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Original Article

Screening a neurotransmitter-receptor-related inhibitor library identifies clomipramine HCl as a potential antiviral compound against Japanese encephalitis virus

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ABSTRACT

Background: Japanese encephalitis virus (JEV) is a leading cause of viral encephalitis worldwide. JEV exhibits significant neuroinvasiveness and neurotoxicity, resulting in considerable damage to the nervous system. Japanese encephalitis is associated with high morbidity and mortality rate, seriously harming both human health and livestock production. The current lack of specific antiviral drugs means that the development of new therapeutic agents for JEV has become urgent.

Methods: Anti-JEV drugs were screened from 111 inhibitors of neurotransmitter receptor-related molecules by high content technology. The antiviral effects of clomipramine HCl were evaluated through plaque assay, real-time quantitative PCR, immunofluorescence assay and western blotting assay. Bioinformatic tools were utilized to cluster the altered signaling pathway members after clomipramine HCl treatment. Finally, the anti-JEV mechanism was deeply resolved in *vivo* via such molecular biology and virological detection techniques.

Results: In this study, we screened nine compounds with significant anti-JEV activity, of which clomipramine HCl demonstrated the most potent antiviral effect and exhibited dose-dependent activity. Mechanistically, clomipramine HCl may activate endoplasmic reticulum stress and modulate the unfolded protein response, thus inhibiting the assembly stage of JEV infection.

Conclusion: This study highlights the importance of clomipramine HCl as a promising approach for JEV infection protection, which may lead to new host-directed antiviral approaches to such mosquito-borne viruses.

1. Introduction

Japanese encephalitis virus (JEV) is a globally prevalent mosquito-borne viral encephalitis, with a 20% fatality rate, and approximately 50% of survivors develop permanent neuropsychiatric complications [1,2]. Targeting the central nervous system (CNS) and causing fatal encephalitis characterize the pathogenesis of JEV. JEV can penetrate the blood–brain barrier, allowing it to infiltrate the CNS. Previous studies have shown that JEV is mainly distributed in the striatum, amygdala, hippocampus, and medial nucleus of the midbrain [3], indicating that JEV infects specific areas and cell types of the brain. Neurons are the main cellular targets for JEV infection and replication [4–6]. The efficient replication and proliferation of JEV in neurons is the direct cause of

Abbreviations: BIP/GRP78, binding protein for immunoglobulins /glucose-regulated protein of 78 kDa; CNS, central nervous system; ER, endoplasmic reticulum; JEV, Japanese encephalitis virus; PERK, protein-kinase-like endoplasmic reticulum kinase; SERT, serotonin transporter; UPR, unfolded protein response; 4-PBA, 4-Phenylbutyric acid; XBP1, X-box binding protein 1; ATF4, activating transcription factor 4.

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neuronal damage. Neuronal damage caused by infection activates glial cells to produce a large number of inflammatory cytokines, triggering excessive host inflammatory responses and ultimately promoting the destruction of neuronal cells [7,8]. At the same time, astrocytes and neuronal microglia are also susceptible to JEV infection. They will directly promote the occurrence of neuroinflammatory responses once they are infected.

Despite significant progress in understanding the biology of JEV, only four clinical studies have been conducted, with no improvements in treatment [2]. This may be attributed to the rapid invasion of the CNS after JEV infection, together with the difficulty of drug penetration to the infection site. Therefore, novel antiviral medications are urgently required.

Neurotransmitters are crucial to the chemical communication between neurons and are also essential in the pathogenesis of neuroleptic viruses. Several studies have demonstrated that JEV regulates its life cycle via neurotransmitter-mediated signaling, with evidence of tissue tropism. In a previous study, we showed that JEV increases N-methyl-D-aspartate receptor phosphorylation, leading to neuro-excitotoxicity and damaging neuronal cells [9]. Dopaminergic neurons in brain regions highly sensitive to JEV have also been shown to correlate with the tissue tropism of the virus. JEV promotes dopamine release and activates dopamine D2 receptor (D2R)-phospholipase C signaling in D2R-expressing cells, facilitating JEV invasion and increasing the extent of infection [10]. Mosquitoes, the primary vectors of flaviviruses, activate the GABAergic system to suppress the innate immune system and facilitate infection [11]. Identifying other neurotransmitter pathways that also influence JEV infection may offer a novel approach to the discovery of specific drugs for JEV.

Here, we used high-content screening to evaluate 111 neurotransmitter-receptor-related molecular inhibitors in primary neurons. Clomipramine HCl, an antagonist of serotonin transporter (SERT), was here identified as an inhibitor of JEV infection, and acts by activating endoplasmic reticulum (ER) stress and modulating the protein-kinase-like endoplasmic reticulum kinase (PERK)-mediated unfolded protein response (UPR). This research offers a crucial scientific foundation for investigating the pathogenesis of JEV and other neurotropic viruses and for developing novel therapeutic interventions for them.

2. Methods

2.1. Cell cultures and viruses

Mouse neuroblastoma (N2a) cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate. Human neuroblastoma (SH-SY 5Y) cells was maintained in a medium composed of DMEM with high glucose (Servicebio, Wuhan, Hubei, China) and F12 (Servicebio) (1:1), containing 10% FBS (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate. Baby hamster kidney (BHK-21) cells were cultured and maintained in DMEM supplemented with 10% FBS (Every Green, Hangzhou, Zhejiang, China), streptomycin sulfate (100 mg/mL), and penicillin (100 U/mL). The experiments were performed at 37 ˚C under a 5% CO_2 atmosphere. JEV strain P3 (GenBank: U47032.1) is stored in our laboratory.

2.2. Isolation of mouse primary neurons

Primary neurons were prepared from the cerebral cortices of 14-day-old Kunming mouse embryos. The plates were coated with 20 mg/mL polylysine (Sigma-Aldrich, Saint louis, Missouri, USA) and inoculated with the primary cells at a density of 5×10^5 cells/mL in DMEM supplemented with 5% FBS (Gibco). Six hours after seeding, the culture medium was replaced with neurobasal medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 2% B-27 Supplement (Thermo Fisher Scientific). The cells were used in subsequent experiments after a 4-day incubation period.

2.3. Cell viability assay

Cell viability was assessed with the CellTiter-Lumi™ Luminescent 3D Cell Viability Assay Kit (Beyotime, Shanghai, China), according to the manufacturer's instructions. After treatment, the luminescent signals were detected with Infinite F200 Fluorescence Microplate Reader (Tecan, Meilen, Zurich, Switzerland).

2.4. Western blotting

Cells were harvested and lysed with RIPA buffer (Sigma-Aldrich) supplemented with cOmplete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland). The Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) was used to determine the protein concentration in each sample. The samples were then boiled at 95 ˚C for 10 min. Equivalent amounts of protein were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride membrane (Millipore, Bedfordshire, Massachusetts, USA) with a Mini Trans-Blot® Cell (Bio-Rad, Hercules, California, USA). The membranes were then blocked by incubating them in the blocking buffer for 1 h at room temperature, and probed with the relevant primary antibodies at 4 ˚C overnight. After the blots were washed

three times with TBS-Tween (50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% [v/v] Tween 20, pH 7.4), they were incubated with the appropriate secondary antibody at room temperature for 45 min. The membranes were washed three times again and the proteins visualized with Pierce® ECL Western Blotting Substrate (Thermo Fisher Scientific). Monoclonal antibodies that specifically target the JEV E and NS5 proteins were produced in our laboratory. Monoclonal antibody DDDDK-Tag (AE005), and monoclonal antibodies directed against PERK (A21255), phospho-PERK (AP1420), binding protein for immunoglobulins (BIP)/glucose-regulated protein of 78 kDa (GRP78) (A11366), activating transcription factor 6 (ATF6) (A0202), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (AC002), horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (AS003) and HRP-conjugated goat anti-rabbit antibody (AS014) were purchased from ABclonal Technology (Wuhan, Hubei, China).

2.5. Plaque assay

The harvested cell supernatants were serially diluted 10-fold with DMEM, and then used to inoculate BHK-21 cell monolayers. After any unbound viral particles were removed, the cells were incubated with DMEM supplemented with 2% FBS and 1.5% sodium carboxymethyl cellulose (Sigma-Aldrich, USA) for 5 days. After incubation, the cells were fixed with 10% formaldehyde solution. They were then stained with crystal violet to aid in the quantification of apparent plaques.

2.6. Screening FDA-approved inhibitors of neurotransmitter-receptor-related molecules

Inhibitors of neurotransmitter-receptor-related molecules in an FDA-approved drug library were purchased from Selleck (https://www.selleckchem.com/screening/fdaapproved-drug-library.html). Primary neurons were infected with JEV (multiplicity of infection [MOI] = 1), and incubated with each compound at a concentration 10 μM. At 24 h post-infection, the cells were fixed with 4% paraformaldehyde polyoxymethylene for 10 min, then washed with phosphate-buffered saline (PBS). After permeabilization by incubation with saponin for 30 min, the cells were blocked for 1 h. They were then incubated with mouse anti-JEV E and rabbit anti-MAP2 antibodies, followed by an Alexa-Fluor-488-conjugated anti-mouse IgG antibody (Invitrogen, USA) and 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). The plates were imaged with Opera Phenix High Content Screening System (PerkinElmer) with a 10 × objective. The percentages of infected and DAPI-positive cells were calculated with an automated image analysis software (Harmony R4.9, PerkinElmer).

2.7. Reverse-transcription (RT)-quantitative PCR (qPCR)

Total cellular RNA was extracted with TRIZOL Reagent (Invitrogen) and reverse transcribed to complementary DNA (cDNA) with the ABscript II cDNA First-Strand Synthesis Kit (ABclonal, China). qPCR was performed with the QuantStudio™ 6 Flex PCR system (Applied Biosystems) and 2X Universal SYBR Green Fast qPCR Mix (ABclonal, China). The data were normalized to the expression of endogenous β-actin (*ACTB*) in each sample. The primers used are listed in Supplementary Table 1.

2.8. Time-of-addition assay

Pre-infection: SH-SY 5Y cells were incubated with clomipramine HCl (10 μM) for 2 h. The cell medium was removed and the cells were infected with JEV (MOI = 1) for 1 h. The cells were washed with DMEM and incubated in fresh medium for 24 h. Co-infection: SH-SY 5Y cells were co-incubated with clomipramine HCl (10 μM) and JEV (MOI = 1) for 1 h. The cells were then washed with DMEM and incubated with fresh medium for 24 h. Post-infection: SH-SY 5Y cells were infected with JEV (MOI = 1) for 1 h. The cells were then washed with DMEM and incubated with clomipramine HCl (10 μM) for 24 h.

2.9. Statistical analysis

The experiments were performed at least thrice under similar conditions. All analyses were performed with the GraphPad Prism (version 8.0.1) (GraphPad Software, USA). The statistical analysis of differences between two groups was performed using two-tailed Student's *t*-test. In this manuscript, statistical significance is indicated: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; or ns, no significance.

3. Results

3.1. Screening a library of inhibitors of neurotransmitter-receptor-related molecules for anti-JEV compounds

To identify neurotransmitter molecules associated with JEV infection, we used fluorescence-based high-content screening to assess the effects of 111 inhibitors on the JEV infection rate in primary neurons (Supplementary Fig. 1). The results showed that 24 inhibitors reduced JEV infection by > 50%. (Fig. 1A). A subsequent plaque assay identified with the nine inhibitors with the strongest inhibitory effects. Clomipramine HCl treatment produced the greatest reduction in JEV titers and was selected for subsequent experiments (Fig. 1B).

A luminescence-based viability assay was used to evaluate the cytotoxicity of clomipramine HCl in primary

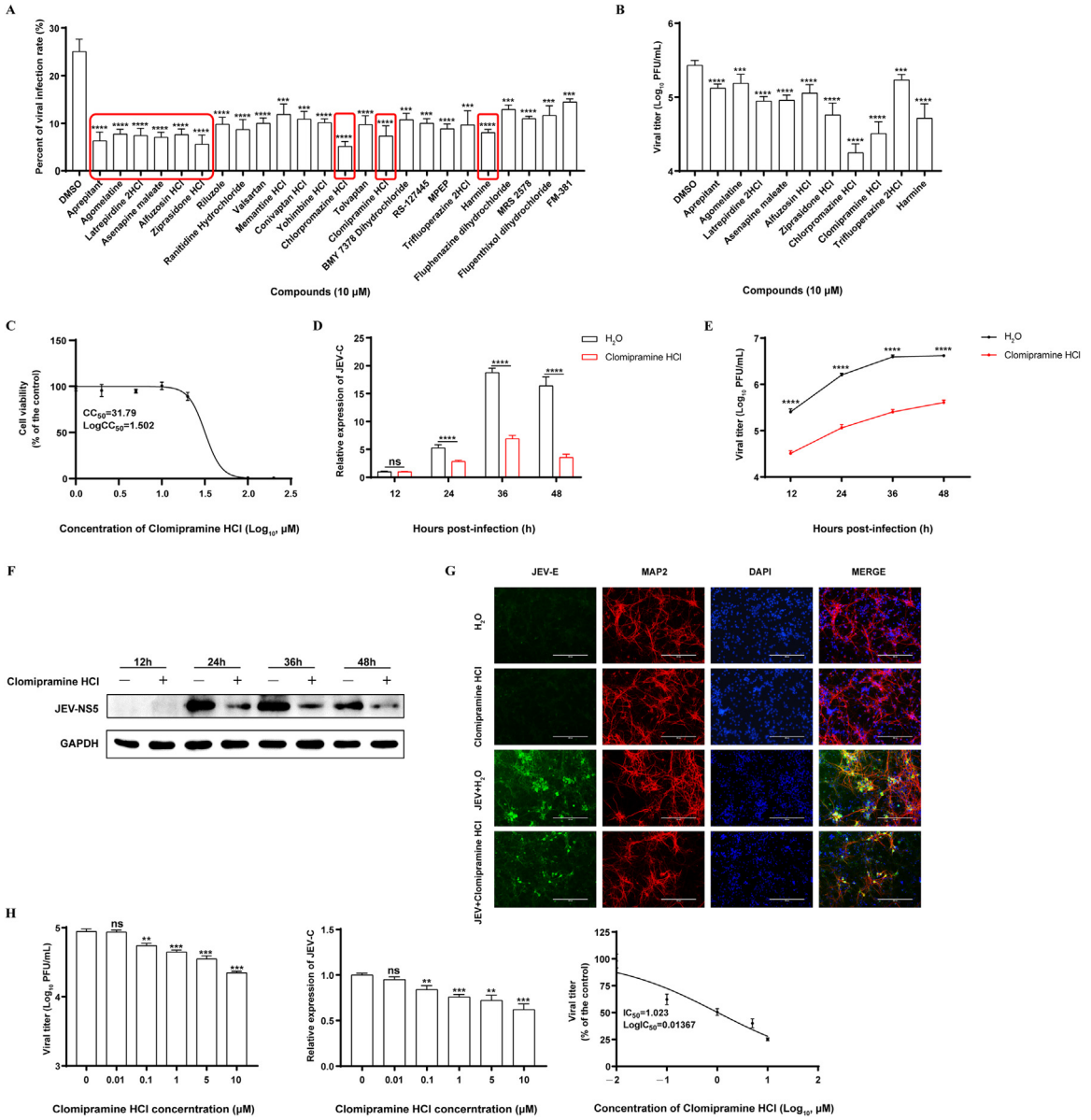

Fig. 1. Screening for antiviral compounds against JEV in a library of inhibitors of neurotransmitter-receptor-related molecules. **(A)** JEV-positive cells were quantified with a high-content screening instrument. Inhibitors with significant anti-JEV effects were selected. **(B)** Effects of potential anti-JEV compounds on JEV replication. Viral titers were measured with plaque assays. **(C)** Effects of clomipramine HCl at the indicated concentrations on the viability of primary neurons treated for 48 h. **(D-G)** Effect of clomipramine HCl on JEV replication. Primary neurons were infected with JEV (MOI = 1) and incubated with clomipramine HCl (10 μM). Viral titers, RNA copies, and E protein expression were measured with plaque assays (D), RT-qPCR (E), western blotting (F), and IFA (G) at indicated time points. Scale bar, 200 μm. **(H)** Primary neurons were infected with JEV (MOI = 1), and incubated with various concentrations of clomipramine HCl (0, 0.01, 0.1, 1, 5, or 10 μM). At 24 h postinfection, the supernatants and cells were collected. Viral titers and mRNA levels were measured with plaque assay and RT-qPCR, respectively, and IC_{50} was calculated. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; or ns, no significance.

neurons (Fig. 1C). The maximum nontoxic concentration (10 μM) was used for subsequent experiments. Primary neurons were infected with JEV and treated with 10 μM clomipramine HCl. As shown in Fig. 1D and E, clomipramine HCl treatment significantly reduced viral RNA replication and the number of infectious viral particles by > 10-fold at 24 h post infection. Consistent with this, western blotting and an immunofluorescence assay (IFA) demonstrated a notable reduction in viral protein expression after the clomipramine HCl treatment (Fig. 1F and G). The level of JEV replication also gradually decreased as the concentration of clomipramine HCl increased, indicating that clomipramine HCl inhibits JEV replication in a dose-dependent manner, with a half maximal inhibitory concentration (IC_{50}) of 1.023 μM (Fig. 1H).

3.2. *Clomipramine HCl inhibits JEV replication* in vitro

To confirm the anti-JEV effects of clomipramine HCl, several *in vitro* experiments were performed in the SH-SY 5Y and N2a cell lines. Clomipramine HCl at concentrations < 10 μM showed no significant cytotoxicity

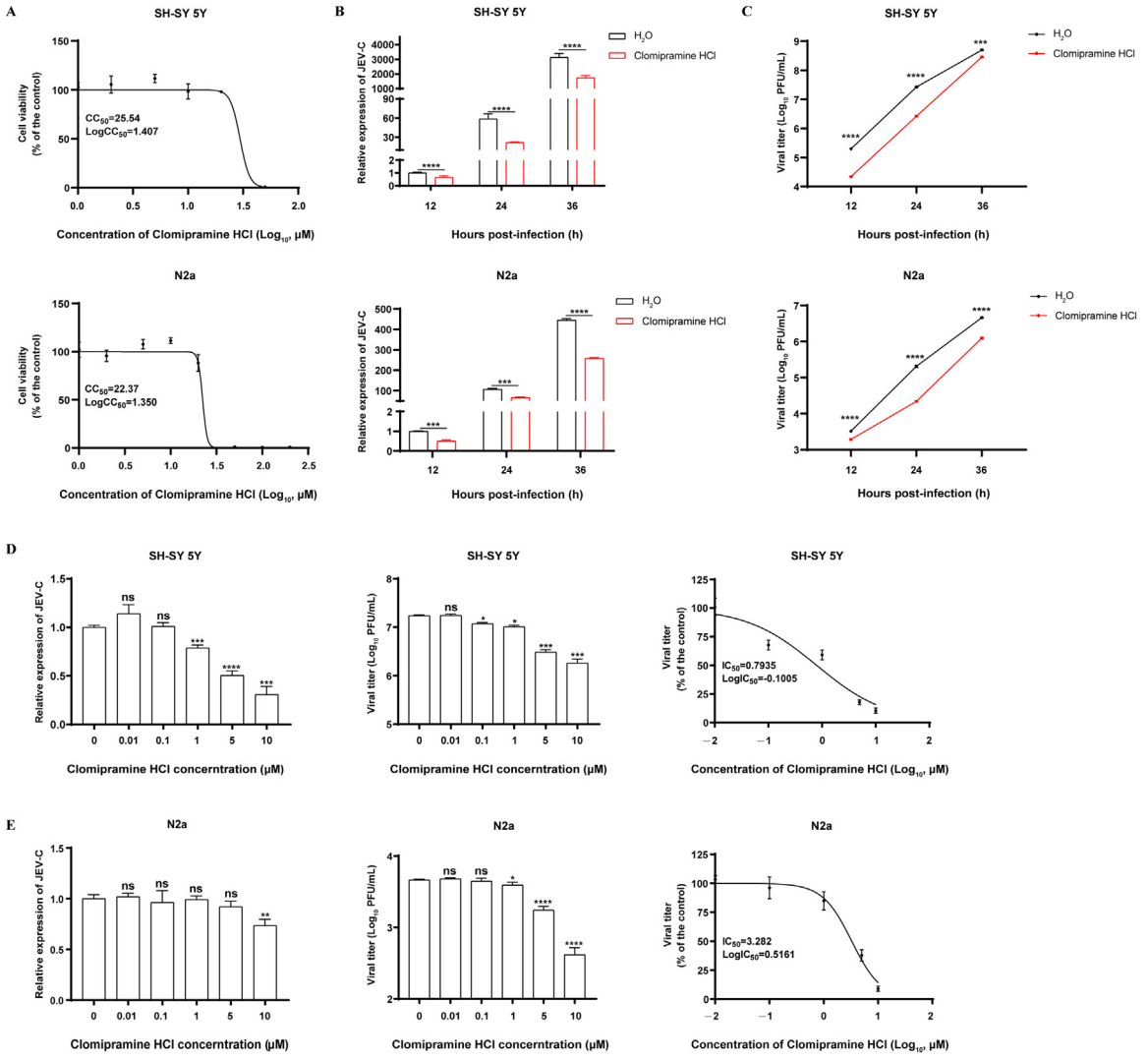

Fig. 2. Clomipramine HCl inhibits JEV replication *in vitro*. **(A)** Viability of SH-SY 5Y (upper panel) and N2a cells (lower panel) treated with clomipramine HCl at the indicated concentrations for 48 h. **(B and C)** SH-SY 5Y and N2a cells were infected with JEV at MOI = 1, and incubated with 10 μM clomipramine HCl. At the indicated times post-infection, the supernatants and cells were collected, and the viral titers and RNA copies were measured with plaque assays (B) and RT-qPCR, respectively (C). **(D and E)** SH-SY 5Y cells and N2a cells were infected with JEV (MOI = 1) and incubated with various concentrations of clomipramine HCl (0, 0.01, 0.1, 1, 5, or 10 μM). At 24 h post-infection, the supernatants and cells were collected. Viral titers and RNA copies were measured with plaque assays (D) and RT-qPCR, respectively (E), and IC_{50} was calculated. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; or ns, no significance.

in SH-SY 5Y cells or N2a cells (Fig. 2A). A plaque assay and RT-qPCR analysis showed that treatment with clomipramine HCl considerably hindered JEV replication in both cell lines (Fig. 2B and C). The effects of various concentrations of clomipramine HCl on JEV replication in these two cell lines were also assessed with RT-qPCR and plaque assays. The results demonstrated that the inhibitory effects of clomipramine HCl on JEV on these two cell lines were also dose-dependent, and that the IC_{50} of clomipramine HCl against JEV was 0.7935 μM in SH-SY 5Y cells and 3.282 μM in N2a cells (Fig. 2D and E).

3.3. Clomipramine HCl inhibits JEV assembly

We then conducted a time-of-addition assay to determine the stage in the JEV life cycle at which the compound exerts its antiviral effect. SH-SY 5Y cells were treated with clomipramine HCl at distinct time points: pre-infection, co-infection, and post-infection (Fig. 3A). As shown in Fig. 3B and C, treating cells with clomipramine HCl after JEV infection significantly inhibited the increase in viral RNA copies and the production of infectious progeny virus, whereas pretreating cells before or during infection had no obvious effect. We also examined the effect of clomipramine HCl treatment on JEV adsorption and invasion. The results showed that clomipramine HCl treatment had no obvious effect on these processes (Fig. 3D and E). Taken together, these findings suggest that clomipramine HCl exerts its antiviral effect in the post-invasion phase of JEV infection.

To analyze the effect of clomipramine HCl on virion assembly and release, we measured the intracellular and

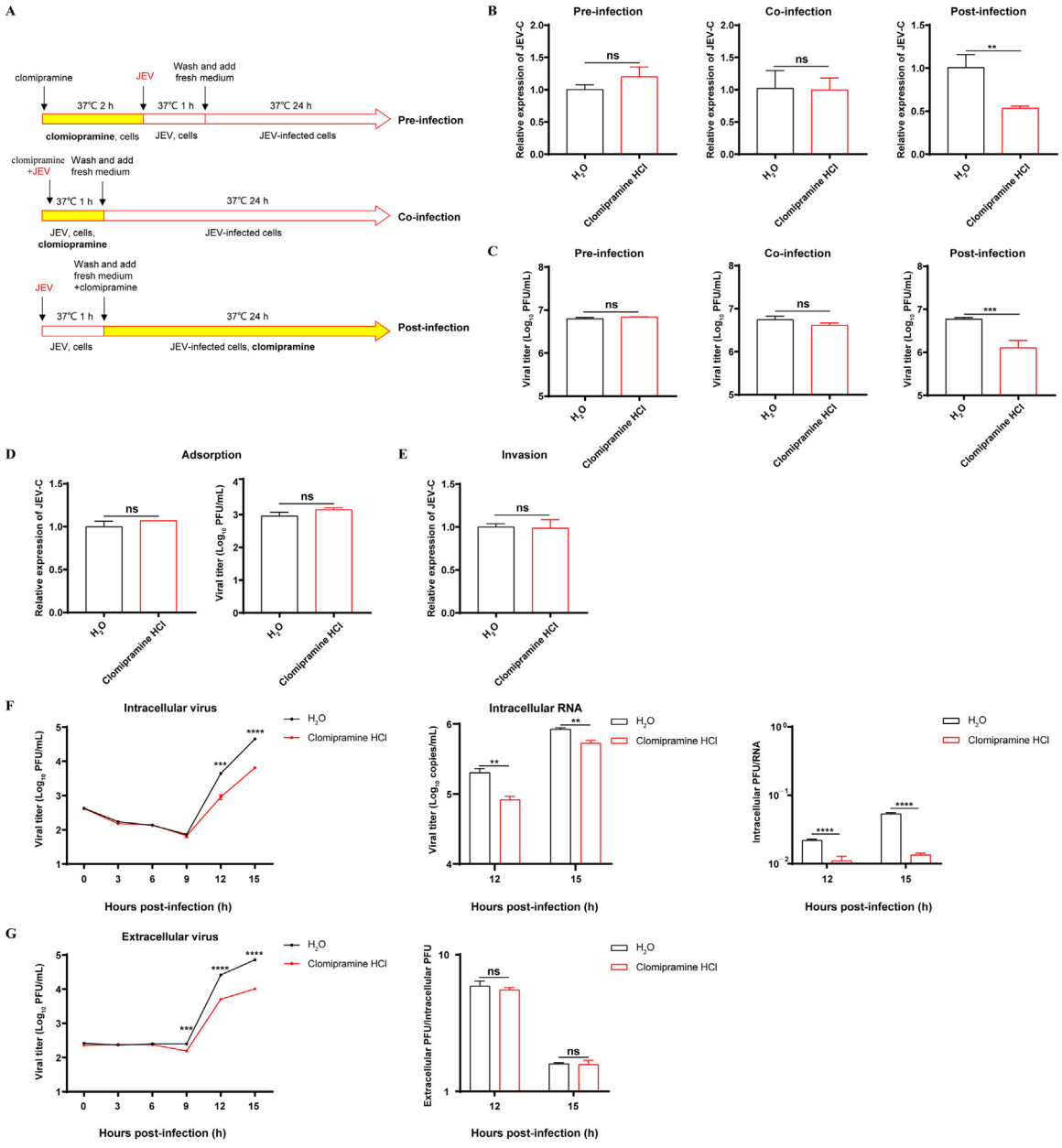

Fig. 3. Clomipramine HCl inhibits JEV assembly. **(A)** Brief schematic diagram of time-of-addition experiment. **(B and C)** Viral titers and RNA copies in each group were determined with plaque assays (B) and RT-qPCR, respectively (C). **(D and E)** JEV adsorption and invasion assay in SH-SY 5Y cells. SH-SY 5Y cells were incubated with JEV (MOI = 1) or JEV (MOI = 1) + clomipramine HCl at 4 °C for 1 h. The viral titers were determined with a plaque assay, and the RNA levels were determined with RT-qPCR. (D) SH-SY 5Y cells were incubated with JEV (MOI = 1) or JEV (MOI = 1) + clomipramine HCl at 4 °C for 1 h. The cells were then washed three times with cold alkaline high-salt solution and treated with H_2O or clomipramine HCl (10 μM) at 37 °C for another 10 min. The cells were collected for a plaque assay to determine the viral titer (E). **(F and G)** Assembly and release assays in SH-SY 5Y cells. SH-SY 5Y cells were incubated with JEV (MOI = 1) at 4 °C for 1 h. Unattached virus was removed by washing three times with PBS. The cells were then incubated with H_2O or clomipramine HCl (10 μM) at 37 °C. The supernatants and cells were harvested at the indicated time points. Intracellular and extracellular viral titers were determined with plaque assays. Viral RNA was measured with RT-qPCR. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; or ns, no significance.

extracellular levels of viral RNA and infectious virions. Genomic RNA is packaged into viral particles during viral assembly. The intracellular viral RNA/plaque-forming unit (PFU) ratio was calculated to assess the efficiency of intracellular virion assembly [12]. The intracellular RNA/PFU ratio was significantly lower in the clomipramine HCl-treated group than in the control group at 12 and 15 h post-infection (Fig. 3F), indicating that clomipramine HCl reduced the efficiency of viral particle assembly. During viral release, viral particles are released from the intracellular to the extracellular environment. Therefore, we calculated the extracellular PFU/intracellular PFU ratio to evaluate the effect of clomipramine HCl on virion release. There were no significant differences in the extracellular PFU/intracellular PFU ratios between the infected cells treated and

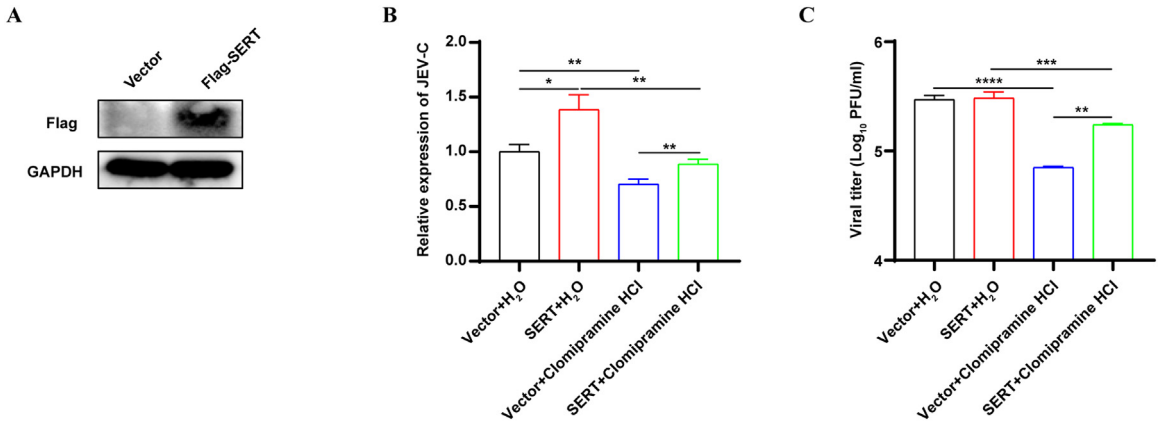

Fig. 4. Clomipramine HCl inhibits JEV replication through SERT. **(A)** SH-SY 5Y cells were transfected with 3 × Flag-SERT-expressing plasmid or empty vector. Expression levels of SERT were determined with an immunoblotting assay at 48 h post-transfection. **(B and C)** At 24 h post-transfection, SH-SY 5Y cells were infected with JEV at MOI = 1 and incubated with H_2O or clomipramine HCl (10 μM). At 24 h post-infection, the viral titers and viral RNA copies were measured with plaque assay (B) and RT-qPCR, respectively (C). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; or ns, no significance.

untreated with clomipramine HCl (Fig. 3G), suggesting that clomipramine HCl does not influence viral release.

In summary, these results indicate that the anti-JEV activity of clomipramine HCl occurs during viral assembly.

3.4. Clomipramine HCl inhibits JEV replication through SERT

Clomipramine HCl is a tricyclic antidepressant (TCA) with strong affinity for SERT [13]. It functions by blocking the reuptake of serotonin and reducing the sensitivity of SERT and norepinephrine transporter (NET) to increase serotonin, norepinephrine, and dopamine levels in the brain, ultimately exerting an effective antidepressant effect [14,15]. To confirm whether the anti-JEV effect of clomipramine HCl is attributable to the inhibition of SERT, we overexpressed SERT and investigated whether it rescued the reduction of JEV infection induced by clomipramine HCl (Fig. 4A). Plaque assays and RT-qPCR showed that the overexpression of SERT attenuated the antiviral activity of clomipramine HCl (Fig. 4B and C). These findings imply that clomipramine HCl exerts its anti-JEV activity through SERT.

3.5. Clomipramine HCl activates ER stress and downstream PERK-mediated UPR

To investigate the antiviral mechanism of clomipramine HCl, RNA sequencing (RNA-seq) was used to evaluate the transcriptome of SH-SY 5Y cells treated with clomipramine HCl. The analysis identified 3346 differentially expressed genes ($p < 0.05$, fold change ≥ 2), including 112 downregulated and 36 upregulated genes, after clomipramine HCl treatment. Heatmaps (Fig. 5A) and volcano plots (Fig. 5B) were generated with the R package to show the global regulatory patterns of these differentially expressed genes. A pathway enrichment analysis indicated that the majority of the differentially regulated genes were involved in protein processing in the ER (Fig. 5C). Given the significance of ER stress in the pathogenesis of JEV [16,17], we investigated the connection between clomipramine HCl and ER stress. To this end, a subset of genes related to ER protein processing was analyzed, and showed that several ER biomarkers were significantly upregulated after clomipramine HCl treatment, including BIP/GRP78, glucose regulated protein 94 (GRP94), PERK, X-box binding protein 1 (XBP1), activating transcription factor 4 (ATF4), and C/eBP homologous protein (CHOP) (Fig. 5D and E). These results indicate that clomipramine HCl activates ER stress.

To maintain ER homeostasis, the ER chaperone BIP/GRP78 dissociates from three ER stress sensors, PERK, inositol-requiring enzyme 1 (IRE1), and ATF6, and initiates downstream signaling to trigger the UPR [18,19]. We analyzed the expression of BIP/GRP78 in clomipramine-HCl-treated SH-SY 5Y cells with western blotting, which showed that clomipramine HCl dramatically increased the expression of BIP/GRP78 (Fig. 5F), indicating the clomipramine HCl treatment activates the UPR. To investigate how the compound activates the UPR, we examined its effect on three major pathways of UPR signaling. As shown in Fig. 5G, the splicing of XBP1 was unaffected by clomipramine HCl. However, western blotting showed that clomipramine HCl markedly increased the expression and phosphorylation of PERK (Fig. 5H). We also assessed the mRNA expression levels of several downstream factors, but only growth arrest and DNA damage-inducible 34 (GADD34), a crucial target gene of the PERK pathway, was significantly increased by clomipramine HCl treatment (Fig. 5I). These results suggest that clomipramine HCl exerts its antiviral effect by inducing ER stress and the downstream PERK-mediated UPR.

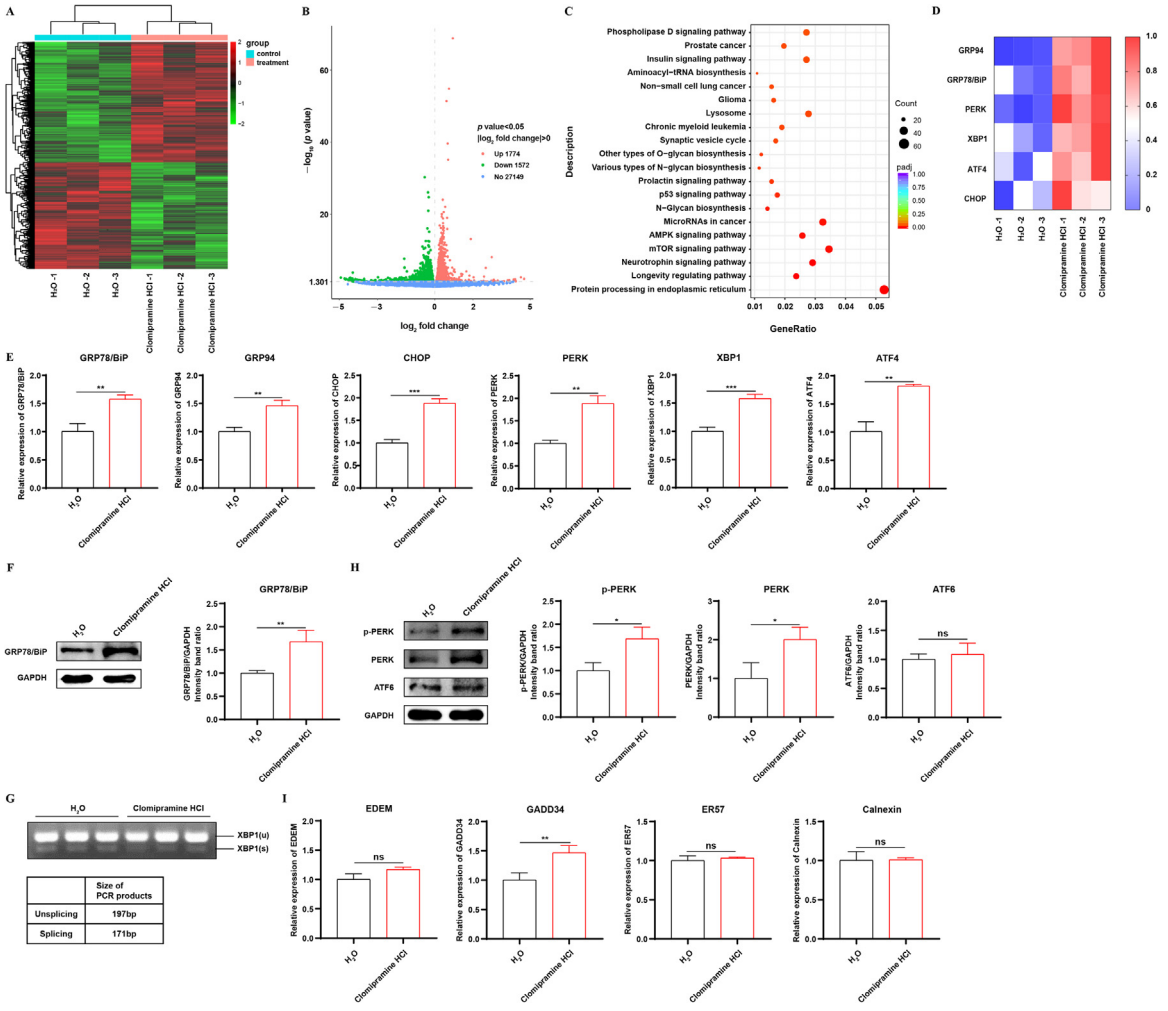

Fig. 5. Clomipramine HCl activates ER stress and downstream PERK-mediated UPR. **(A)** Hierarchical clustering of differentially expressed genes. Red indicates upregulated genes and green indicates downregulated genes. **(B)** Volcano plot represents modified genes. Blue dots denote non-significantly expressed genes. Up-regulated genes are shown as red dots and downregulated genes as green dots. **(C)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the differentially expressed genes **(D)** Heatmap of genes related to ER stress. **(E)** RT-qPCR was used to analyze the expression of ER-stress-related genes. **(F)** SH-SY 5Y cells were treated with clomipramine HCl for 24 h. Levels of BIP protein were determined with an immunoblotting assay (left panel). Quantitative analysis of optical band densitometry (right panel). **(G)** Whole-cell mRNA was isolated from clomipramine-HCl-treated cells and analyzed with RT-qPCR, which amplified XBP1u and XBP1s. XBP1u, inactive unspliced form; XBP1s, active spliced form. **(H)** Expression levels of p-PERK, PERK, and ATF6 were determined with immunoblotting assays (left panel). Quantitative analysis of optical band densitometry (right panel). **(I)** RT-qPCR was used to analyze the expression of EDEM, GADD34, ER57, and calnexin. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; or ns, no significance.

3.6. Clomipramine HCl prevents JEV replication by activating ER stress

4-Phenylbutyric acid (4-PBA) is an inhibitor of ER stress that attenuates the UPR and UPR-mediated apoptosis by regulating molecular chaperones [20]. To further clarify the anti-JEV activity of clomipramine HCl in stimulating ER stress, we used 4-PBA to inhibit the ER stress triggered by clomipramine HCl. Cytotoxicity assays showed that the appropriate concentration of 4-PBA for application to SH-SY 5Y cells was 500 μM (Fig. 6A). The altered mRNA levels of BIP/GRP78 and CHOP confirmed the inhibitory effect of 4-PBA on ER stress (Fig. 6B). These observations indicated that treating cells with 4-PBA, but not DMSO, rescued the halted JEV replication caused by clomipramine HCl, as determined with a plaque assay and western blotting (Fig. 6C and D) These findings suggest that ER stress plays a crucial role in mediating the antiviral activity of clomipramine HCl.

4. Discussion

JEV is considered one of the major infectious diseases prevalent during the summer and autumn seasons in Asia [21,22]. Supportive measures are the main treatment for JEV because no specific therapy is currently available [23]. Therefore, there is an urgent need to develop potent therapeutic agents against JEV infection. In this study, clomipramine HCl was identified as a potential anti-JEV agent.

We specifically isolated mouse brain neurons to screen for neurotransmitter-receptor-related molecules. Among

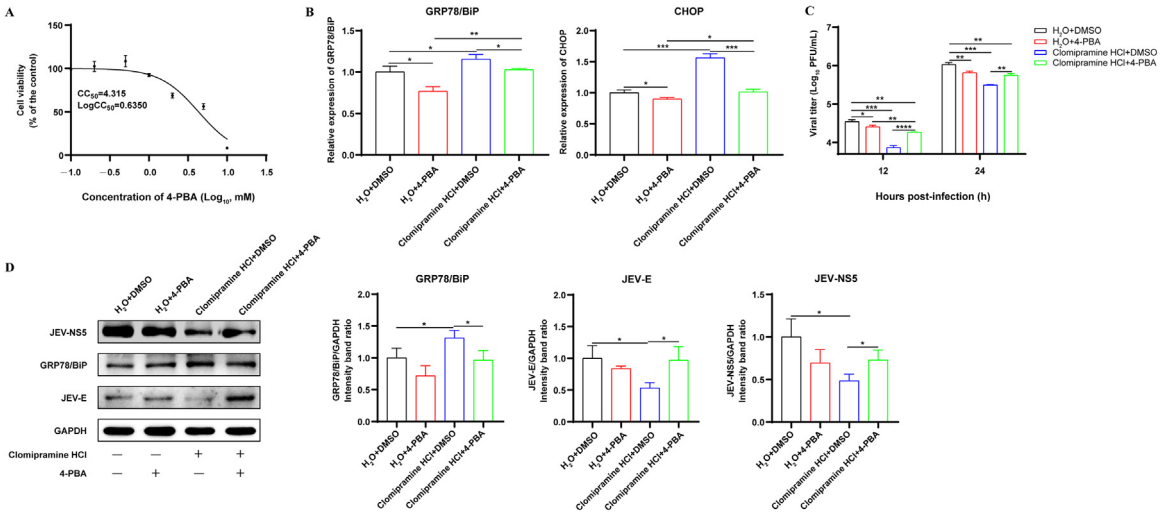

Fig. 6. Clomipramine HCl prevents JEV replication by activating ER stress. **(A)** Viability of SH-SY 5Y cells after treatment with 4-PBA at the indicated concentrations for 48 h. **(B and C)** SH-SY5Y cells were infected with JEV at MOI = 1 and incubated with 4-PBA and clomipramine HCl at the indicated concentrations. At 12 h postinfection, the mRNA levels of BIP/GRP78 and CHOP were measured with RT-qPCR (B). Viral titers were determined with plaque assays at 12 and 24 h postinfection (C). **(D)** Expression levels of BIP/GRP78, viral E protein, and viral NS5 protein were determined with western blotting (left panel). Quantitative analysis of optical band densitometry (right panel). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; or ns, no significance.

these, chlorpromazine HCl and clomipramine HCl showed the most potent inhibitory effects on JEV infection. This finding is consistent with previous reports that have demonstrated that chlorpromazine HCl exerts antiviral effects on flaviviruses, including Zika virus, Dengue virus, and JEV, by suppressing lattice-protein-mediated endocytosis [24–26]. However, the antiviral effects and mechanisms of clomipramine HCl required further study.

Clomipramine HCl is an FDA-approved drug. It primarily exerts its antidepressant effects through the inhibition of 5-hydroxytryptamine (5-HT) reuptake by presynaptic neurons and the consequent elevation of inter-synaptic 5-HT levels [27]. The antiviral properties of clomipramine HCl are not yet fully understood, although previous studies have indicated its inhibitory effects on the replication of hepatitis C virus, similar to those of other antidepressant, such as fluoxetine [28]. Furthermore, the recent finding that fluvoxamine inhibits the replication of SARS-CoV2 by modulating the ER stress response also supports the potential application of antidepressant in antiviral treatment, especially for neurotropic viruses [29]. Here, our findings demonstrate that clomipramine HCl potentially impedes the virion assembly stage of JEV. RNA-seq suggested that clomipramine HCl regulates protein processing in the ER, and plays a central role in mediating viral assembly and the formation of immature viral particle [30]. On the basis of these findings, we hypothesize that clomipramine HCl interferes with viral assembly in the ER.

The ER regulates various biological processes, including protein folding and posttranslational modifications [31]. When the genus *Flavivirus* replicates and forms non-infectious, immature viral particles in the ER and Golgi membranes, it disrupts the normal host protein synthesis and triggers ER stress [32–34]. ER stress functions as a "double-edged sword", mediating the different outcomes of cell survival and death [35,36]. During JEV infection, the activation of the ER stress sensors XBP1 and ATF6 promotes cellular autophagy, which induces antiviral effects [17]. Moreover, PERK activation triggers the formation of stress granules, thereby reducing JEV-induced apoptosis. On the contrary, the JEV NS4B protein induces PERK dimerization and triggers apoptosis through the PERK-ATF4-CHOP pathway, leading to neuronal apoptosis and encephalitis [16]. We examined three pathways of the UPR and demonstrated that clomipramine HCl therapy activates ER stress and dramatically increases the expression of the PERK protein. Moreover, inhibiting this ER stress with 4-PBA rescued the clomipramine-induced suppression of JEV replication. These findings indicate that clomipramine exerts its antiviral effects by activating ER stress. However, we also found that 4-PBA inhibits JEV replication, consistent with the report that 4-PBA suppresses JEV-activated ER stress and subsequently reduces the expression of the NS5 protein [37]. Therefore, investigating the modulation of ER stress during JEV infection and the flexible use ER stress drugs to prolong UPR signaling and inhibit the onset of infection-induced apoptosis may offer a promising therapeutic approach to JEV infection.

ER stress induces the oligomerization and autophosphorylation of PERK, leading to the phosphorylation of eukaryotic initiation factor 2A (EIF2α) and the induction of ATF4 expression [38,39]. ATF4 activation further stimulates the expression of its downstream effector, GADD34, maintaining steady intracellular protein translation. The activation of this process inhibits the 5′-cap-dependent protein translation process, leading to the widespread

suppression of protein synthesis in the ER [40,41]. Because clomipramine HCl activates the PERK signaling pathway, the potential involvement of PERK in the antiviral mechanism of clomipramine HCl warrants further investigation.

ER stress has also been shown to affect the replication of other flaviviruses, and the general deceleration of protein synthesis regulated by the PERK-EIF2α pathway restricts West Nile virus replication [42]. The IRE1-XBP1 pathway activated by ZIKV infection enhances the virus's replication [43]. This is further evidence of the dual role of ER stress in viral replication. However, further determinants must be investigated to determine whether clomipramine HCl and other antidepressants exert broad-spectrum antiviral effects on flaviviruses by modulating ER stress and the UPR.

A lack of experiments *in vivo* was a limitation of this study. However, finding that clomipramine HCl exerts an anti-JEV effect is encouraging because as an FDA-approved antidepressant, it targets the CNS and therefore has great potential utility in effectively inhibiting JEV replication in the CNS. However, we realize that the cytotoxicity of clomipramine HCl is relatively high, as has been reported in many previous studies. For example, rCMEC cells treated with 1 μM clomipramine HCl for 24 h showed significantly reduced cellular bioenergetics, significantly increased production of reactive oxygen species, and significantly increased cell apoptosis and DNA damage [44]. Treatment with clomipramine HCl at concentrations of 5 μM for 24 h inhibited the autophagic flux in primary neurons [45]. Although lower doses of clomipramine HCl have shown significant antiviral effects (Fig. 2D and E), caution must be exercised because of its potential side effects as an antidepressant and its cytotoxicity. We refrained from conducting animal experiments hastily for these reasons. By integrating the pharmacokinetics of clomipramine HCl with the dynamic distribution of JEV in future studies, we plan to effectively validate the antiviral efficacy of the compound and gain valuable insights into its potential applications.

5. Conclusion

Clomipramine HCl was selected as a possible anti-JEV medication from the 111 compounds screened in an inhibitor library. Our *in vitro* study demonstrates that clomipramine HCl effectively inhibits viral assembly. Clomipramine HCl inhibits viral replication by affecting ER stress and the modulation of the UPR. Therefore, clomipramine HCl could be used as a therapeutic agent to inhibit the replication of JEV.

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Author contributions

Yixin Liu: Methodology, Validation, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. **Xugang Wang:** Methodology, Formal analysis. **Qi Li:** Methodology, Formal analysis. **Shuo Zhu:** Validation, Investigation. **Wenjing Zhu:** Validation, Investigation. **Huanchun Chen:** Methodology. **Youhui Si:** Methodology, Formal analysis. **Bibo Zhu:** Methodology. **Shengbo Cao:** Conceptualization, Supervision. **Zikai Zhao:** Investigation, Validation, Writing – original draft, Writing – review & editing. **Jing Ye:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Validation, Writing – original draft.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data available statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Ethics statement

An ethical statement is not required as there were no human subjects involved in this study.

Informed consent

An informed consent is not required as there were no human subjects involved in this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imj.2024.100130.

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