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Identification of the corticotropin-releasing factor receptor 1 antagonists as inhibitors of Chikungunya virus replication using a *Gaussia* luciferase–expressing subgenomic replicon

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ABSTRACT

The Chikungunya virus (CHIKV), an enveloped RNA virus that has been identified in over 40 countries and is considered a growing threat to public health worldwide. However, there is no preventive vaccine or specific therapeutic drug for CHIKV infection. To identify a new inhibitor against CHIKV infection, this study constructed a subgenomic RNA replicon expressing the secretory *Gaussia* luciferase (Gluc) based on the CHIKV SL11131 strain. Transfection of *in vitro*-transcribed replicon RNA to BHK-21 cells revealed that Gluc activity in culture supernatants was correlated with the intracellular replication of the replicon genome. Through a chemical compound library screen using the Gluc reporter CHIKV replicon, we identified several compounds that suppressed CHIKV infection in Vero cells. Among the hits identified, CP-154,526, a non-peptide antagonist of the corticotropin-releasing factor receptor type-1 (CRF-R1), showed the strongest anti-CHIKV activity and inhibited CHIKV infection in Huh-7 cells. Interestingly, other CRF-R1 antagonists, R121919 and NGD 98-2, also exhibited inhibitory effects on CHIKV infection. Time-of-drug addition and virus entry assays indicated that CP-154,526 suppressed a post-entry step of infection, suggesting that CRF-R1 antagonists acted on a target in the intracellular replication process of CHIKV. Therefore, the Gluc reporter replicon system established in this study would greatly facilitate the development of antiviral drugs against CHIKV infection.

1. Introduction

The Chikungunya virus (CHIKV) is an enveloped virus belonging to the *Alphavirus* genus in the *Togaviridae* family, causing Chikungunya fever (CHIKF), which is generally characterized by fever, myalgia, and arthralgia [1]. Its viral genome is a single-stranded, positive-sense RNA approximately 12 kb in length, which encodes four non-structural (nsP1, nsP2, nsP3, and nsP4) and five structural (C, E3, E2, 6k, and E1) proteins flanked by 5′ and 3′ non-translated regions (NTRs) and has a 5′ cap structure and 3′ poly A tail [2]. The entry of CHIKV into target cells is mediated by receptor-mediated endocytosis, and after disassembly of the internalizing virion, the non-structural precursor proteins are synthesized from the viral RNA genome, yielding nsP1–4, which are involved in intracellular viral RNA amplification and protein processing [3]. In contrast to the nonstructural proteins, structural proteins are expressed from 26S subgenomic RNA, which is transcribed under the internal promoter present in the negative-sense viral RNA [2]. After the full-length viral RNA and capsid proteins are assembled, the nucleocapsid core buds out from the plasma membrane with glycosylated E proteins [4].

CHIKV is transmitted to humans through the bite of infected

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Aedes mosquitos. Although CHIKF had been considered an endemic disease in tropical areas of Africa and Asia, a major outbreak, which started in 2004 in coastal Kenya, spread to the islands in the Indian Ocean, and reached India and Southeast Asia, has changed its recognition as a re-emerging disease [5]. Importantly, genetic adaptations of CHIKV to *Aedes albopictus*, which is found in temperate regions, have been implicated in the spread of CHIKV to non-endemic regions, including Europe [6]. Since *Aedes albopictus* is more widely distributed than *Aedes aegypti*, CHIKV is currently regarded as an increasing threat to public health worldwide. In addition, there is a risk that the virus will be imported to new areas by infected travelers [7]. However, no approved vaccine or antiviral therapeutics exist. Treatment of CHIKV infection is directed primarily at relieving the symptoms, including joint pain using antipyretics, optimal analgesics, and fluids [8]. Therefore, developing anti-CHIKV drugs is needed to prepare for an outbreak.

In this study, we aimed to identify small chemical inhibitors against CHIKV by newly generating a self-replicative subgenomic RNA (i.e., replicon system) expressing the *Gaussia* luciferase (Gluc), a secretory luciferase that enabled rapid and quantitative evaluation of the level of intracellular CHIKV RNA replication. Through screen of a chemical library consisting of 80 agonists and antagonists for cellular signaling pathways using the Gluc replicon, CP-154,526, a non-peptide corticotropin-releasing factor receptor type-1 (CRF-R1) antagonist was identified as an inhibitor of the replication of CHIKV. Interestingly, structurally similar (R121919) and dissimilar (NGD 98-2) CRF-R1 antagonists also exhibited antiviral activity against CHIKV, indicating that the CHIKV reporter replicon harboring a secretory luciferase gene is a useful tool for the development of anti-CHIKV drugs.

2. Materials and methods

2.1. Cells

All mammalian cell cultures were maintained at 37 °C in the presence of 5% CO_2. BHK-21 (baby hamster kidney) and Vero (green monkey kidney) cells were maintained in Eagle's minimum essential medium (MEM) containing Earle's salts and L-glutamine (Nacalai Tesque, Inc.) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Huh-7 (human hepatoma) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Nacalai Tesque, Inc.) supplemented with 10% FBS and antibiotics.

2.2. Construction of plasmid for expression of the CHIKV reporter replicon

A cassette containing puromycin resistance (pac), the foot-and-mouth disease virus 2A cleavage site (FMDV2A), and Gluc genes was synthesized by overlapping PCR using pLKO.1-puro (Sigma-Aldrich) and DGL2 [9] plasmid DNAs and inserted into *Bam*HI (in the nsP1 gene) and *Hind*III (in 3′NTR) sites of a T7 promoter-driven CHIKV Ross strain infectious clone expression plasmid (pT7-Ross [10]) using an In-Fusion HD Cloning Kit (Clontech) with appropriate PCR primers. After sequence confirmation of the pac-FMDV2A-Gluc cassette, the vector sequence from the ATG start codon of puromycin-*N*-acetyl-transferase (pac) gene to the end of 5′NTR was amplified by inverse PCR and joined with a PCR product cDNA encompassing the nsP1–4 genes and the junction (J) region of the CHIKV SL11131 strain [10] using an In-Fusion HD Cloning Kit (designed as pT7/SL-Gluc).

A mutant CHIKV reporter construct, in which the catalytic Gly-Asp-Asp (GDD) motif in the RNA-dependent RNA polymerase (RdRp) of nsP4 was changed to Gly-Ala-Ala (GAA) [11], was generated by PCR-based site-directed mutagenesis (designated as pT7/SL-Gluc-nsP4^{GAA}).

The sequence of plasmid constructs was confirmed by an automated DNA sequencer. All plasmids were prepared using *Escherichia coli* strain DH5α, and PCR was performed using PrimeSTAR Max DNA Polymerase (Takara).

2.3. Production and validation of CHIKV reporter replicon RNA

The 5′-capped replicon RNA was transcribed *in vitro* from pT7/SL-Gluc and pT7/SL-Gluc-nsP4^{GAA}, which had been linearized with *Not*I (just downstream of a poly(A) tail [10]) using the mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific) and purified using a FastGene RNA Basic Kit (Nippon Genetics Co., Ltd.).

BHK-21 cells were seeded in a 24-well plate at a density of 5×10^4 cells/well one day prior to transfection and transfected with 200 ng of SL-Gluc or SL-Gluc-nsP4^{GAA} RNA using 0.3 μl of Lipofectamine MessengerMAX (Thermo Fisher Scientific). Two days after transfection, 20 μl of culture supernatant was mixed with 50 μl of a luciferase substrate of the Renilla Luciferase Assay System (Promega), which was compatible with the detection of Gluc activity, and the luciferase activity was measured using a GloMax-Multi$^+$ Detection System (Promega). In a parallel experiment, BHK-21 cells were co-transfected with 100 ng of SL-Gluc RNA and 25 pmol of an siRNA duplex targeting CHIKV nsP3 [12] or a negative control siRNA duplex [13].

For an immunofluorescent analysis (IFA) of CHIKV replicon, BHK-21 cells (5×10^4) were cultured on poly-L-lysine-treated glass coverslips in a 12-well plate one day before transfection and transfected with 1 μg of SL-Gluc RNA. Two days after transfection, cells on the coverslips were subjected to IFA in the procedure described previously [10] and observed under a BZ-X700 fluorescent microscope (Keyence). The first antibodies used were anti-double-stranded (ds)RNA mouse monoclonal (clone J2, English & Scientific Consulting Bt.) and in-house-generated anti-nsP2 rabbit polyclonal antibodies. The nsP2 antibody was produced through immunization of a rabbit with a synthetic peptide from CHIKV Ross nsP2 (amino acid position 121–139 [14]) and affinity-purified using a bacterially expressed maltose-binding protein-nsP2 helicase domain fusion protein and a HiTrap NHS-activated HP column (Cytiva). The secondary antibodies were Alexa Fluor 555–conjugated anti-mouse and Alexa Fluor 488–conjugated antibodies (Thermo Fisher Scientific). The slide was mounted using ProLong Glass Antifade Mountant containing NucBlue (Thermo Fisher Scientific).

2.4. Chemical library screening

BHK-21 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well one day prior to transfection and transfected with 100 ng of SL-Gluc RNA using 0.15 μl of Lipofectamine MessengerMAX. On the next day, the culture medium was exchanged with a fresh one containing 2% FBS and 10 μM compounds (Sigma-Aldrich, Cat. No. S990043-SIG1) or 0.1% DMSO control; two days after treatment, the Gluc activity of the culture supernatant was measured. The replicon RNA–transfected cell culture was further subjected to a cell viability assay using a CellTiter-Glo Luminescent Cell Viability Assay (Promega). Note that Gluc expression does not interfere with the CellTiter-Glo Luminescent Cell Viability Assay using a firefly luciferase (Fluc) because of the difference in substrate specificity between Gluc and Fluc [15].

2.5. Antiviral activity assays

Production and titration of infectious CHIKV were performed as described previously [10]. For the validation of candidate compounds obtained through chemical library screening, Vero cells were seeded in a 96-well plate at a density of 1×10^4 cells/well one day prior to infection and incubated with pyrilamine maleate salt (Sigma-Aldrich), CP-154526 hydrochloride (Sigma-Aldrich), veratridine (Calbiochem), tolcapone (Sigma-Aldrich), protriptyline hydrochloride (MedChemExpress), R121919 hydrochloride (Sigma-Aldrich), NGD 98-2 hydrochloride (Sigma-Aldrich), or 0.1% DMSO for 1 h, followed by exposure to CHIKV at a multiplicity of infection (MOI) of 1 for an additional 1 h. After infection, cells were cultured with 100 μl of MEM containing 10% FBS and respective compounds, and culture supernatants collected 24 h after infection were subjected to the plaque assay to measure the virus titer as described previously [10]. As for the assessment of CP-154,526 in human cells, Huh-7 cells were seeded in a 24-well plate at a density of 1×10^5 cells/well one day prior to infection and infected with CHIKV at an MOI of 0.1. After infection, cells were cultured with DMEM containing 2% FBS in the presence of CP-154,526 (or 0.1 DMSO), and culture supernatants collected 48 h after infection were subjected to the plaque assay.

In a time-of-addition assay, Vero cells were exposed to CHIKV (MOI of 1) in the presence (for the simultaneous treatment condition) or absence (for the post-treatment condition) of CP-154,526, and after 1 h, cells were cultured with CP-154,526. The virus titer in culture supernatants 24 h after infection was measured by the plaque assay.

The concentration of compounds used in each experiment is indicated in the corresponding figures.

2.6. Virus entry assay

Vero cells, seeded in a 24-well plate at a density of 2×10^5/well one day before assay, were inoculated with CHIKV at an MOI of 5 in the presence of 1 μM CP-154,526 or 0.1% DMSO at 37 °C for 1 h. Uninternalized virus particles were removed by washing the cells twice with cold PBS, followed by 0.25% trypsin-EDTA treatment at 37 °C for 5 min. After washing with cold PBS three more times, total RNA was extracted, and cell-associated CHIKV RNA was analyzed via RT-qPCR analysis using a CellAmp Direct Probe RT-qPCR Kit (Takara Bio) with CHIKV-specific primers and a fluorescent probe [16].

2.7. Statistical analysis

The average values were obtained using a representative set of at least three independent experiments and are shown with error bars indicating the standard deviation (SD) in the figures. Where indicated in the figures, statistical significance was determined using JMP Pro software (SAS Institute), and P values below 0.05 were considered significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3. Results

3.1. Establishment of a Gluc reporter CHIKV replicon system

To perform a screening assay for a search of chemicals that potentially inhibited CHIKV replication, we developed a subgenomic CHIKV replicon-expressing secretory Gluc reporter whose activity can be directly measured using culture supernatants [9]. In this study, the reporter replicon plasmid (pT7/SL-Gluc) generated based on a previously reported infectious clone of the CHIKV SL11131 strain [10] contained the 5′ half (5′NTR, nsP1–4, and J region) and 3′ terminus (3′NTR and a poly(A) tail) of the CHIKV genome [2], which were separated by a pac-FMDV2A-Gluc gene cassette (Fig. 1A). The CHIKV reporter replicon RNA genome was transcribed *in vitro* under the control of a T7 RNA promoter (Fig. 1A) and used for transfection experiments. Although we initially attempted to establish a cell line in which the CHIKV replicon RNA was persistently replicated using a *pac* gene (i.e., under the selection with puromycin), a stable cell line could not be created with any of the vertebrate cells (derived from humans and rodents) tested, presumably due to the cytopathic effects of CHIKV nsPs [17,18].

When the RNA transcripts were transiently transfected to BHK-21 cells, a time-dependent increase in Gluc activity was detected in the culture supernatants of wild-type (i.e., SL-Gluc)-transfected cells, whereas no increase in Gluc activity was observed with a mutant replicon, in which the GDD motif in the active site of the nsP4 RdRp had been changed to GAA (SL-Gluc-nsP4^{GAA}, Fig. 1A) [11], indicating that the increased Gluc activity was accompanied by the self-replication of SL-Gluc RNA (Fig. 1B). Co-transfection of SL-Gluc transcripts with an siRNA targeting the CHIKV nsP3 gene [12] revealed that the Gluc activity was decreased by the nsP3 siRNA, confirming that the inhibition of intracellular replicon RNA amplification was reflected by the level of Gluc expression in culture supernatants (Fig. 1C). Supporting this, a dose-dependent reduction in Gluc activity was observed in the transfected BHK-21 cells that were treated with ribavirin (Supplemental Fig. S1) [19]. In addition, positive signals of RNA amplification and nsP expressions were detected in the SL-Gluc RNA-transfected BHK-21 cells via IFA using anti-dsRNA and in-house-generated anti-nsP2 antibodies (Supplemental Fig. S2, Fig. 1D). These results demonstrate that the subgenomic CHIKV reporter replicon is suitable for the evaluation of intracellular replication of CHIKV.

3.2. Identification of anti-CHIKV compounds using a reporter replicon system

Employing the CHIKV reporter replicon system, we performed a primary screening assay using a chemical library consisting of 80 agonists and antagonists of cellular signaling pathways (purchased from Sigma-Aldrich, Japan). In this screening assay, 100 ng of SL-Gluc RNA was transfected to BHK-21 cells seeded in a 96-well plate, 10 μM compounds were added to the cultures, and Gluc activity was measured two days after chemical treatment. As a parallel experiment, cell viability of the CHIKV-infected and compound-treated cultures was evaluated using CellTiter-Glo. The results showed that a total of six compounds (pyrilamine maleate salt, CP-154,526 hydrochloride, veratridine, tolcapone, protriptyline hydrochloride, and imipramine hydrochloride) exhibited an approximately 50% or more reduction in Gluc activity with more than 90% cell viability when compared to the 0.1% DMSO-treated control culture (Fig. 2A).

Of the hit compounds, imipramine has been reported to inhibit CHIKV [20]. Hence, the anti-CHIKV activity of the other five candidate compounds was further validated using infectious CHIKV. Vero cells were infected with a CHIKV SL11131 infectious clone [10] at an MOI of 1 and cultured in the presence of 10 μM compounds. Measurements of the virus titer in culture supernatants via plaque assay and of the cell viability using CellTiter-Glo 24 h after infection showed that the five candidate compounds significantly inhibited the replication of CHIKV in Vero cells (Fig. 2B) without a loss of cell viability (Fig. 2C). However, among these candidate compounds, we focused on CP-154,526 in the following experiments, as this compound exhibited the strongest antiviral activity against CHIKV (Fig. 2B).

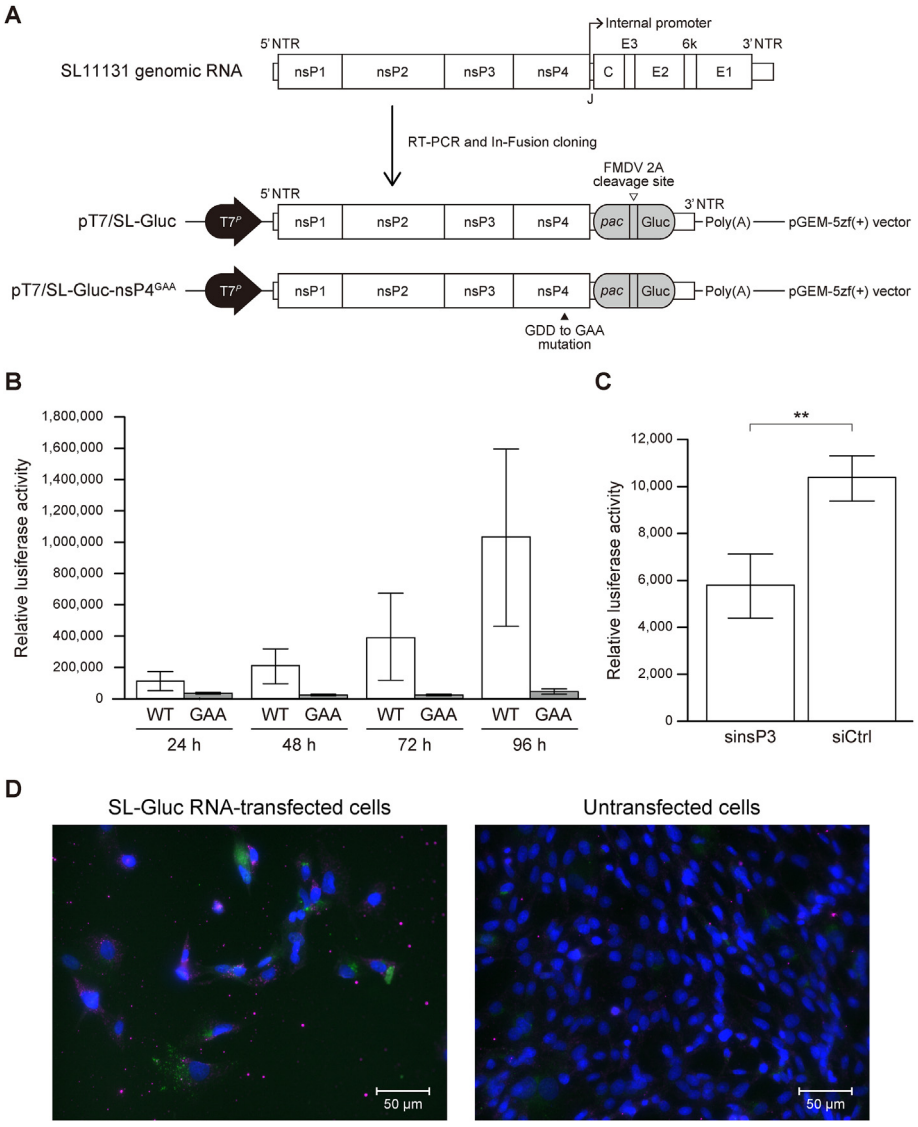

Fig. 1. Development of CHIKV reporter replicon system. (A) Schematic representations of CHIKV Gluc replicons. The viral sequence covering the nsP1 to the J region of the CHIKV SL11131 genome and a cassette sequence containing puromycin resistance (pac), the FMDV2A cleavage site, and Gluc genes were generated via PCR and inserted between the 5′NTR and 3′NTR in a pGEM-5Zf(+) vector. As a replication-defective mutant replicon, the catalytic GDD motif in nsP4 was changed to GAA in the pT7/SL-Gluc-nsP4^{GAA} construct. (B) Activity of the CHIKV reporter replicon. Replicon RNA was transcribed *in vitro* from *Not*I-digested pT7/SL-Gluc (wild-type [WT], white bars) or pT7/SL-Gluc-nsP4^{GAA} (GAA, gray bars) and transfected to BHK-21 cells. The culture supernatant of the transfected cells was collected every 24 h and subjected to a Gluc activity assay. The average values of the Gluc (relative luciferase) activity from three transfections are shown with the SD. (C) Reduction of replicon activity by CHIKV-targeting siRNA. WT replicon (SL-Gluc) RNA was co-transfected with an siRNA duplex against CHIKV nsP3 (sinsP3) or nonspecific siRNA (siCtrl), and two days after transfection, Gluc activity in the culture supernatant was measured. The average values of the Gluc activity from three experiments are shown with the SD. Statistical significance was determined using Student's *t*-test. (D) IFA to detect dsRNA and nsP2 expressions in CHIKV replicon RNA-transfected cells. SL-Gluc RNA-transfected (left panel) and untransfected (right panel) BHK-21 cells were subjected to IFA using anti-dsRNA (magenta) and CHIKV nsP2 (green) antibodies two days after transfection. Cell nuclei were stained with NucBlue (Blue).

3.3. Inhibition of CHIKV replication by CP-154,526

CP-154,526 (IUPAC name: *N*-butyl-*N*-ethyl-2,5-dimethyl-7-(2,4,6-trimethylphenyl)pyrrolo [2,3-d]pyrimidin-4-amine, Fig. 3A) is a non-peptide corticotropin-releasing factor receptor type-1 (CRF-R1) antagonist, which has been developed for the treatment of depression [21,22]. However, the antiviral activity of CP-154,526 has not been reported. When CHIKV-infected Vero cells were treated with different concentrations (10−0.1 μM) of CP-154,526, dose-dependent inhibition of CHIKV replication was observed 24 h after infection, while cell viability remained over 90% with these concentrations when compared to the control (i.e., 0.1% DMSO)-treated Vero cells (Fig. 3B). The inhibitory concentration of 50% (IC_{50}) of CP-154,526 in the inhibition of CHIKV was determined to be 0.39 ± 0.20 μM. Furthermore, the anti-CHIKV activity of CP-154,526 was also confirmed in a human Huh-7 cell line (Fig. 3C).

3.4. Anti-CHIKV activity of CRF-R1 antagonists

The CRF pathway is proposed as a future therapeutic target for the treatment of patients with anxiety and depressive disorders, and several CRF-R1 antagonists other than CP-154,526 have been developed [23]. Hence, we evaluated the anti-CHIKV activities of other CRF-R1 antagonists. When CHIKV-infected Vero cells were treated with R121919 [24], a compound structurally similar to CP-154,526, a significant reduction in viral titer was observed

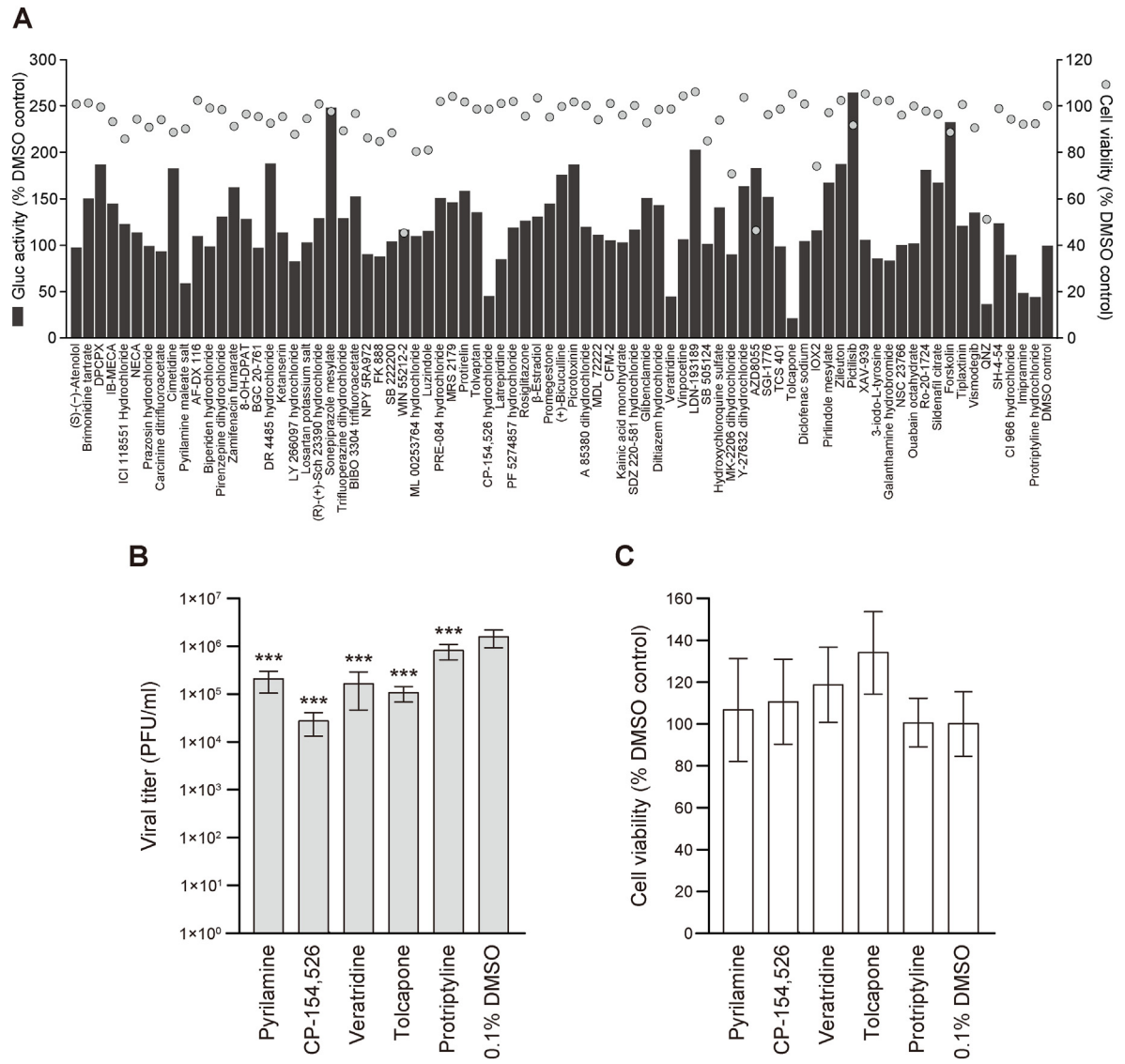

Fig. 2. Identification of anti-CHIKV compounds. (A) Primary screen using reporter replicon. BHK-21 cells were transfected with SL-Gluc RNA in a 96-well plate, and on the next day, 80 chemical compounds were added to each well at a final concentration of 10 μM in the presence of 0.1% DMSO. Two days after treatment, the Gluc activity in culture supernatants was measured, followed by a cell viability assay of the compound-treated cells using a CellTiter-Glo Luminescent Cell Viability Assay Kit. The Gluc activity (black bars) and cell viability (gray circles) of each compound-treated cell are expressed as relative percentages over that of the DMSO-treated control condition (rightmost bar and circle). (B and C) Validation of candidate compounds in the inhibition of CHIKV replication. Vero cells were pretreated with the five compounds (10 μM) identified in the reporter replicon–based screening (A) and exposed to infectious CHIKV (SL11131 strain) at an MOI of 1, followed by cultivation with the respective compounds. The virus titer in culture supernatants at 24 h after infection was measured via plaque assay. The average values of the infectious titer (PFU/ml) from eight independent infections are shown with the SD. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparison test (B). Also at 24 h after infection, the viability of the CHIKV-infected and compound-treated cells was analyzed (C). Cell viability was expressed as relative mean percentages with SD over that of DMSO-treated control cells.

without cell toxicity (Fig. 4A). Interestingly, another CRF-R1 antagonist, NGD 98-2 [25], which was structurally different from CP-154,526, from compounds, also exhibited a significant suppressive effect against CHIKV in a dose-dependent manner (Fig. 4B), suggesting that CRF-R1 antagonists had a common mechanism of action for the inhibition of CHIKV infection.

To investigate whether the anti-CHIKV activity of CP-154,526 was the result of inhibition against viral entry or the post-entry step, a time-of-addition assay was conducted by treating Vero cells with CP-154,526 at the time of virus infection (simultaneous treatment condition [S]) or after virus infection (post-treatment condition [P]). The results showed that CP-154,526 still significantly inhibited CHIKV replication in Vero cells even at the treatment after 1 h of infection, and no significant difference was observed in the inhibition efficiency between simultaneous treatment and post-treatment of CP-154,526 (Fig. 4C). To confirm the observation that the post-entry process of CHIKV replication was inhibited by CP-154,526, an RT-PCR-based virus entry assay was performed. Vero cells were exposed to CHIKV with an MOI of 5 at 37 °C for 1 h in the presence of 1 μM CP-154,526, which allowed binding and internalization of the virions. Then cells were treated with trypsin to remove uninternalized viruses and washed with cold PBS. RT-qPCR analysis to measure the level of viral RNA associated with cells showed that the binding and entry steps of CHIKV infection were not influenced by treatment with CP-154,526 (Fig. 4D). These results indicated that CP-154,526 suppressed CHIKV at a post-entry

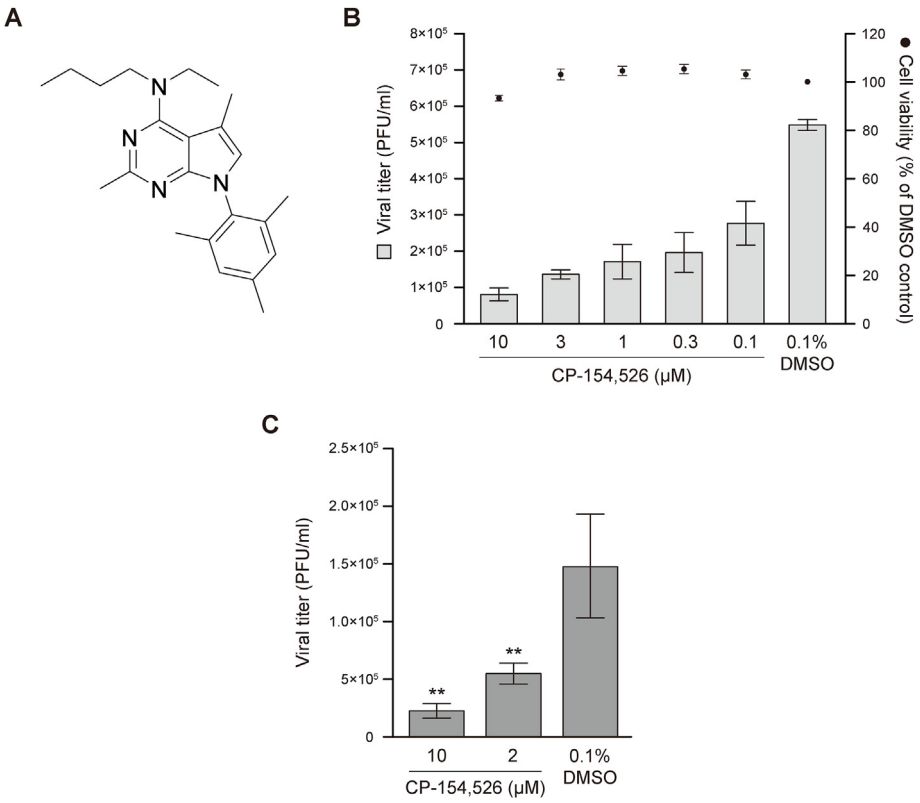

Fig. 3. Antiviral activity of CP-154,526 against CHIKV. (A) Chemical structure of CP-154,526. (B) Dose-dependent inhibition of CHIKV infection by CP-154,526. Vero cells were infected with CHIKV at an MOI of 1 and cultured in the presence of serial dilutions of CP-154,526 or 0.1% DMSO. Twenty-four hours after infection, culture supernatants were harvested, and the virus titer was measured via plaque assay (gray bars). As a parallel experiment, the cell viability in respective cultures was analyzed (black circles). The average values from three independent infections are shown with the SD. (C) Anti-CHIKV activity of CP-154,526 on human cells. Huh-7 cells were infected with CHIKV at an MOI of 1 and cultured in the presence of CP-154,526 (10 and 2 μM) or 0.1% DMSO. The virus titer in the culture supernatant 48 h after infection was measured via plaque assay. The average values of the infectious titer from three independent infections are shown with the SD. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparison test.

step of infection, which was in accordance with the data that identified CP-154,526 as an inhibitor against the activity of a subgenomic replicon lacking structural genes (Fig. 2).

4. Discussion

Subgenomic replicon systems, which are engineered by replacing structural genes with reporter genes, have been widely used for drug discovery studies for many RNA viruses [26]. Since the replicon RNA does not produce infectious virions, it is particularly useful when manipulating of the infectious virus that requires higher biosafety levels for containment, such as for the BSL-3 pathogen CHIKV. As such, a reporter replicon has also been used to evaluate antiviral agents against CHIKV [17,20,27–29]. In this study, we generated a CHIKV subgenomic replicon harboring a Gluc gene (Fig. 1) and performed an inhibitor screening with the Gluc reporter secreted into the supernatant of replicon RNA-transfected BHK-21 cells as an assay readout (Fig. 2A). The result showed that six chemical compounds (pyrilamine maleate salt, CP-154,526 hydrochloride, veratridine, tolcapone, protriptyline hydrochloride, and imipramine hydrochloride) reduced the Gluc activity (Fig. 2A). Importantly, the anti-CHIKV activity of imipramine has been reported using an infectious virus and a reporter replicon system [20]. In addition, the remaining five compounds were shown to suppress the replication of infectious CHIKV (Fig. 2B). These data demonstrated that our Gluc-expressing replicon was appropriate for the identification of antiviral agents inhibiting CHIKV infection.

Among the other candidate compounds inhibiting CHIKV, tolcapone, an FDA (U.S. Food and Drug Administration)-approved drug for the treatment of Parkinson's disease, was reported to inhibit the protease activity of dengue virus (DENV) and West Nile virus (WNV) [30] and also block the entry of the Ebola virus (EBOV) [31]. Another FDA-approved drug, pyrilamine, which is a selective histamine H1 receptor antagonist, was shown to have a suppressive effect on the endothelial lesions of the cornea produced by Newcastle Disease Virus (NDV) on rabbit eyes, though the suppression might not be a consequence of direct inhibition of NDV replication [32]. Although the antiviral activity of veratridine, protriptyline, and CP-154,526 has not been reported, this study focused on CP-154,526 since this compound exhibited the strongest antiviral activity among the hit compounds (Fig. 2A) and revealed that CP-154,526 efficiently inhibited the replication of CHIKV (Fig. 3). CP-154,526 blocks the binding of CRF (also known as the corticotropin-releasing hormone [CRH]) to its G-protein coupled receptors, CRF-R1 and CRF-R2. In the central nervous system (CNS), the CRF-CRF-R interaction induces the release of the adrenocorticotropin hormone (ACTH) from the anterior pituitary, which stimulates the adrenal glands to produce corticosteroids [33]. Since the over-production of CRF was shown to induce physiological and behavioral phenotypes of anxiety and depressive disorders in rodents, the CRF-CRF-R signaling system was suggested to be related to stress-related disorders [22].

Of particular interest was that other CRF-R1 antagonists, regardless of whether they were structurally similar to (R121919) or different from (NGD 98-2) CP-154,526, also exhibited significant inhibitory activity against CHIKV (Fig. 4A and B). Hence, one might

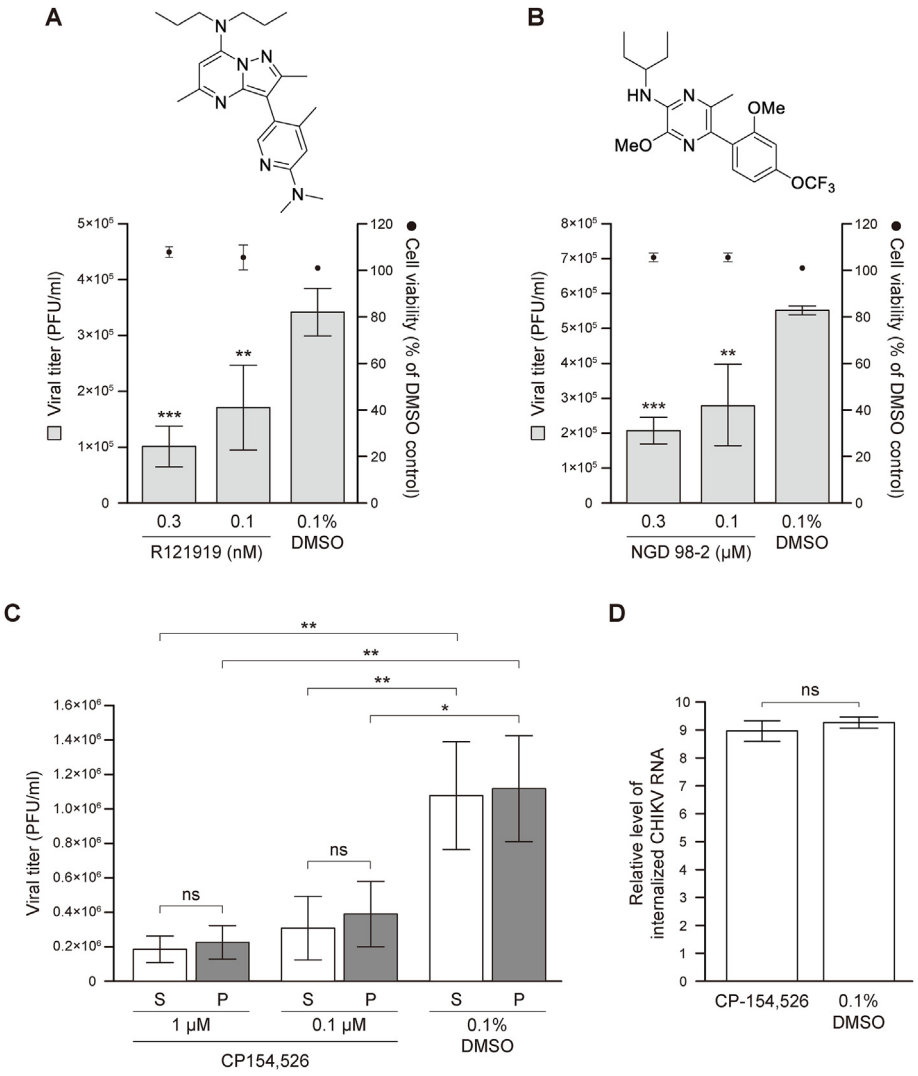

Fig. 4. Effect of CRF-R1 antagonists on CHIKV replication. (A and B) Inhibitory activities of CRF-R1 antagonists against CHIKV. Vero cells were infected with CHIKV and cultured in the presence of 0.1 and 0.3 μM R121919 (A), NGD 98-2 (B), or 0.1% DMSO. The virus titer in culture supernatants (gray bars) and cell viability (black circles) 24 h after infection are shown. Chemical structures of R121919 and NGD 98-2 are also indicated. (C) Time-of-addition assay of CP-154,526. Vero cells were exposed to CHIKV in the presence (simultaneous treatment [S]) or absence (post-treatment [P]) of CP-154,526 (1 and 0.1 μM) for 1 h, and after removing the virus, cells were cultured with CP-154,526. The virus titer in culture supernatants 24 h after infection was measured via plaque assay. (D) Entry assay of CHIKV. Vero cells were exposed to CHIKV at an MOI of 5 in the presence of 1 μM CP-154,526 or 0.1% DMSO at 37 °C for 1 h. After washing with ice-cold PBS and trypsin-EDTA, total RNA was extracted from the cells and subjected to RT-qPCR analysis using CHIKV-specific primers and a fluorescent probe. All data are shown as the average values from three independent experiments with SD. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparison test (A, B, and C) and Student's *t*-test (D). ns, no significance.

assume that CRF-R1 was involved in the entry process of CHIKV, and CRF-R1 antagonists including CP-154,526 interfered with the binding of the CHIKV virion to target cells. However, the result of the time-of-addition experiment showed no difference in the inhibitory effect of CP-154,526 between the treatment at the time of CHIKV infection and that post-infection (Fig. 4C). In addition, the RT-qPCR-based virus entry assay revealed that CP-154,526 treatment did not influence the CHIKV binding/entry process (Fig. 4D). Supporting this, the replication efficiency of CHIKV in Huh-7 cells was not changed by the transfection of CRF-R1-specific siRNA (Supplemental Fig. S3). These data demonstrated that CP-154,526 inhibited a post-entry step of CHIKV infection, and CRF-R1 was not likely to be involved in the inhibitory mechanism. Therefore, a plausible explanation for the anti-CHIKV activity of CRF-R1 antagonists would be that these compounds (CP-154,526, R121919, and NGD 98-2) may intracellularly recognize and interfere with another (cellular or viral) molecule, which plays an important role in CHIKV replication, thereby blocking a step during viral RNA and functional protein events. Future studies will be needed to clarify the common mechanism by which CRF-R1 antagonists inhibit the post-entry process of CHIKV infection.

In conclusion, this study presented the generation of a novel reporter CHIKV replicon system and its successful use for the evaluation of anti-CHIKV agents. Importantly, compound screening using the replicon system enabled us to identify several new antiviral compounds whose inhibitory activity against CHIKV replication has not been previously reported. Further analysis of the identified candidate inhibitors revealed that CRF-R1 antagonists potentially suppressed CHIKV infection. In addition, our Gluc-based replicon reflected the aspects of intracellular replication of CHIKV, which will have broad applications for investigating in the virological features of CHIKV and the understanding of host-virus interactions during CHIKV infection.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Youichi Suzuki reports financial support was provided by Japan Agency for Medical Research and Development.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.11.013.

References

[1] T. Couderc, M. Lecuit, Chikungunya virus pathogenesis: from bedside to bench, Antivir. Res. 121 (2015) 120–131. https://10.1016/j.antiviral.2015.07.002.
[2] M. Solignat, B. Gay, S. Higgs, L. Briant, C. Devaux, Replication cycle of chikungunya: a re-emerging arbovirus, Virology 393 (2009) 183–197. https://10.1016/j.virol.2009.07.024.
[3] T. Ahola, G. McInerney, A. Merits, Alphavirus RNA replication in vertebrate cells, Adv. Virus Res. 111 (2021) 111–156. https://10.1016/bs.aivir.2021.07.003.
[4] R. Abdelnabi, J. Neyts, L. Delang, Chikungunya virus infections: time to act, time to treat, Curr. Opin. Virol. 24 (2017) 25–30. https://10.1016/j.coviro.2017.03.016.
[5] S.C. Weaver, N.L. Forrester, Chikungunya: evolutionary history and recent epidemic spread, Antivir. Res. 120 (2015) 32–39. https://10.1016/j.antiviral.2015.04.016.
[6] F. Amraoui, A.B. Failloux, Chikungunya: an unexpected emergence in Europe, Curr. Opin. Virol. 21 (2016) 146–150. https://10.1016/j.coviro.2016.09.014.
[7] L.H. Chen, M.E. Wilson, Dengue and chikungunya infections in travelers, Curr. Opin. Infect. Dis. 23 (2010) 438–444. https://10.1097/QCO.0b013e32833c1d16.
[8] R. Abdelnabi, J. Neyts, L. Delang, Towards antivirals against chikungunya virus, Antivir. Res. 121 (2015) 59–68. https://10.1016/j.antiviral.2015.06.017.
[9] F. Kato, T. Kobayashi, S. Tajima, T. Takasaki, T. Miura, T. Igarashi, T. Hishiki, Development of a novel Dengue-1 virus replicon system expressing secretory Gaussia luciferase for analysis of viral replication and discovery of antiviral drugs, Jpn. J. Infect. Dis. 67 (2014) 209–212. https://10.7883/yoken.67.209.
[10] Y. Suzuki, A. Tanaka, Y. Maeda, A. Emi, Y. Fujioka, S. Sakaguchi, S.G. Vasudevan, T. Kobayashi, C.K. Lim, T. Takasaki, H. Wu, T. Nakano, Construction and characterization of an infectious clone generated from Chikungunya virus SL11131 strain, Virology 552 (2021) 52–62. https://10.1016/j.virol.2020.09.006.
[11] A. Utt, T. Quirin, S. Saul, K. Hellstrom, T. Ahola, A. Merits, Versatile trans-replication systems for Chikungunya virus allow functional analysis and tagging of every replicase protein, PLoS One 11 (2016), e0151616. https://10.1371/journal.pone.0151616.
[12] P.K. Dash, M. Tiwari, S.R. Santhosh, M. Parida, P.V. Lakshmana Rao, RNA interference mediated inhibition of Chikungunya virus replication in mammalian cells, Biochem. Biophys. Res. Commun. 376 (2008) 718–722. https://10.1016/j.bbrc.2008.09.040.
[13] Y. Suzuki, W.X. Chin, Q. Han, K. Ichiyama, C.H. Lee, Z.W. Eyo, H. Ebina, H. Takahashi, C. Takahashi, B.H. Tan, T. Hishiki, K. Ohba, T. Matsuyama, Y. Koyanagi, Y.J. Tan, T. Sawasaki, J.J. Chu, S.G. Vasudevan, K. Sano, N. Yamamoto, Characterization of RyDEN (C19orf66) as an interferon-stimulated cellular inhibitor against dengue virus replication, PLoS Pathog. 12 (2016), e1005357. https://10.1371/journal.ppat.1005357.
[14] S. Chattopadhyay, A. Kumar, P. Mamidi, T.K. Nayak, I. Das, J. Chhatai, I. Basantray, U. Bramha, P.K. Maiti, S. Singh, A.R. Suryawanshi, S. Chattopadhyay, Development and characterization of monoclonal antibody against non-structural protein-2 of Chikungunya virus and its application, J. Virol. Methods 199 (2014) 86–94. https://10.1016/j.jviromet.2014.01.008.
[15] A.A. Capul, J.C. de la Torre, A cell-based luciferase assay amenable to high-throughput screening of inhibitors of arenavirus budding, Virology 382 (2008) 107–114. https://10.1016/j.virol.2008.09.008.
[16] Y. Suzuki, M. Kotoura, S. Yashima, H. Wu, T. Nakano, K. Sano, Measuring dengue virus RNA in the culture supernatant of infected cells by real-time quantitative polymerase chain reaction, JoVE (2018), https://doi.org/10.3791/58407.
[17] L. Pohjala, A. Utt, M. Varjak, A. Lulla, A. Merits, T. Ahola, P. Tammela, Inhibitors of alphavirus entry and replication identified with a stable Chikungunya replicon cell line and virus-based assays, PLoS One 6 (2011), e28923. https://10.1371/journal.pone.0028923.
[18] A. Utt, P.K. Das, M. Varjak, V. Lulla, A. Lulla, A. Merits, Mutations conferring a noncytotoxic phenotype on chikungunya virus replicons compromise enzymatic properties of nonstructural protein 2, J. Virol. 89 (2015) 3145–3162. https://10.1128/JVI.03213-14.
[19] P. Leyssen, J. Balzarini, E. De Clercq, J. Neyts, The predominant mechanism by which ribavirin exerts its antiviral activity in vitro against flaviviruses and paramyxoviruses is mediated by inhibition of IMP dehydrogenase, J. Virol. 79 (2005) 1943–1947. https://10.1128/JVI.79.3.1943-1947.2005.
[20] S. Wichit, R. Hamel, E. Bernard, L. Talignani, F. Diop, P. Ferraris, F. Liegeois, P. Ekchariyawat, N. Luplertlop, P. Surasombatpattana, F. Thomas, A. Merits, V. Choumet, P. Roques, H. Yssel, L. Briant, D. Misse, Imipramine inhibits Chikungunya virus replication in human skin fibroblasts through interference with intracellular cholesterol trafficking, Sci. Rep. 7 (2017) 3145. https://10.1038/s41598-017-03316-5.
[21] D.W. Schulz, R.S. Mansbach, J. Sprouse, J.P. Braselton, J. Collins, M. Corman, A. Dunaiskis, S. Faraci, A.W. Schmidt, T. Seeger, P. Seymour, F.D. Tingley 3rd, E.N. Winston, Y.L. Chen, J. Heym, CP-154,526: a potent and selective non-peptide antagonist of corticotropin releasing factor receptors, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 10477–10482. https://10.1073/pnas.93.19.10477.
[22] M.B. Muller, M. Uhr, F. Holsboer, M.E. Keck, Hypothalamic-pituitary-adrenocortical system and mood disorders: highlights from mutant mice, Neuroendocrinology 79 (2004) 1–12. https://10.1159/000076041.
[23] S.R. Spierling, E.P. Zorrilla, Don't stress about CRF: assessing the translational failures of CRF1antagonists, Psychopharmacology (Berl) 234 (2017) 1467–1481. https://10.1007/s00213-017-4556-2.
[24] C. Chen, K.M. Wilcoxen, C.Q. Huang, Y.F. Xie, J.R. McCarthy, T.R. Webb, Y.F. Zhu, J. Saunders, X.J. Liu, T.K. Chen, H. Bozigian, D.E. Grigoriadis, Design of 2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylaminopyrazolo [1,5-a]pyrimidine (NBI 30775/R121919) and structure–activity relationships of a series of potent and orally active corticotropin-releasing factor receptor antagonists, J. Med. Chem. 47 (2004) 4787–4798. https://10.1021/jm040058e.
[25] K.J. Hodgetts, P. Ge, T. Yoon, S. De Lombaert, R. Brodbeck, M. Gulianello, A. Kieltyka, R.F. Horvath, J.H. Kehne, J.E. Krause, G.D. Maynard, D. Hoffman, Y. Lee, L. Fung, D. Doller, Discovery of N-(1-ethylpropyl)-[3-methoxy-5-(2-methoxy-4-trifluoromethoxyphenyl)-6-methyl-pyra zin-2-yl]amine 59 (NGD 98-2): an orally active corticotropin releasing factor-1 (CRF-1) receptor antagonist, J. Med. Chem. 54 (2011) 4187–4206. https://10.1021/jm200365y.
[26] R.S. Fernandes, M. Freire, R.V. Bueno, A.S. Godoy, L. Gil, G. Oliva, Reporter replicons for antiviral drug discovery against positive single-stranded RNA viruses, Viruses 12 (2020) 598. https://10.3390/v12060598.
[27] F.S. Varghese, P. Kaukinen, S. Glasker, M. Bespalov, L. Hanski, K. Wennerberg, B.M. Kummerer, T. Ahola, Discovery of berberine, abamectin and ivermectin as antivirals against chikungunya and other alphaviruses, Antivir. Res. 126 (2016) 117–124. https://10.1016/j.antiviral.2015.12.012.
[28] R. Lani, P. Hassandarvish, C.W. Chiam, E. Moghaddam, J.J. Chu, K. Rausalu, A. Merits, S. Higgs, D. Vanlandingham, S. Abu Bakar, K. Zandi, Antiviral activity of silymarin against chikungunya virus, Sci. Rep. 5 (2015) 11421. https://10.1038/srep11421.
[29] I.C. Albulescu, M. van Hoolwerff, L.A. Wolters, E. Bottaro, C. Nastruzzi, S.C. Yang, S.C. Tsay, J.R. Hwu, E.J. Snijder, M.J. van Hemert, Suramin inhibits chikungunya virus replication through multiple mechanisms, Antivir. Res. 121 (2015) 39–46. https://10.1016/j.antiviral.2015.06.013.
[30] A. Balasubramanian, M. Manzano, T. Teramoto, R. Pilankatta, R. Padmanabhan, High-throughput screening for the identification of small-molecule inhibitors of the flaviviral protease, Antivir. Res. 134 (2016) 6–16. https://10.1016/j.antiviral.2016.08.014.
[31] M. Qiu, Z. Li, Y. Chen, J. Guo, W. Xu, T. Qi, Y. Qiu, J. Pang, L. Li, S. Liu, S. Tan, Tolcapone potently inhibits seminal amyloid fibrils formation and blocks entry of ebola pseudoviruses, Front. Microbiol. 11 (2020) 504. https://10.3389/fmicb.2020.00504.
[32] J.O. Oh, C.A. Evans, Suppressive effects of pyrilamine maleate and d-lysergic acid diethylamide (LSD-25) on early corneal lesions produced *in vitro* by Newcastle disease virus (NDV) and compound 48/80, Virology 10 (1960) 127–143. https://10.1016/0042-6822(60)90011-8.
[33] J.J. Bonfiglio, C. Inda, D. Refojo, F. Holsboer, E. Arzt, S. Silberstein, The corticotropin-releasing hormone network and the hypothalamic-pituitary-adrenal axis: molecular and cellular mechanisms involved, Neuroendocrinology 94 (2011) 12–20. https://10.1159/000328226.