------|-----------|--------|------------|
|                             | EC50 (μmol/L) | 95% CI (μmol/L) | EC50 (μmol/L) | 95% CI (μmol/L) | EC50 (μmol/L) | 95% CI (μmol/L) |
| LASV-GP/HIV-luc             | 10.0      | 8.0−12.4 | 0.0024     | 0.0014−0.0043 | >10        | /         |
| LCMV-GP/HIV-luc             | >30       | /       | >10        | /            | 0.23       | 0.19−0.28 |
| LASV-GP-(SSP-GP2TM)LCMV/HIV-luc | >30       | /       | >10        | /            | 0.44       | 0.39−0.49 |
| LCMV-GP-(SSP-GP2TM)LASV/HIV-luc | 6.1       | 5.1−7.4 | 0.00089    | 0.00073−0.0011 | >10        | /        |

95% CI, 95% confidence intervals. /, not applicable.
results showed that capsaicin lost its activity against LASV pseudotypes bearing V431M, F434L or V435M mutation, indicating that Val431, Phe434, and Val435 in GP2TM of LASV are activity determinants of capsaicin, and might play important roles in SSP-GP2 interaction. The reference compounds ST-193 and F3406-2010 displayed a loss of activity consistent with previous reports.

4. Discussion

In this study, we discovered capsaicin as a LASV entry inhibitor that functions by blocking the LASV-GP mediated viral fusion (Fig. 2B and C) and binding directly to the LASV pseudovirions (Fig. 2D). Further mechanism study showed that it targets the SSP-GP2 interface, a crucial functioning unit of LASV GPC mediated fusion (Table 2). Site-directed mutagenesis study revealed single residues in the SSP and GP2TM that affect anti-LASV activity of capsaicin (Tables 3–5). These results implied a possible non-covalent interaction between residues Ala25 in SSP and Val431, Phe434, and Val435 in GP2TM, and that a hydrophobic pocket may be formed. The two reference compounds we used in the mutational study, ST-193 and F3406-2010, showed a consistent pattern of activity variation with previous studies as in mutations V431M and V435M, meanwhile displayed new sensitivities to mutations such as L23I and F434L. These results

Table 3 Activities of capsaicin against LASV-GPs with fragment substitution in SSP mediated viral entry on A549 cells.

| Pseudovirus                  | Capsaicin | ST-193 | F3406-2010 |
|-----------------------------|-----------|--------|------------|
|                             | EC50 (µmol/L) 95% CI (µmol/L) | EC50 (µmol/L) 95% CI (µmol/L) | EC50 (µmol/L) 95% CI (µmol/L) |
| LASV-GP/HIV-luc             | 9.3       | 7.6–11.5 | 0.0024       | 0.0016–0.0037 | >10 | / |
| LASV-GP-(SSP)LCMV/HIV-luc   | >30       | /       | 0.0029       | 0.0021–0.0040 | 0.16 | 0.13–0.19 |
| LASV-GP-(SSP)LCMV/HIV-luc   | 8.3       | 6.4–10.7 | 0.0021       | 0.0012–0.0038 | 5.2 | 3.1–8.6 |
| LASV-GP-(SSP)LCMV/HIV-luc   | >30       | /       | 0.19         | 0.089–0.41 | 1.1 | 0.47–2.5 |
| LASV-GP-(SSP)LCMV/HIV-luc   | 16.9      | 11.8–24.3 | 0.0057         | 0.0038–0.0085 | >10 | / |
| LASV-GP-(SSP)LCMV/HIV-luc   | 17.3      | 11.3–26.4 | 0.0054         | 0.0031–0.0093 | >10 | / |

95% CI, 95% confidence intervals. /, not applicable.

Figure 3 Fragment replacements and residue substitutions between LASV-GP (orange) and LCMV-GP (blue). (A) The domains of LASV-GP, LCMV-GP, LASV-GP-(SSP-GP2TM)LCMV, and LCMV-GP-(SSP-GP2TM)LASV are showed in diagram. (B)–(D) Amino acid sequence alignment of SSP (B), SSP_TM1 (C) and GP2_TM region (D) between LASV-GP (strain Josiah) and LCMV-GP (strain Arm53b) along with their composition cartoons. The role of the replaced fragments and the residue substitutions which marked with black triangles is investigated in this study.
There has been a report on the anti-herpes simplex virus activity of sensitive residues in the GP2TM, and identified two residues, Ala25 in validated Val431 and Val435, two previously reported drug sen-

timentary therapeutics 45,46, pruritus 47, and cannabinoid hyper-
dietary preservative for capsaicin’s bactericidal properties 49.

mains to be clarified. Historically peppers have been used as a effect of capsaicin on these non-neuropathic syndromes re-

den, and is used widely as a spice in the world. It also has a long chile peppers, which is one of the most popular and historical effect and application. Capsaicin is the major active component of antiviral, which is notably distinct from its traditional biological indications such as obesity 43, osteoarthritis44, cancer comple-

mentation of TRPV128, a non-selective cation channel on neuron cells. Capsaicin was also investigated for a wide range of pharmacology revealed that the traditional medical usage of gastrointestinal diseases in China since the 15th century. Modern history in medical usage, as it was recorded as a medicine for

arenavirus fusion inhibitors design or discovery based on the de novo drug design: compounds coming from natural products of developing therapeutic treatments from natural products over de novo drug design: compounds coming from natural products over

desensitization of TRPV1 11, a non-selective cation channel on neuron cells. Capsaicin was also investigated for a wide range of indications such as obesity12, osteoarthritis13, cancer comple-

mentary therapeutics14, pruritus15, and cannabinoid hyper-

e syndrome (CHS)16; however, the underlying mechanism of the effect of capsaicin on these non-neuropathic syndromes re-

mains to be clarified. Historically peppers have been used as a dietary preservative for capsaicin’s bactericidal properties17. There has been a report on the anti-herpes simplex virus activity of Capsicum annuum extract18; however, the anti-infective mechanism has been unclear. To the best of our knowledge, our study is the first report of the single molecular entity capsaicin as a direct acting antiviral.

The discovery of capsaicin as an antiviral agent not only offered a novel category of structural backbone in targeting LASV glycoprotein, but also disclosed a new potential medical usage for this historically used natural product and commonly known dietary supplement. There are two prominent advantages of developing therapeutic treatments from natural products over

de novo design: compounds coming from natural products over de novo drug design: compounds coming from natural products over

Table 4 Activities of capsaicin against LASV-GP-SSP TM mutation mediated viral entry on A549 cells.

| Pseudovirus            | Capsaicin  | ST-193   | F3406-2010 |
|------------------------|------------|----------|------------|
|                        | EC50 (µmol/L) | 95% CI (µmol/L) | EC50 (µmol/L) | 95% CI (µmol/L) | EC50 (µmol/L) | 95% CI (µmol/L) |
| LASV-GP/HIV-luc        | 9.3        | 6.4–13.6 | 0.0044     | 0.0029–0.0067   | >10         | /          |
| LASV-GP/AIV/HIV-luc    | 8.2        | 5.8–11.5 | 0.00040    | 0.00020–0.00078 | 3.7         | 3.2–4.2    |
| LASV-GP/TPA/HIV-luc    | 14.3       | 10.5–19.5| 0.0096     | 0.0048–0.019    | 0.47        | 0.29–0.77  |
| LASV-GP/TPA/IV/HIV-luc | >30        | /        | 0.0022     | 0.0011–0.0044   | 9.1         | 6.8–12.0   |
| LASV-GP/TPA/IV/HIV-luc | 21.0       | 13.7–32.1| 0.090      | 0.032–0.25      | 9.5         | 5.5–16.3   |
| LASV-GP/TPA/IV/HIV-luc | 8.5        | 5.4–13.5 | 0.067      | 0.050–0.15      | 7.4         | 5.7–9.7    |
| LASV-GP/TPA/IV/HIV-luc | 8.3        | 6.2–11.2 | 0.022      | 0.014–0.034     | 8.5         | 3.8–18.7   |
| LASV-GP/TPA/IV/HIV-luc | 9.7        | 7.0–13.3 | 0.051      | 0.028–0.096     | 7.1         | 5.9–8.4    |
| LASV-GP/TPA/IV/HIV-luc | 11.4       | 7.6–17.1 | 0.032      | 0.019–0.052     | >10         | /          |

95% CI, 95% confidence intervals.
/ , not applicable.

Table 5 Effects of capsaicin on LASV-GP-GP 2 TM mutation mediated viral entry on A549 cells.

| Pseudovirus            | Capsaicin  | ST-193   | F3406-2010 |
|------------------------|------------|----------|------------|
|                        | EC50 (µmol/L) | 95% CI (µmol/L) | EC50 (µmol/L) | 95% CI (µmol/L) | EC50 (µmol/L) | 95% CI (µmol/L) |
| LASV-GP/HIV-luc        | 8.3        | 6.9–10.0 | 0.0096     | 0.0051–0.018   | >10         | /          |
| LASV-GP/AIV/HIV-luc    | 11.0       | 6.9–17.5 | 0.0076     | 0.0051–0.011   | >10         | /          |
| LASV-GP/TPA/IV/HIV-luc | >30        | /        | 0.73       | 0.52–1.0       | 1.5         | 1.2–1.9    |
| LASV-GP/TPA/IV/HIV-luc | >30        | /        | 0.014      | 0.0060–0.031   | >10         | /          |
| LASV-GP/TPA/IV/HIV-luc | >30        | /        | 1.6        | 0.91–2.7       | >10         | /          |
| LASV-GP/TPA/IV/HIV-luc | 14.0       | 10.0–19.7| 0.0093     | 0.0032–0.027   | >10         | /          |
| LASV-GP/TPA/IV/HIV-luc | 16.6       | 9.2–29.9 | 0.0095     | 0.0044–0.021   | >10         | /          |

95% CI, 95% confidence intervals.
/ , not applicable.
Taken together, this study identified capsaicin as a LASV fusion inhibitor, providing a lead compound derived from natural dietary supplements with a distinct chemical scaffold that targets LASV glycoprotein. This study also highlighted the role of SSP1_TM1 in the interaction between SSP and GP2, and identified important residues in both SSP1_TM1 and GP2_TM1 that affect antiviral activity, providing framework for the discovery and design of arenavirus fusion inhibitors based on the SSP—GP2 interface of mammarenavirus glycoprotein.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (Nos. 81473256 and 81273561), the CAMS Innovation Fund for Medical Sciences (No. 2016-12M-014, China), the Science and Technology Program of Beijing (No. Z151100000115008, China), the Beijing Key Laboratory of New Drug Mechanisms and Pharmacological Evaluation Study (No. BZ0150, China), and the Drug Innovation Major Project (Nos. 2015ZX09102-023 and 2018ZX09711001-003-002, China), the Disciplines Construction Project (No. 201920200802, China).

Author contributions

Ying Guo conceptualized and supervised this study. Ke Tang designed the experiments. Ke Tang and Xiaoyu Zhang carried out the experiments, performed data analysis, and drafted the manuscript. All authors revised the manuscript, and have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interests.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.aphs.2020.02.014.

References

1. Bhadelia N. Understanding Lassa fever. Science 2019;363:30.
2. Richmond JK, Baglole DJ. Lassa fever: epidemiology, clinical features, and social consequences. BMJ 2003;327:1271–5.
3. Nigeria Centre for Disease Control. 2018 Lassa fever outbreak situation report [EB/OL]. Available from: http://ncdc.gov.ng/diseases/sitreps; 31 December 2018.
4. Nigeria Centre for Disease Control. 2019 Lassa fever outbreak situation report [EB/OL]. Available from: http://ncdc.gov.ng/diseases/sitreps; 15 December 2019.
5. Hastie KM, Saphire EO. Lassa virus glycoprotein: stopping a moving target. Curr Opin Virol 2018;31:52–8.
6. Messina EL, York J, Nunberg JH. Dissection of the role of the stable signal peptide of the arenavirus envelope glycoprotein in membrane fusion. J Virol 2012;86:6138–45.
7. Kanz S, Rojek JM, Perez M, Spiropoulou CF, Oldstone MB. Characterization of the interaction of lassa fever virus with its cellular receptor alpha-dystroglycan. J Virol 2005;79:5970–87.
8. Jae LT, Raaben M, Herbert AS, Kuehne AI, Wirchunianski AS, Soh TK, et al. Virus entry. Lassa virus entry requires a trigger-induced receptor switch. Science 2014;344:1506–10.
9. White JM, Whitaker GR. Fusion of enveloped viruses in endosomes. Traffic 2016;17:593–614.

10. York J, Romanowski V, Lu M, Nunberg JH. The signal peptide of the Junin arenavirus envelope glycoprotein is myristoylated and forms an essential subunit of the mature G1–G2 complex. J Virol 2004;78:10783–92.
11. Saunders AA, Ting JP, Meisner J, Neuman BW, Perez M, de la Torre JC, et al. Mapping the landscape of the lymphocytic choriomeningitis virus stable signal peptide reveals novel functional domains. J Virol 2007;81:5649–57.
12. York J, Nunberg JH. Distinct requirements for signal peptide processing and function in the stable signal peptide subunit of the Junin virus envelope glycoprotein. Virology 2007;359:72–81.
13. Bederka LH, Bonhomme CJ, Ling EL, Buchmeier MJ. Arenavirus stable signal peptide is the keystone subunit for glycoprotein complex organization. mBio 2014;5:e02063.
14. York J, Nunberg JH. Intersubunit interactions modulate pH-induced activation of membrane fusion by the Junin virus envelope glycoprotein GPC. J Virol 2009;83:4121–6.
15. York J, Nunberg JH. Myristoylation of the arenavirus envelope glycoprotein stable signal peptide is critical for membrane fusion but dispensable for virion morphogenesis. J Virol 2016;90:8341–50.
16. Shankar S, Whity LR, Casquiello-Gray HE, York J, Boger DL, Nunberg JH. Small-molecule fusion inhibitors bind the pH-sensing stable signal peptide—GP2 subunit interface of the Lassa virus envelope glycoprotein. J Virol 2016;90:6799–807.
17. Houlihan C, Behrens R. Lassa fever. BMJ 2017;358:j2986.
18. Larson RA, Dui D, Hosack VT, Tan Y, Bolken TC, Hruby DE, et al. Identification of a broad-spectrum arenavirus entry inhibitor. J Virol 2008;82:10768–75.
19. Lee AM, Rojek JM, Spiropoulou CF, Gundersen AT, Jin W, Shaginian A, et al. Unique small molecule entry inhibitors of hemorrhagic fever arenaviruses. J Biol Chem 2008;283:18734–42.
20. Spence JS, Melnick LI, Badani H, Wimley WC, Garry RF. Inhibition of arenavirus infection by a glycoprotein-derived peptide with a novel mechanism. J Virol 2014;88:8556–64.
21. Shrivastava-Ranjpan P, Bergeron E, Chakrabarti AK, Albarino CG, Flint M, Nichol ST, et al. 25-Hydroxycholesterol inhibition of Lassa virus infection through aberrant GPI glycosylation. BioMed 2016;7:e01808–16.
22. Wang MK, Ren T, Liu H, Lim SY, Lee K, Honko A, et al. Critical role for cholesterol in Lassa fever virus entry identified by a novel small molecule inhibitor targeting the viral receptor LAMP1. PLoS Pathog 2018;14:e1007322.
23. Wang P, Liu Y, Zhang G, Wang S, Guo J, Cao J, et al. Screening and identification of Lassa virus entry inhibitors from an FDA-approved drug library. J Virol 2018;92:e00548-18.
24. Zhang G, Cao J, Cai Y, Liu Y, Li Y, Wang P, et al. Structure—activity relationship optimization for lassa virus fusion inhibitors targeting the transmembrane domain of GP2. Protein Cell 2019;10:137–42.
25. Madu IG, Files M, Gharaibeh DN, Moore AL, Jung KH, Gowen BB, et al. A potent Lassa virus antiviral targets an arenavirus virulence determinant. PLoS Pathog 2018;14:e1007439.
26. Rautiainen S, Manson JE, Lichtenstein AH, Sesso HD. Dietary supplements and disease prevention—a global overview. Nat Rev Endocrinol 2016;12:407–20.
27. Abdel-Salam OM, Szolcsanyi J, Mozskik G. Capsaicin and the stomach. A review of experimental and clinical data. J Physiol Paris 1997;91:151–71.
28. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 1997;389:816–24.
29. Blair HA. Capsaicin 8% dermal patch: a review in peripheral neuropathic pain. Drugs 2018;78:1489–500.
30. Simpson DM, Estanislao L, Brown SJ, Sampson J. An open-label pilot study of high-concentration capsaicin patch in painful HIV neuropathy. J Pain Symptom Manag 2008;35:299–306.
31. Yong YL, Tan LT, Ming LC, Chan KG, Lee LH, Goh BH, et al. The Effectiveness and safety of topical capsaicin in postherpetic neuralgia: a systematic review and meta-analysis. Front Pharmacol 2016;7:538.
32. Tang K, He S, Zhang X, Guo J, Chen Q, Yan F, et al. Tangeretin, an extract from Citrus peels, blocks cellular entry of arenaviruses that cause viral hemorrhagic fever. *Antivir Res* 2018;160:87–93.

33. Zhang X, Yan F, Tang K, Chen Q, Guo J, Zhu W, et al. Identification of a clinical compound losmapimod that blocks Lassa virus entry. *Antivir Res* 2019;167:68–77.

34. Chen Q, Tang K, Zhang X, Chen P, Guo Y. Establishment of pseudovirus infection mouse models for in vivo pharmacodynamics evaluation of filovirus entry inhibitors. *Acta Pharm Sin B* 2018;8:200–8.

35. Labudova M, Pastorek J, Pastorekova S. Lymphocytic choriomeningitis virus: ways to establish and maintain non-cytolytic persistent infection. *Acta Virol* 2016;60:15–26.

36. Emonet S, Lemasson JJ, Gonzalez JP, de Lamballerie X, Charrel RN. Phylogeny and evolution of old world arenaviruses. *Virology* 2006;350:251–7.

37. Bowen MD, Rollin PE, Ksiazek TG, Hustad HL, Bausch DG, Demby AH, et al. Genetic diversity among Lassa virus strains. *J Virol* 2000;74:6992–7004.

38. Oloniniyi OK, Unigwe US, Okada S, Kimura M, Koyano S, Miyazaki Y, et al. Genetic characterization of Lassa virus strains isolated from 2012 to 2016 in southeastern Nigeria. *PLoS Neglected Trop Dis* 2018;12:e0006971.

39. Nunberg JH, York J. The curious case of arenavirus entry, and its inhibition. *Viruses* 2012;4:83–101.

40. Torriani G, Galan-Navarro C, Kunz S. Lassa virus cell entry reveals new aspects of virus-host cell interaction. *J Virol* 2017;91:e01902–16.

41. Ngo N, Henthorn KS, Cisneros MI, Cubitt B, Iwasaki M, de la Torre JC, et al. Identification and mechanism of action of a novel small-molecule inhibitor of arenavirus multiplication. *J Virol* 2015;89:10924–33.

42. Wang W, Zhou Z, Zhang L, Wang S, Xiao G. Structure–function relationship of the mammarenavirus envelope glycoprotein. *Virology* 2016;31:380–94.

43. Sanati S, Razavi BM, Hosseinzadeh H. A review of the effects of *Capsicum annuum* L. and its constituent, capsaicin, in metabolic syndrome. *Iran J Basic Med Sci* 2018;21:439–48.

44. Guedes V, Castro JP, Brito I. Topical capsaicin for pain in osteoarthritis: a literature review. *Reumatol Clinica* 2018;14:40–5.

45. Cho SC, Lee H, Choi BY. An updated review on molecular mechanisms underlying the anticancer effects of capsaicin. *Food Sci Biotechnol* 2017;26:1–13.

46. Patowary P, Pathak MP, Zaman K, Raju PS, Chattopadhyay P. Research progress of capsaicin responses to various pharmacological challenges. *Biomed Pharmacother* 2017;96:1501–12.

47. Gooding SM, Canter PH, Coelho HF, Boddy K, Ernst E. Systematic review of topical capsaicin in the treatment of pruritus. *Int J Dermatol* 2010;49:858–65.

48. McConachie SM, Caputo RA, Wilhelm SM, Kelle-Pradhan PB. Efficacy of capsaicin for the treatment of cannabinoid hyperemesis syndrome: a systematic review. *Ann Pharmacother* 2019;53:1145–52.

49. Marin E, Magi G, Mingoa M, Pugnaloni A, Facinelli B. Antimicrobial and anti-virulence activity of capsaicin against erythromycin-resistant, cell-invasive group a *Streptococci*. *Front Microbiol* 2015;6:1281.

50. Hafiz T, Mubarak M, Dkhil M, Al-Quraishy S. Antiviral activities of *Capsicum annuum* methanolic extract against herpes simplex virus 1 and 2. *Pakistan J Zool* 2017;49:251–5.

51. Martinez JP, Sasse F, Bronstrup M, Diez J, Meyerhans A. Antiviral drug discovery: broad-spectrum drugs from nature. *Nat Prod Rep* 2015;32:29–48.