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Discovery of a novel antiviral agent for chikungunya virus

（新規抗チクングニアウイルス化合物の探索と解析）

Yuji WADA

Contents

Abbreviations

BFV	Barmah Forest virus
BHK-21	baby hamster kidney cell line
BSA	bovine serum albumin
BVDV	bovine viral diarrhea virus
cDNA	complementary deoxyribonucleic acid
CC_{50}	50% cytotoxicity concentration
CHIKF	chikungunya fever
CHIKV	chikungunya virus
CPE	cytopathic effect
DAPI	4′,6-diamidino-2-phenylindole
DENV	dengue virus
DMEM	Dulbecco's modified eagle medium
E.coli	*Escherichia coli*
ECSA	East/Central/South African
EC_{30}	30% effective concentration
EC_{50}	50% effective concentration
EEEV	eastern equine encephalitis virus
E2	envelope protein 2
FCS	fetal calf serum
GETV	Getah virus
HCl	hydrochloric acid
HCV	hepatitis C virus

HEK-293T	human embryonic kidney cell line
h.p.i.	hours post infection
h.p.t.	hours post transfection
IgG	immunoglobulin G
IOL	Indian Ocean lineage
MAYV	Mayaro virus
MOI	multiplicity of infection
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
nsP	nonstructural protein
ONNV	o'nyong-nyong virus
PBS	phosphate buffered saline
PBS-T	PBS containing 0.01% Tween 20
PCR	polymerase chain reaction
PFU	plaque forming unit
PV	poliovirus
res-CHIKV	resistant CHIKV clone
rCHIKV	recombinant CHIKV
RdRp	RNA-dependent RNA-polymerase
rep-CHIKV	CHIKV replicon
RRV	Ross River virus
RV	rubella virus
SD	standard deviation
SFV	Semliki Forest virus

SINV	Sindbis virus
sP	structural protein
$TCID_{50}$	median tissue culture infective dose
VEEV	Venezuelan equine encephalitis virus
WEEV	western equine encephalitis virus

Preface

Chikungunya virus (CHIKV) is a plus sense, single-stranded RNA virus belonging to the genus *Alphavirus*, family *Togaviridae*. The genome is composed of two open reading flames; one encoding a nonstructural polyprotein which is post-translationally processed to four nonstructural proteins (nsP1-4) that are involved in genome replication, and a second region encoding a structural polyprotein which is also processed to structural proteins (sP) and forms viral particles which are composed of a capsid and two envelope proteins [1-3].

Chikungunya fever (CHIKF) is a re-emerging mosquito-borne zoonosis caused by CHIKV infection [4]. The major symptoms of CHIKF are an acute febrile illness with arthralgia, myalgia, rash, lymphopenia, thrombocytopenia and gastrointestinal symptoms [4-7]. The arthralgia is most typical and characteristic symptom for CHIKF patients and can become chronic in significant numbers of patients persisting over years as a rheumatoid-like disorder [8, 9]. This painful, persistent arthralgia caused by CHIKF is a major factor threatening the quality of life of patients and the economy in affected countries or regions [10-12]. Outbreaks of CHIKF have occurred worldwide since 2004, in Africa, Asia and North, Central and South America [9, 13-18]. Viral genome mutations is thought to be one factor in the re-emergence of CHIKF, which have resulted in an increased efficiency of viral transmission in different mosquito vectors and/or possibly an increased virulence in humans [19, 20].

Currently there are no licensed prophylactic vaccines or chemotherapeutic options available for prevention or treatment [5, 21-23]. Vaccine development is

relatively progressed than drug development because two vaccine candidates have showed promising results in phase I clinical studies [2, 24, 25]. However, those vaccine candidates face to some difficulties being licensed [22]. For example, clinical efficacy trials should be done in unpredictable outbreaks which usually finished short duration for assessment. It should be also considered the co-prevalence of other arboviral diseases including dengue fever and zika fever for proofing vaccine effectiveness. Considering this situation, it would be important to develop alternative strategy countering the burden of CHIKF in addition to vaccine development.

The development of antiviral therapeutics for CHIKF is still in early development [5, 21, 23]. Recently, a lot of candidates which can inhibit CHIKV infection have been reported; however, the basis of their inhibitory effects have not been fully elucidated [2, 21]. Several studies have also suggested that the development of chronic arthralgia is related to the severity of the symptoms during the acute phase [26-29]. Therefore, effective antiviral treatment during the acute phase could potentially reduce symptoms not only in the acute but also the chronic phases.

The age of patients is recognized as risk factor in clinical investigations in recent CHIKF outbreaks [5, 30]. The CHIKF outbreaks in Reunion island and India both in 2006 suggested neurological manifestations are frequently observed in infants and children [31-33]. It was also indicated that adults aged over 65 frequently showed atypical and/or severe CHIKF symptoms, probably due to comorbidities and/or immunosenescence [34, 35]. To protect those risked groups

from CHIKF, the need of effective therapeutics is warranted in addition to vaccine development.

In chapter 1 of this thesis, chemical compound libraries were screened by cell viability assay to find novel antivirals, and a compound (designated Compound-A) inhibiting CHIKV infection at nanomolar concentration was discovered from a benzimidazole compound library. The inhibitory effect of Compound-A was confirmed by other assessments evaluating viral protein expression level and viral growth efficiency. The evaluation of antiviral activity of Compound-A using multiple strains and genera of viruses suggested that the inhibitory effect of Compound-A was specific to alphaviruses. Furthermore, a key chemical structure for anti-CHIKV activity of Compound-A was identified in the investigation of the benzimidazole compound library.

In chapter 2, the mechanism of action of inhibitory effect of Compound-A was investigated, and identified as a potent RNA-dependent RNA-polymerase (RdRp) inhibitor targeting methionine residue on nsP4 of CHIKV. Amino acid sequence analysis revealed that the methionine residue was located in one of functional motifs of RdRp and conserved in alphaviruses.

Chapter 1

Discovery of Compound-A: potential drug candidate for CHIKF

Introduction

CHIKF cause painful arthralgia to patient which sometime persists over months or years [8, 9]. The prolonged arthralgia caused by CHIKF threated the quality of life of the patients and economy in affected countries or regions [10-12]. Infants and adults aged over 65 are recognized as risked groups of CHIKF, and these aged patients have frequently showed severe and/or chronic CHIKF symptoms with some lethal cases in recent outbreaks [31-35]. However, no therapeutic option or prophylactic measure has been established [5, 21-23]. Several studies have suggested that the severity of the symptoms during acute phase would be related to the development of chronic arthralgia [26-29]. Thus, development of antiviral treatments has potential to improve CHIKF symptoms in both the acute and chronic phases.

To explore the novel drug candidate for CHIKF, chemical compound libraries were screened by cell viability assay using an epidemic strain of CHIKV in recent outbreaks. The inhibitory effect of a detected compound was confirmed by multiple methods, and was suggested to be specific for alphaviruses. Moreover, a key chemical structure for anti-CHIKV activity of the compound was identified.

Materials and Methods

Cells and viruses

Vero cells were obtained from the Japan Health Sciences Foundation (Tokyo, Japan). BHK-21 cells were obtained from the Shionogi & Co., LTD. (Osaka, Japan). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 unit/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in 5% CO_2.

CHIKV-SL10571 (AB455494), CHIKV-BaH306, dengue virus (DENV) serotype 1 hu/PHL/10-07 and DENV serotype 2 hu/INDIA/09-74 strains were kindly provided by Dr. Tomohiko Takasaki (National Institute of Infectious Diseases, Tokyo, Japan) [36, 37]. The both of DENV strains were clinically isolated in Japan. CHIKV-S27 strain (NC004162) was kindly provided by Dr. Kouichi Morita (Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan) [38]. Sindbis virus (SINV)-Ar339 strain was obtained from the American Type Culture Collection (Manassas, VA, USA).

Compound libraries

Chemical compound libraries and T-705 were synthesized and provided by the Shionogi & Co., Ltd (Toyonaka, Japan).

Cell viability assays

The MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay was employed to examine cell viability following virus infection using

methods as previously described [39].

In the case of the CHIKVs and SINV, compounds were serially diluted in DMEM containing 2% FCS in 96-well cell culture plates (50 μl /well). Vero cells containing 3×10^4 cells (100 μl) were added to each well containing diluted compound solutions and incubated for 1 hour. Thereafter, 10 median tissue culture infective dose ($TCID_{50}$) of CHIKV or SINV was added into each well and incubated for 3 days (finally 200 μl /well). The MTT reagent (Sigma-Aldrich, St. Louis, MO, USA, 5 mg/ml) was then added to each well and incubated for 1 hour. Culture supernatants (150 μl) were then discarded and 150 μl of formazan dissolution reagent (2-propanol with 10% Triton-X100 and 0.28% HCl) added to each well and incubated overnight at room temperature. The absorbance intensity was measured at a 570 nm wavelength with a 630 nm reference wavelength using a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). T-705 was used as positive control, as it has been previously reported to inhibit CHIKV infection [39]. Each assay was performed in duplicate and independently at least 3 times. The 50% effective concentration (EC_{50}), 30% effective concentration (EC_{30}) and 50% cytotoxicity concentration (CC_{50}) were calculated from the results of MTT assay.

In the case of assays for DENVs, compounds were serially diluted in DMEM containing 5% FCS in 96-well cell culture plates (50 μl /well). BHK-21 cells containing 2×10^4 cells (100 μl) were added to each well containing diluted compound solutions and incubated for 1 hour. Thereafter, 10 $TCID_{50}$ of DENVs was added into each well and incubated for 4 days (finally 200 μl /well).

Generation of recombinant CHIKVs (rCHIKVs) by reverse genetics

Recombinant CHIKVs (rCHIKVs) were synthesized following to previously report [40]. CHIKV-SL10571 genomic RNA was extracted using the High Pure Viral RNA kit (Roche Diagnostics, Basel, Switzerland), and cDNAs generated with random hexamers and SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). The viral genome was amplified by PrimeSTAR Max (Takara Bio, Shiga, Japan), and the T7 promoter with *Not* I restriction sites being added at the 5' and 3' ends of the viral sequence, respectively. The viral genome was inserted into the pSMART-LCKan vector (Lucigen, Middleton, WI, USA) using the In-Fusion HD cloning kit (Takara Bio). The viral cDNA plasmid was transformed in *Escherichia coli* (*E. coli*) HST08 Premium competent cells (Takara Bio), cloned and amplified. Plasmid extraction was performed using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA). Extracted plasmids were linearized with *Not* I and *in vitro* transcribed with the mMESSAGE mMACHINE T7 transcription kit (Life Technologies). Transcribed RNA was transfected to Vero cells with the TransIT-mRNA transfection kit (Mirus Bio LLC, Madison, WI, USA). The supernatant from the RNA-transfected cells was harvested when cytopathic effect (CPE) was observed; thereafter cell debris was removed by brief centrifugation and supernatant stored at -80°C

Immunefluorescent assay

rCHIKV-SL10571 (multiplicity of infection: MOI = 0.001) was inoculated onto

Vero cells in cell culture dish (35 × 10 mm, CORNING, Corning, NY, USA). After the virus attachment described as above, DMEM containing 2% FCS (2 ml) with or without 2 μM of Compound-A was added in each dish and incubated for 24 hours. The CHIKV-infected cells were once washed by phosphate buffered saline (PBS), and fixed by methanol for 10 minutes at -30°C. Methanol was completely removed and dried up after the fixation. The fixed cells were washed by PBS containing 0.01% Tween 20 (PBS-T) 3 times. Blocking was performed by PBS containing 1% bovine serum albumin (1%BSA-PBS) for 30 minutes at room temperature. Then, 100 times diluted mouse-monoclonal anti-Alphavirus Immunoglobulin G (IgG, Santa Cruz, Dallas, TX, USA) was putted on the blocked cells over night at 4°C. After washing by PBS-T 3 times, 500 times diluted Alexa-Fluor-488-conjugated secondary antibodies (Life Technologies) was putted on the cells for 1 hour at room temperature with shading. All antibodies were diluted by 1%BSA-PBS.

Plaque assay

Viral dilutions were inoculated onto Vero cells in 24-well cell culture plates and incubated for 1 hour to allow virus attachment. After removal of virus-containing supernatants, the cells were overlaid with culture media containing 1.25% methyl cellulose and incubated for 2 days. Cells were then fixed by buffered-formalin, stained by crystal violet, and plaque numbers was counted. All of the plaque assays were performed in duplicate.

Evaluation of viral replication efficiency in the presence or absence of Compound-A

rCHIKV-SL10571 (MOI = 0.001) was inoculated onto Vero cells in 24-well cell culture plates and incubated for 1 hour to allow virus attachment. After removing virus-containing supernatants, DMEM containing 2% FCS (500 μl) with or without 1 μM of Compound-A was added in each well and incubated for 12, 24 or 36 hours. At each time point, the supernatants from the CHIKV-inoculated cells were harvested and stored at -80°C after removing cell debris by brief centrifugation. Viral titers of the supernatants were calculated by plaque assay. Each assay was performed in duplicate and independently at least 3 times.

Statistical analysis

Statistical significance was calculated by Student's *t*-test from at least three independent experiments (*: $p < 0.05$, **: $p < 0.01$).

Results

MTT screening of chemical compound libraries

To identify novel chemical compounds which could inhibit CHIKV infection, MTT screening assays was performed using chemical compound libraries. The conditions of the MTT assay was optimized referring to the EC_{50} value of T-705 against CHIKV as previously reported (Table 1) [39]. The CHIKV-SL10571 strain belonging to the Indian Ocean Lineage (IOL), which is one of epidemic strains in recent outbreaks, was used in this assay. I evaluated 63 compounds from the chemical compound library and identified a group of compounds which possessed a benzimidazole structure that inhibited CHIKV infection at low concentrations. Based on this result, 103 compounds which also possessed a benzimidazole central structure with different chemical side chains were then evaluated. Finally, a compound (designated Compound-A) was identified to inhibit CHIKV infection at nanomolar concentrations (Figure 1 and Table 1). There was no report that benzimidazole compounds showed anti-CHIKV activities at nanomolar concentrations. Therefore, the antiviral activity of Compound-A was further evaluated.

Characterization of the inhibitory effect of Compound-A

To evaluate anti-CHIKV activity of Compound-A, viral replication was visualized by immunefluorescent assay. Recombinant CHIKV (rCHIKV)-SL10571 (rCHIKV-SL10571) generated by reverse genetics was used in this assay to exclude biases caused by quasispecies. The CHIKV proteins were detected in

almost all cells at 24 hours post infection (h.p.i.) in the absence of Compound-A. On the other hands, quite small population of cells expressed CHIKV proteins in the presence of 2 μM of Compound-A (Figure 2).

Viral growth efficiency in the presence of Compound-A was also assessed using rCHIKV-SL10571. Supernatants from the rCHIKV-SL10571-inoculated cells were collected at 12, 24 and 36 h.p.i. and the viral titers were assessed by plaque assay. It was found that Compound-A significantly inhibited CHIKV replication at each time point with reductions of viral titers (plaque forming unit: PFU/ml) by 14.5, 81.6 and 75.2 times compared to the untreated controls (Figure 3).

Investigation of specificity of inhibitory effect of Compound-A

Additional MTT assays were performed using two different CHIKV strains and another alphavirus, SINV. CHIKV-S27 and CHIKV-BaH306 strains belong to the East/Central/South African (ECSA) linage and the Asian linage, respectively. SINV is a member of the genus *Alphavirus*, but is classified in a different complex (western equine encephalitis virus: WEEV complex) from that of CHIKV (semliki forest virus: SFV complex) [41]. Consistent with the prior experiments with CHIKV-SL10571, Compound-A inhibited infection by all three viruses at nanomolar concentrations (Table 1). These findings suggest that Compound-A has the potential to inhibit not only different lineages of CHIKV strains but potentially other alphavirus infections. The inhibitory effect of Compound-A was also evaluated against DENV serotype 1 and 2 belonging to genus *Flavivirus*, family of *Flaviviridae*; however, Compound-A did not inhibit infection by these

viruses (Table 2).

Investigation of key chemical structures for anti-CHIKV activity of Compound-A

In the 103 benzimidazole compounds whose anti-CHIKV activities were evaluated at the screening as above, a compound (designated Compound-D) was found to have a similar chemical structure with Compound-A (Figure 1). An only different structure between them is that Compound-A has a 2-Pyrrolidone at the terminal region (Figure 1, asterisk). The anti-CHIKV activity of Compound-D assessed by cell viability assay was under the detection limit of EC_{50}, and only EC_{30} value could be calculated (Table 3). From this result, it was revealed that anti-CHIKV activity of Compound-D was much weaker than that of Compound-A.

Discussion

In this study, a benzimidazole compound designated Compound-A was discovered as a novel antiviral agent for CHIKV infection. Compound-A inhibited CHIKV-SL10571, CHIKV-S27 and CHIKV-BaH306 strains (Table 1). CHIKV-SL10571 strain is classified as IOL and is derived from the ECSA lineage and representative of recently circulating CHIKV strains endemic in Asia and Africa [13, 42]. CHIKV-S27 and CHIKV-BaH306 strains are classified as classical ECSA lineage and Asian lineages, respectively [13, 37, 38]. The ECSA and Asian lineages of CHIKV are now endemic in North, Central and South America [14, 18, 43]. Therefore, it can be expected that Compound-A should inhibit CHIKV strains worldwide.

Compound-A also inhibited SINV infection at nanomolar concentrations (Table 1). SINV is classified within the WEEV complex in the genus *Alphavirus* and is distinct from the SFV complex to which CHIKV belongs [41]. On the other hand, Compound-A failed to inhibit DENV infections, which belong to the family of *Flaviviridae* (Table 2). These observations suggest that the inhibitory effect of Compound-A was specific for alphaviruses.

Furthermore, it may be possible that Compound-A has the potential to inhibit other pathogenic alphaviruses in addition to CHIKV and SINV. There are currently no approved chemotherapeutic options for the treatment of all alphavirus infections; therefore, the broad spectrum of anti-alphaviral activity exhibited by Compound-A is important.

Unfortunately, the safety index (CC_{50} / EC_{50}) of Compound-A was not as high

when compared to other approved antivirals (Table 1) and as such it may be necessary for this to be chemically modified [39, 44]. It would be useful to consider the strategy for chemical modification of Compound-A because anti-CHIKV activities of Compound-A and -D were different (Figure 1, Table 3). The structure of Compound-D is the same as that of Compound-A except for a 2-Pyrrolidone at the terminal region. However, the anti-CHIKV effect of Compound-D against CHIKV-SL10571 was much weaker than that of Compound-A. This results suggest that the 2-Pyrrolidone at the terminal region of the benzimidazole structure is important for anti-CHIKV activity. It is supposed that modification of other branches in Compound-A with keeping a terminal 2-Pyrrolidone structure may improve its selectivity index.

Summary

CHIKF has been causing severe damage to human society in worldwide since 2004, and currently no specific therapeutic option is available. Drug development is necessary to control the burden of CHIKF, because effective treatment could improve both of acute and chronic state of symptoms and would be also useful to treat risked age groups, young and elderly.

In this study, a novel drug candidate for CHIKF, designated Compound-A, was discovered in the screening of small compound libraries. Compound-A inhibited CHIKVs and a SINV infections at nanomolar concentrations, and it was suggested antiviral activity was specific to alphaviruses. The safety index of Compound-A was not as high when compared to that of other approved antivirals, thus chemical modification of Compound-A would be required for further development as drug candidate. Here, a key structure for anti-CHIKV activity of Compound-A was also identified and this information would be valuable for chemical modifications of Compound-A in the future.

Table 1 Efficacy and cell toxicity of the chemical compounds against alphaviruses

	EC_{50} (μM)				CC_{50} (μM)
	CHIKV-SL10571	CHIKV-S27	CHIKV-BaH306	SINV-Ar339	
Compound-A	0.54 ± 0.08	0.98 ± 0.08	0.77 ± 0.02	0.38 ± 0.29	3.70 ± 0.30
T-705	23.86 ± 1.28	67.27 ± 0.94	34.38 ± 1.36	23.98 ± 3.63	> 100

Table 2 Efficacy and cell toxicity of the chemical compounds against flaviviruses

	EC_{50} (μM)		CC_{50} (μM)
	DENV-1 hu/PHL/10-07	DENV-2 hu/INDIA/09-74	
Compound-A	ND	ND	8.02 ± 0.17
T-705	18.52 ± 2.02	22.10 ± 4.02	> 100

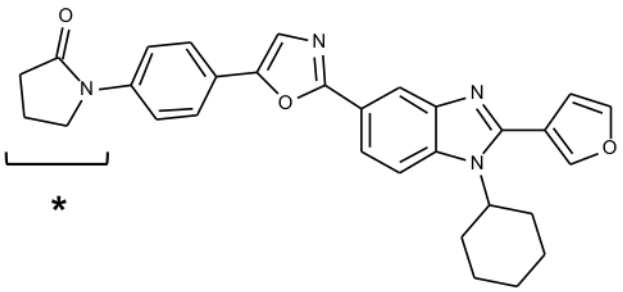
ND: not detected

Table 3 Efficacy and cell toxicity of the chemical compounds against CHIKV-SL10571

	Compound-A	Compound-D	T-705
EC_{50} (μM)	0.54 ± 0.08	ND	23.86 ± 1.28
EC_{30} (μM)	0.40 ± 0.07	2.18 ± 0.75	15.95 ± 1.19
CC_{50} (μM)	3.70 ± 0.32	> 10	> 100

ND: not detected

Compound-A



Compound-D

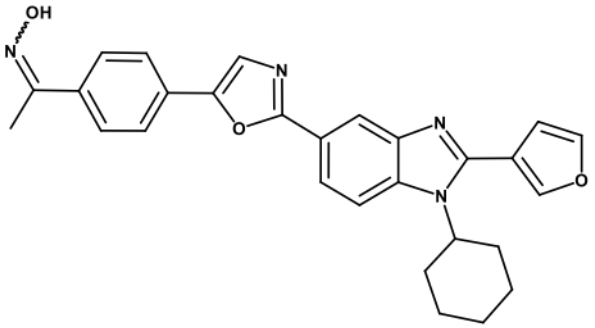


Figure 1. Chemical structure of Compound-A and -D.

Both of Compound-A and -D possess a benzimidazole structure. An asterisk indicates a 2-Pyrrolidone of Compound-A.

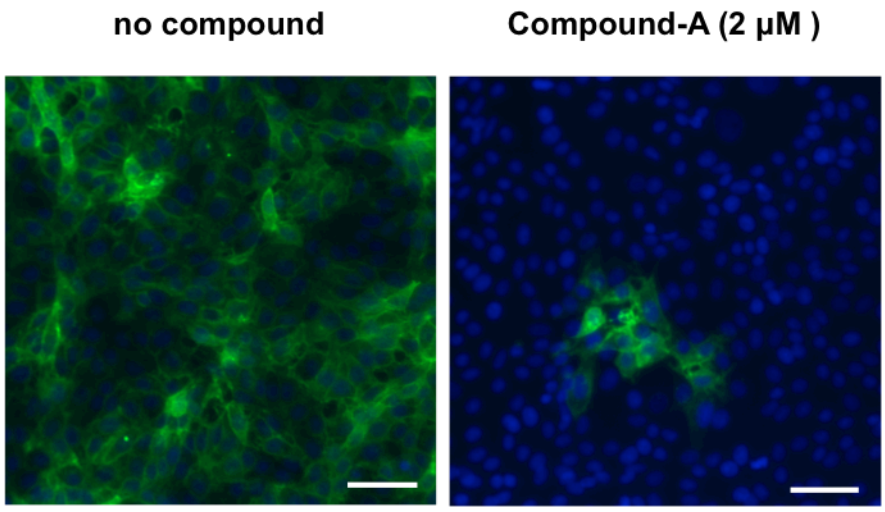


Figure 2. Detection of CHIKV infection by immunofluorescence in the presence or absence of Compound-A.

CHIKV infection in Vero cells was visualized by immunofluorescence at 24 h.p.i.. rCHIKV-SL10571 was inoculated at an MOI of 0.001. Growth media containing 2 μM of Compound-A was added after virus attachment. CHIKV protein (green) and cell nuclei (blue) were stained with mouse-monoclonal anti-Alphavirus IgG and 4′,6-diamidino-2-phenylindole (DAPI), respectively. The figures showed representative results in multiple assessments. Scale bars: 50 μm.

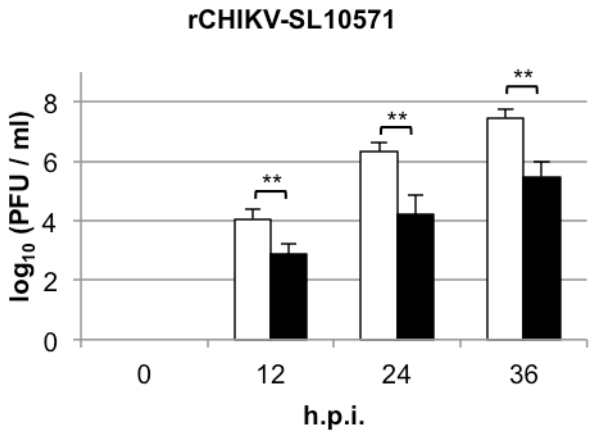


Figure 3. Compound-A significantly inhibits CHIKV infection.

The replication efficiency of rCHIKV-SL10571 in the presence (black bars) or absence (white bars) of Compound-A was evaluated by plaque assay at 12, 24 and 36 h.p.i.. rCHIKV-SL10571 was inoculated at an MOI of 0.01. Growth media containing 1 μM of Compound-A was added after virus attachment. The viral titers are shown (logarithmic scale) on the y-axis. Data represent mean values ± standard deviation (SD) of at least three independent experiments. Significance was analyzed by the Student's *t*-test and indicated by asterisks (** $p < 0.01$).

Chapter 2

Identification of inhibitory mechanism of action of Compound-A as RdRp inhibitor

Introduction

CHIKF causes severe damage to the quality of life of patients and the economy in affected countries or regions as one of re-emerging zoonotic diseases [10-12]. No effective therapeutic option is available for CHIKF in current situation, thus drug development is urgently required to control this disease [5, 21, 23]. A lot of drug candidates to CHIKF have been reported; however, their inhibitory mechanism of action have not been fully elucidated [2, 21].

Compound-A, discovered in a series of this research, is a novel drug candidate for CHIKF with efficient antiviral activity and was suggested to be specific for alphaviruses. To investigate the inhibitory mechanism of action, Compound-A resistant-CHIKVs were isolated and were analyzed by their whole genome sequences. Subsequent analysis using the recombinant CHIKVs with mutations revealed that Compound-A targeted a methionine residue on nsP4 of CHIKV. Further assessment using CHIKV replicon system identified Compound-A worked as a potent RdRp inhibitor. Moreover, amino acid sequence analysis found the methionine residue was located in one of functional motif of RdRp and conserved throughout alphaviruses.

Materials and Methods

Cells and viruses

Vero cells were obtained from the Japan Health Sciences Foundation (Tokyo, Japan), and maintained in DMEM containing 10% FCS, 2 mM L-glutamine, 100 unit/ml penicillin and 100 μg/ml streptomycin. HEK-293T cells were obtained from the RIKEN BRC (Tsukuba, Japan), and maintained in DMEM containing 10% FCS, 4,500 mg/L of glucose and antibiotics as above. Cells were cultured at 37°C in 5% CO_2.

Isolation of Compound-A resistant CHIKV clones

Vero cells (3×10^5 cells) in 12-well cell culture plates were inoculated with CHIKV-SL10571 (MOI = 1) in the presence of Compound-A (2 μM). When CPEs were detected by microscopy, the supernatants from the culture (500 μl) were transferred to fresh Vero cells in the presence of Compound-A. Passages of the cells under the same conditions were repeated 12 times in duplicate.

After twelve passages, the CHIKVs were cloned by limiting dilution. Briefly, the passaged viruses were serially diluted 10-fold to 10^{-6} and inoculated onto Vero cells (3×10^4 cells) in 96-well cell culture plates in the presence of Compound-A (2 μM). The supernatants from the cells with CPE were inoculated onto fresh Vero cells in the presence of Compound-A (2 μM). Finally, Compound-A-resistant viruses were isolated from supernatants of the cell cultures after a total of 14 passages in the presence of Compound-A.

The supernatants from CHIKV-SL10571-inoculated cells after 20 passages in

the absence of Compound-A were used as controls (passaged-CHIKV).

Plaque assay

Plaque assay was performed following the procedure described in Chapter 1.

Briefly, viral dilutions were inoculated onto Vero cells and incubated for 1 hour to allow virus attachment. After removal of virus-containing supernatants, the cells were overlaid with culture media containing 1.25% methyl cellulose and incubated for 2 days. Cells were then fixed, stained and plaque numbers counted. All of the plaque assays were performed in duplicate.

Evaluation of viral replication efficiency in the presence or absence of Compound-A

Evaluation of viral replication efficiency was performed following the procedure described in Chapter 1.

Briefly, rCHIKVs (MOI = 0.001) were inoculated onto Vero cells and incubated for 1 hour to allow virus attachment. After removing virus-containing supernatants, DMEM containing 2% FCS (500 μl) with or without 1 μM of Compound-A was added in each well and incubated for 12, 24 or 36 hours. The supernatants from the CHIKV-inoculated cells were harvested and stored at -80°C. Viral titers of the supernatants were calculated by plaque assay. Each assay was performed in duplicate and independently at least 3 times.

Genome sequence analysis

Full genome sequence analysis was performed using the Ion PGM system (Life Technologies) following to previous report [45]. Viral genomic RNA was extracted using the High Pure Viral RNA kit (Roche Diagnostics) and reverse transcribed to double-stranded cDNA (ds-cDNA) using random hexamers with tag-sequences using a cDNA Synthesis kit (Takara Bio). Synthesized ds-cDNA was purified by Agencourt AMPure XP (Beckman Coulter, Pasadena, CA, USA) and amplified by the PrimeSTAR Max (Takara Bio) using tag-sequence primers. PCR products were fragmented using the Covaris S2 focused-ultrasonicator (Covaris, Woburn, MA, USA) and used to prepare a 400-base-read library using the Ion Plus Fragment Library kit (Life Technologies) and E-Gel SizeSelect 2% agarose gels (Life Technologies). Emulsion PCR was performed by the Ion PGM Template Hi-Q OT2 400 kit (Life Technologies). Sequencing was performed using the Ion PGM sequencer with the Ion PGM Sequencing 400 kit and Ion 318 Chip V2 (Life Technologies). Data analysis was performed with the CLC Genomics Workbench ver 7.5.1 (CLC bio Japan, Tokyo, Japan).

Sanger sequencing was performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the Applied Biosystems 3130 and 3130xl Genetic Analyzers (Applied Biosystems). Data analysis was carried out with the GENETYX software v.10 (Genetyx Corporation, Tokyo, Japan).

Generation of rCHIKVs with amino acid mutations by reverse genetics

Generation of rCHIKVs was performed following the procedure described in

Chapter 1.

Briefly, rCHIKV-SL10571 genomic RNA was extracted and cDNAs were generated. The viral genome was amplified by PrimeSTAR Max (Takara Bio), and was inserted into the pSMART-LCKan vector (Lucigen) using the In-Fusion HD cloning kit (Takara Bio). The viral cDNA plasmid was transformed in *E. coli* HST08 Premium competent cells (Takara Bio), cloned and amplified. Extracted plasmids were linearized and *in vitro* transcribed with the mMESSAGE mMACHINE T7 transcription kit (Life Technologies). Transcribed RNA was transfected to Vero cells and their supernatants f were harvested when CPE was observed.

Non-synonymous mutation(s) associated with resistance for Compound-A were introduced by the In-Fusion HD cloning kit (Takara Bio).

Luciferase assays using CHIKV replicon

A CHIKV replicon was constructed as previously described [46]. Genome recombination was performed as described above. The sequences encoding structural polyprotein in CHIKV cDNA plasmid, which had been constructed to produce rCHIKV, were replaced by a NanoLuc luciferase sequence derived from pNL2.1 vector (Promega, Madison, WI, USA) using the In-Fusion HD cloning kit. Transformation, extraction of plasmids, *in vitro* mutagenesis (GDD to GAA and M2295I) and *in vitro* transcription were performed as described in the generation of rCHIKVs.

The transcribed RNA (100 ng) was transfected into HEK-293T cells (5×10^4 cells), presheeted in Poly-L-Lysine coated-96-well cell culture plates, by the Lipofectamine Messenger MAX (Life Technologies) and incubated for 1 hour.

After removing replicon RNA-containing supernatants, DMEM containing 10% FCS and 4,500 ml/L of glucose (100 μl) with or without 1 μM of Compound-A was added in each well and incubated for 6, 12, 24 or 36 hours. At each time point, NanoLuc activities were measured using the Nano-Glo luciferase assay kit (Promega) and GloMax 96 microplate luminometer (Promega). Each assay was performed in duplicate and independently at least 3 times.

Sequence alignment analysis

The amino acid sequences of CHIKV-SL10571, CHIKV-S27, CHIKV-BaH306, o'nyong-nyong virus (ONNV: AF079456), Mayaro virus (MAYV: AF237947), Semliki Forest virus (SFV: X04129), Ross River virus (RRV: M20162), Getah virus (GETV: EU015061), Barmah Forest virus (BFV: U73745), western equine encephalitis virus (WEEV: GQ287640), eastern equine encephalitis virus (EEEV: EF151503), Venezuelan equine encephalitis virus (VEEV: L01442), rubella virus (RV: AAB81187), DENV serotype 1 (AAB40695), hepatitis C virus (HCV: AAA45676), bovine viral diarrhea virus (BVDV) genotype 1 (AAA42854) and Poliovirus (PV: AAA46910) were downloaded from GenBank for multiple sequence alignments. Multiple alignment analysis was performed by GENETYX software v.10.

Since the genomic sequence of CHIKV-BaH306 has not been reported, the partial amino acid sequence of the viral strain was estimated from the genome sequence obtained by myself.

Statistical analysis

Statistical significance was calculated by Student's *t*-test or Tukey-Kramer test based on One-way ANOVA analysis from at least three independent experiments (*: $p < 0.05$, **: $p < 0.01$).

Results

Viral genome sequence analysis of Compound-A resistant CHIKV clones

To investigate the inhibitory effect of Compound-A on CHIKV infection, an isolation of CHIKV clones with reduced susceptibility to Compound-A was attempted in duplicate, and a total of 12 Compound-A-resistant CHIKV clones (9 clones from one well and 3 clones from another) were analyzed by full genome sequence analysis. The complete genome sequence of a control CHIKV passaged 20 times in the absence of Compound-A (passaged-CHIKV) and the resistant CHIKV clones were analyzed. Amino acid mutations which were detected in both the passaged-CHIKV and the resistant CHIKV clones were omitted from the analysis, as it was assumed these were unrelated to resistance to Compound-A. Furthermore, mutations in the resistant CHIKV clones whose frequency was <20% by deep sequencing were also omitted from the analysis.

Three resistant CHIKV clones (res-CHIKV-1) which were isolated from the same single well were picked up for further investigation. These 3 resistant CHIKV clones possessed same set of four amino acid mutations (R1158S in nsP2, M1751I and S1804P in nsP3, and M2295I in nsP4 with frequencies >98% for each mutation, Table 4). These four amino acid mutations in the res-CHIKV were also confirmed by Sanger sequence analysis and were not detected in the untreated passaged-CHIKV control or other nine resistant CHIKV clones.

Next, the replication efficiency of the res-CHIKV-1 in the presence of Compound-A was evaluated, and which revealed that the res-CHIKV-1 showed no significant difference of viral replication efficiency either in the presence or

absence of Compound-A (data not shown). Based on the findings, it was hypothesized Compound-A targeted a single or a combination of these four amino acid mutations.

On the other hand, different amino acid mutations were detected in nsP2, nsP3, nsP4 and envelope protein 2 (E2) in nine resistant CHIKV clones which were isolated from the other single well. Eight of nine resistant CHIKV clones (res-CHIKV-2) possessed same set of five mutations (T1002A in nsP2, G1519D and L1827P in nsP3, V2072I in nsP4, and E349Q in E2), and remained one clone (res-CHIKV-3) possessed four amino acid mutations (T1210M in nsP2, L1827P in nsP3, V2072I in nsP4, and E349Q in E2). Three amino acid mutations (L1827P in nsP3, V2072I in nsP4, and E349Q in E2) were shared among res-CHIKV-2 and -3. Unlike res-CHIKV-1, res-CHIKV-2 and -3 didn't show clear resistant abilities against Compound-A in preliminary assessments, therefore I didn't investigate those mutations further in this research (data not shown).

Susceptibility of CHIKV against Compound-A evaluated by reverse-engineered CHIKV

To specifically identify the target region(s) in CHIKV of Compound-A, recombinant CHIKVs containing the amino acid mutation(s) detected in the res-CHIKV were generated using reverse genetics. Firstly, the susceptibility to Compound-A of a rCHIKV containing all four amino acid mutations in the nsPs (rCHIKV-R1158S-M1751I-S1804P-M2295I) was examined. Consistent with the analysis using the res-CHIKV, the rCHIKV-R1158S-M1751I-S1804P-M2295I

showed no significant decrease in viral replication efficiency in the presence of Compound-A (Figure 4A).

Next, the susceptibility of rCHIKVs which had three of four introduced mutations (rCHIKV-R1158S-M1751I-S1804P, rCHIKV-R1158S-M1751I-M2295I, rCHIKV-R1158S-S1804P-M2295I and rCHIKV-M1751I-S1804P-M2295I) was evaluated whether these mutations were associated with reduced susceptibility to Compound-A. Notably, each rCHIKV which had the M2295I mutation showed no significant difference in replication efficiency in the presence or absence of Compound-A, although the rCHIKV without the M2295I mutation had significant inhibition of viral replication in the presence of Compound-A (Figure 4B-4E).

Based on these results, I then evaluated the rCHIKVs containing the M2295I in combination with the other amino acid mutations (rCHIKV-R1158S-M2295I, rCHIKV-M1751I-M2295I, rCHIKV-S1804P-M2295I) (Figure 4F-4H) and single mutations (rCHIKV-R1158S, rCHIKV-M1751I, rCHIKV-S1804P, rCHIKV-M2295I) (Figure 4I-4L). Consistently, only the rCHIKVs which had the M2295I mutation in nsP4 showed no significant difference in viral replication efficiency in the presence or absence of Compound-A. In contrast the rCHIKVs not harboring the M2295I showed significant differences in viral replication efficiency in the presence or absence of Compound-A (Figure 4I-4K).

On the basis of these findings, it can be concluded that the M2295I mutation was critical for the inhibitory effect of Compound-A and the target of Compound-A is the M2295 in the viral nonstructural protein, nsP4 (Figure 4 and Table 5).

Characterization of rCHIKV-M2295I

To further understand the interaction between M2295I mutation in CHIKV and resistance to Compound-A, the phenotype of rCHIKV-M2295I was further analyzed. The rCHIKV-M2295I exhibited a significant decrease in viral growth efficiency compared to rCHIKV-SL10571, rCHIKV-R1158S, rCHIKV-M1751I and rCHIKV-S1804P in the absence of Compound-A (Figure 5A). The decrease of viral growth efficiency is certainly a disadvantage for viral replication cycle; however, the res-CHIKV possessing the mutation of the M2295I became dominant in the presence of Compound-A. It was also performed competition assays with rCHIKV-SL10571 and rCHIKV-M2295I. In these, rCHIKV-SL10571 became dominant in the absence of Compound-A; on the other hand, the rCHIKV-M2295I became dominant in the presence of Compound-A (Figure 5B and 5C). These results suggest that the M2295I mutation is an advantage to overcome the selective pressure of Compound-A and also supports our conclusion that Compound-A targets M2295.

CHIKV genome replication efficiency in the presence of Compound-A evaluated by a CHIKV replicon

Generally, it is well known that the main role of the alphavirus nsP4 is RdRp which regulates viral genome replication. Therefore, it was assessed whether Compound-A can inhibit viral genome replication by using CHIKV encoding NanoLuc luciferase whose activity represents replication of the virus.

To evaluate the replication efficiency of CHIKV in the presence of

Compound-A, a wild type replicon and a RdRp-inactivated replicon were constructed. The sequence alignment of the wild type replicon is based on CHIKV-SL10571, but sequences encoding structural polyprotein were replaced by the sequences encoding NanoLuc luciferase (rep-CHIKV-SL10571). The RdRp-inactivated replicon had introduced amino acid mutations from GDD to GAA in the motif C of rep-CHIKV-SL10571, which is the catalytic core of RdRp and also known as the GDD motif (rep-CHIKV-SL10571-GAA). At 6, 12, 24 and 36 hours post transfection (h.p.t.) of these replicons, NanoLuc activities were measured using the cell lysates. Genome replication of rep-CHIKV-SL10571 was observed from 12 h.p.t. and found to be significantly inhibited in the presence of Compound-A at 24 and 36 h.p.t. with reductions of luminescence by 106.0 and 216.7 times compared to that in the absence of Compound-A (Figure 6A). The NanoLuc activity of the rep-CHIKV-SL10571-GAA was stable from 6 to 36 h.p.t., therefore, this demonstrated that the RdRp function of rep-CHIKV-SL10571-GAA was precisely inactivated and the RNAs of rep-CHIKV-SL10571-GAA were translated until 6 h.p.t.. In addition, genome translation efficiency of rep-CHIKV-SL10571-GAA was not affected by Compound-A. From these observations, it was confirmed that Compound-A significantly inhibited CHIKV genome replication, but did not inhibit genome translation.

I also analyzed the replicons with the introduced M2295I mutation in rep-CHIKV-SL10571 or rep-CHIKV-SL10571-GAA (rep-CHIKV-M2295I and rep-CHIKV-M2295I-GAA). Notably, the genome replication of rep-CHIKV-M2295I was significantly inhibited at 24 and 36 h.p.t. in the presence of Compound-A;

however, the reductions of luminescence were quite different from that of rep-CHIKV-SL10571, being only 4.5 and 5.1 times at each time point compared to the luminescence in the absence of Compound-A (Figure 6B). From this analysis, it was confirmed that the M2295I mutation was critical for resistance to the inhibitory effect of Compound-A.

From these observation, I hypothesize Compound-A inhibits the nsP4 function of CHIKV targeting to M2295 at nsP4 but does not inhibit genome translation.

nsP4 amino acid sequence analysis

The consensus of amino acid sequence frequencies around M2295 in nsP4 was assessed by aligning alphaviral nsP4 proteins together with other RNA viruses (rubivirus, flavivirus, hepacivirus, pestivirus and enterovirus). This analysis revealed that M2295 is located in motif B (SGxxxT), which is one of the functional domains of the viral RdRp coding region (Figure 7A) [47]. The M2295 and its adjacent amino acids were highly conserved throughout the genus alphavirus (Figure 7A); however, these were not conserved in other RNA viruses except for motif B (Figure 7B). The R1158, M1751, S1804 and their surrounding sequences were also not seen to be conserved in the alphaviruses (data not shown).

These multiple sequence alignments further support the hypothesis that Compound-A inhibits the nsP4 function of CHIKV *in vitro* by targeting the M2295 residue in the nsP4.

Sensitivity of rCHIKV-M2295I against benzimidazole compounds

To investigate whether anti-CHIKV activity targeting M2295 of Compound-A was specific or not, sensitivity of rCHIKV-M2295I against two other benzimidazole compounds was investigated (designated Compound-B and -C). These benzimidazole compounds inhibited CHIKV-SL10571 infection at a relatively low concentration at MTT screening assay (Figure 8 and Table 6). Firstly, inhibitory effect of Compound-A and T-705 (as an assay control) against rCHIKV-M2295I was evaluated (Table 7). The EC_{50} value against rCHIKV-M2295I for Compound-A was 2.39 times higher than that against CHIKV-SL10571 even though that for T-705 was slightly (0.80 times) decreased. In this condition, inhibitory effects of Compound-B and -C against rCHIKV-M2295I were evaluated (Figure 8 and Table 7). As a result, the EC_{50} values for Compound-B and -C against rCHIKV-M2295I were similar to or lower than the corresponding against CHIKV-SL10571 (0.99 and 0.82, respectively).

From these observations, I conclud that the anti-CHIKV activity targeting M2295 is specific for Compound-A rather than broadly present in compounds containing a benzimidazole structure similar to that of Compound-A, such as Compound-B and -C. In addition, these results also support our conclusion that the M2295I mutation is critical to the anti-CHIKV activity of Compound-A.

Discussion

In this study, the inhibitory mechanism of action of Compound-A was suggested as a RdRp inhibitor targeting M2295 in motif B. This is the first report of a compound which targets CHIKV RdRp with the exception of certain nucleotide analogues [39, 44]. It is unlikely that Compound-A operates as nucleotide analog, because the chemical structure of Compound-A is completely different [39, 48]. Notably, Compound-A did not inhibit infections by dengue virus described as Chapter 1. It is known that ribo-nucleotide analogs inhibit infection by a broad range of RNA viruses [44, 49-51], and that the RdRp fundamentally forms similar structures in other RNA viruses even if the sequence alignment is very different [47, 52, 53]. In other RNA virus analyses motif B plays a role in ribo- or deoxyribo-nucleotide selection as synthetic substrates and RNA synthesis cooperating with motif C (known as the GDD motif) which is the catalytic core of RdRp and is located near to the motif B [47, 54, 55]. Accordingly, it could be possible that the M2295 residue might be located in the active site of the CHIKV RdRp and Compound-A might disrupt the vital function of RdRp by inhibiting ribo-nucleotide selection. However, it could not be demonstrated that Compound-A directly binds M2295 and inhibits the function of motif B due to a lack of information on the RdRp structure in the genus Alphavirus. Chen, *et al*. have recently established a protein expression system of truncated-nsP4 of CHIKV with keeping a catalytic activity [56]. This assay system would be useful to evaluate direct interaction with CHIKV nsP4 and Compound-A.

The inhibitory mechanism of actions of Compound-B and -C were different

from that of Compound-A, because Compound-B and -C inhibited infections from CHIKV-SL10571 and rCHIKV-M2295I at similar concentrations. This observation suggests that the main structure of Compound-A could be changed to improve selectivity index. In addition to the obtained result in Chapter 1 which identified the 2-Pyrrolidone was a key structure for anti-CHIKV activity of Compound-A, these obtained results would be useful to perform chemical modification of Compound-A in the future to be developed as promising drug candidate.

The present studies also suggest that Compound-A has the potential to contribute to future outbreaks of CHIKF as a preemptive measure. Previous studies have suggested that CHIKV genome mutations are associated with adaptation to new mosquito vector species and/or to an increase in viral pathogenicity [19, 20, 43]. As CHIKV evolves and adapts further, it is possible that additional mutations will arise which may cause an increase efficiency of viral replication and/or viral transmission. Importantly, the M2295 residue in the nsP4 is located in the highly conserved functional motif B (Figure 7) and therefore, it is less likely that mutations might be introduced at this site in a natural situation. This is supported by our findings which assessed the viral growth efficiency and competition of rCHIKVs. The rCHIKV-M2295I showed significant decrease of the viral growth efficiency and became minor population compared to rCHIKV-SL10571 in the absence of Compound-A (Figure 5). If M2295I was accidentally introduced in the CHIKV genome, this virus would probably be eliminated due to its low replication ability. In addition, the M2295 residue is conserved in the genus *Alphavirus*, and Compound-A also inhibited SINV

infection (Table 1 in Chapter 1). It might be also possible that Compound-A could contribute to future outbreaks of other diseases caused by alphaviruses.

Summary

CHIKF is one of re-emerging zoonotic diseases and drug development is urgently required. Drug candidates have been reported in recent studies, but inhibitory mechanism of actions of them were insufficiently investigated.

Here, Inhibitory mechanism of action of Compound-A was investigated by using Compound-A resistant CHIKVs, recombinant CHIKVs generated by reverse genetic method, and CHIKV replicon system. Finally, it was identified that Compound-A targeted the M2295 residue in nsP4 and inhibited viral RNA replication of CHIKV. Further amino acid sequence analysis revealed the methionine residue was located in one of functional motif of RdRp and conserved through alphaviruses. These observations suggested Compound-A could contribute to the development of therapeutic strategies for CHIKF or potentially other diseases caused by pathogenic alphaviruses.

Table 4. Amino acid mutations in res-CHIKVs

Viral protein	non-structural protein nsP2			nsP3				nsP4		structural protein E2
Nucleotide	3080	3550	3705	4632	5347	5486	5556	6290	6961	8611
Residue	1002	1158	1210	1519	1757	1804	1827	2072	2295	349
CHIKV -SL10571	Thr (ACC)	Arg (AGA)	Thr (ACG)	Gly (GGC)	Met (ATG)	Ser (TCA)	Leu (CTA)	Val (GTC)	Met (ATG)	Glu (GAG)
passaged -CHIKV	-	-	-	-	-	-	-	-	-	-
res-CHIKV-1	-	Ser (AGT)	-	-	Ile (ATA)	Pro (CCA)	-	-	Ile (ATA)	-
res-CHIKV-2	Ala (GCC)	-	-	Asp (GAC)	-	-	Pro (CCA)	Ile (ATC)	-	Gln (CAG)
res-CHIKV-3	-	-	Met (ATG)	-	-	-	Pro (CCA)	Ile (ATC)	-	Gln (CAG)

Table 5. Summary of the relationship between amino acid mutations and susceptibility against Compound-A

Number of mutations	4	3				2			1			
Viral regions	nsP2 nsP3 nsP4	nsP2 nsP3	nsP2 nsP3 nsP4		nsP3 nsP4	nsP2 nsP4	nsP3 nsP4		nsP2	nsP3		nsP4
Amino acid mutations	R1158S M1757I S1804P **M2295I**	R1158S M1757I S1804P	R1158S M1757I **M2295I**	R1158S S1804P **M2295I**	M1757I S1804P **M2295I**	R1158S **M2295I**	M1757I **M2295I**	S1804P **M2295I**	R1158S	M1757I	S1804P	**M2295I**
Susceptibility against Compound-A	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**S**	**R**

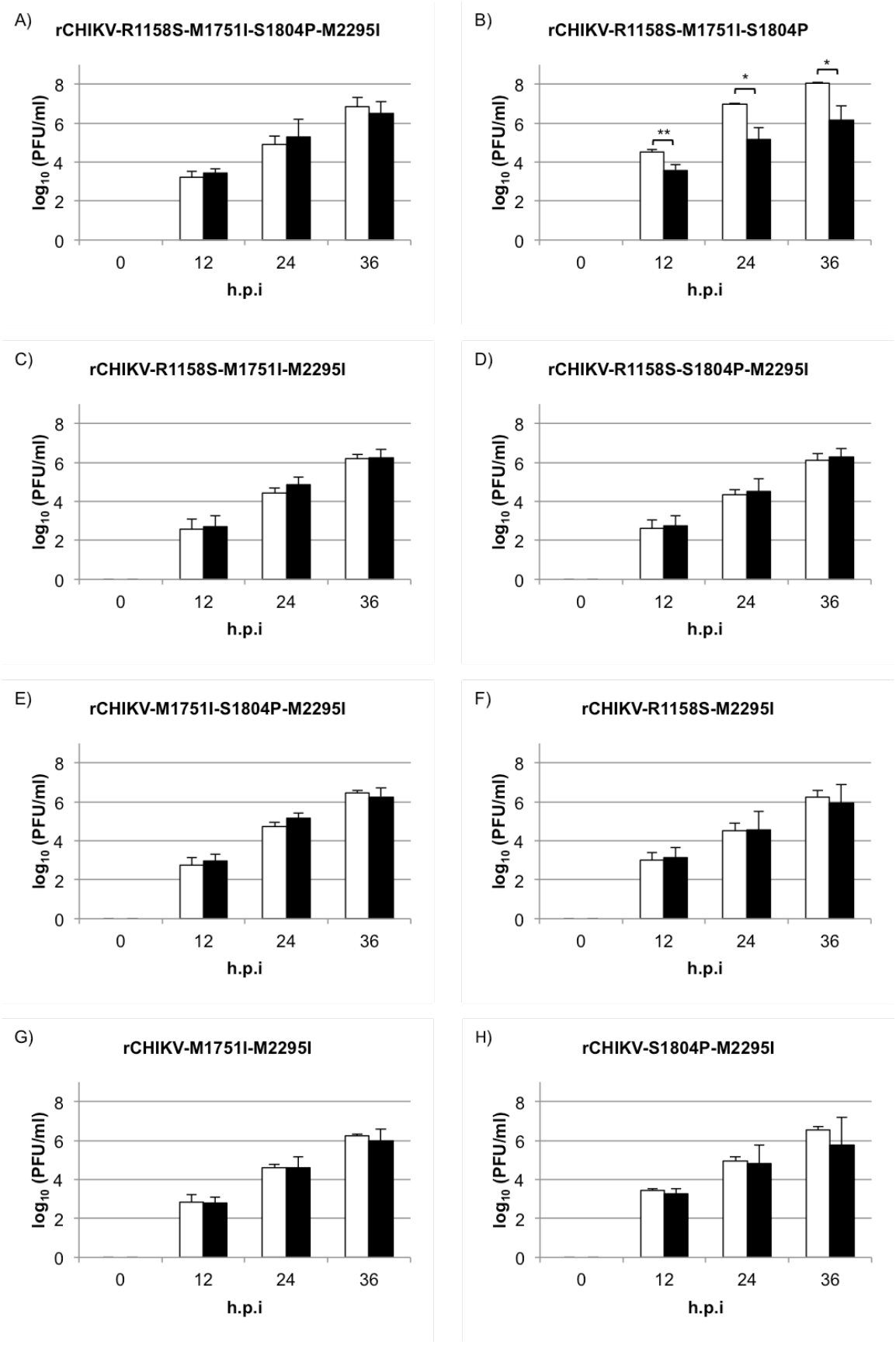
R; resistance, S; sensitive

Table 6 Efficacy and cell toxicity of the chemical compounds against CHIKV-SL10571

	Compound-A	Compound-B	Compound-C	T-705
EC_{50} (μM)	0.54 ± 0.08	2.38 ± 0.80	2.11 ± 1.08	23.86 ± 1.28
CC_{50} (μM)	3.70 ± 0.32	> 10	5.52 ± 1.48	> 100

Table 7 Efficacy and cell toxicity of the chemical compounds against rCHIKV-M2295I

	Compound-A	Compound-B	Compound-C	T-705
EC_{50} (μM)	1.29 ± 0.51	2.38 ± 0.29	1.73 ± 0.39	19.32 ± 2.80
CC_{50} (μM)	5.84 ± 0.17	> 10	4.31 ± 0.30	> 100


A)
rCHIKV-R1158S-M1751I-S1804P-M2295I
B)
rCHIKV-R1158S-M1751I-S1804P
C)
rCHIKV-R1158S-M1751I-M2295I
D)
rCHIKV-R1158S-S1804P-M2295I
E)
rCHIKV-M1751I-S1804P-M2295I
F)
rCHIKV-R1158S-M2295I
G)
rCHIKV-M1751I-M2295I
H)
rCHIKV-S1804P-M2295I
log10 (PFU/ml)
h.p.i
0
12
24
36
0
2
4
6
8
*
**

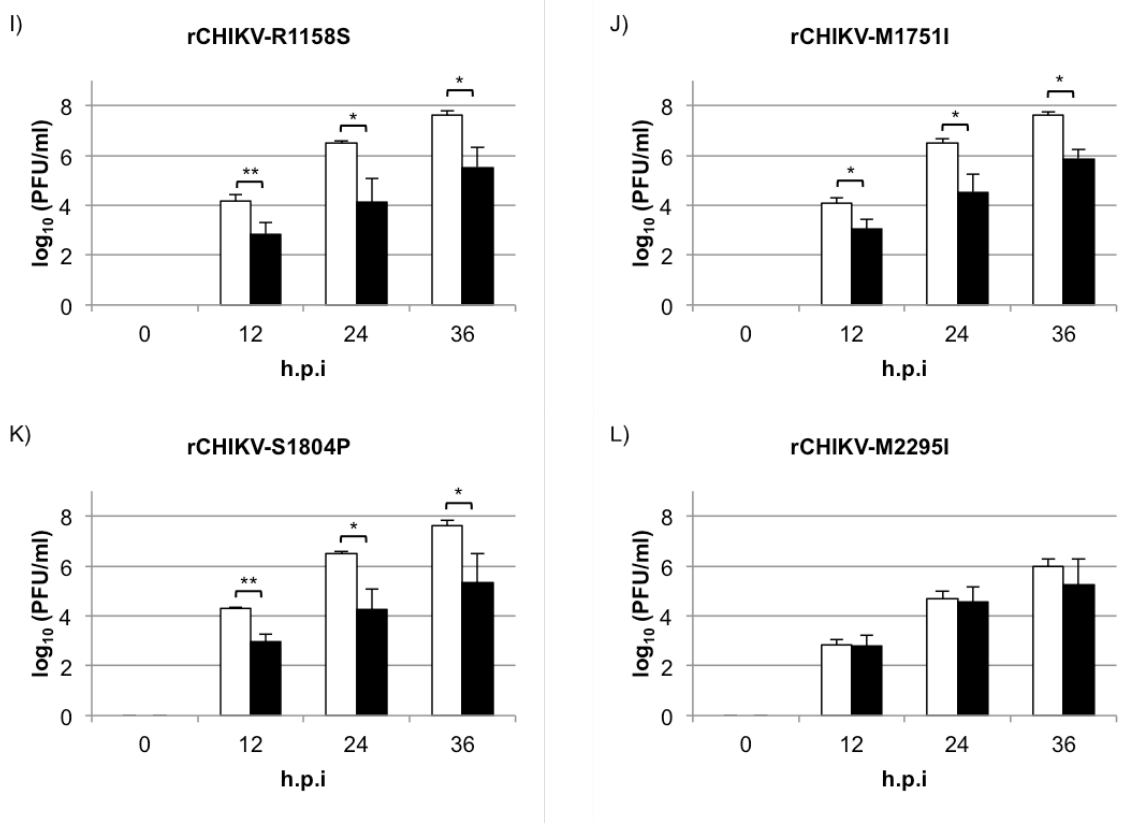


Figure 4. The M2295I mutation in rCHIKVs is related to a decreased susceptibility to Compound-A.

The susceptibility of rCHIKVs with amino acid mutation(s) against Compound-A were assessed. The rCHIKVs were inoculated at MOI of 0.01 and cultivated for 12, 24 and 36 h.p.i. in the presence (black bars) or absence (white bars) of Compound-A. Growth media containing 1 μM of Compound-A was added after virus attachment. (A) The rCHIKV-R1158S-M1751I-S1804P-M2295I was generated with all four amino acid mutations detected in the res-CHIKV (Table 2). (B-E) The rCHIKVs with three of four amino acid mutations (rCHIKV-R1158S-M1751I-S1804P, rCHIKV-R1158S-M1751I-M2295I, rCHIKV-R1158S-S1804P-M2295I, rCHIKV-M1751I-S1804P-M2295I) were also assessed. (F-H) The rCHIKVs with M2295I and other single amino acid mutations

(rCHIKV-R1158S-M2295I, rCHIKV-M1751I-M2295I, rCHIKV-S1804P-M2295I) were assessed. (I-L) The rCHIKVs with single amino acid mutations (rCHIKV-R1158S, rCHIKV-M1751I, rCHIKV-S1804P, rCHIKV-M2295I) were assessed. The viral titers are shown (logarithmic scale) on the y-axis. Data represent mean values ± SD of at least three independent experiments. Significance was analyzed by the Student's *t*-test and indicated by asterisks (* $p < 0.05$, ** $p < 0.01$).

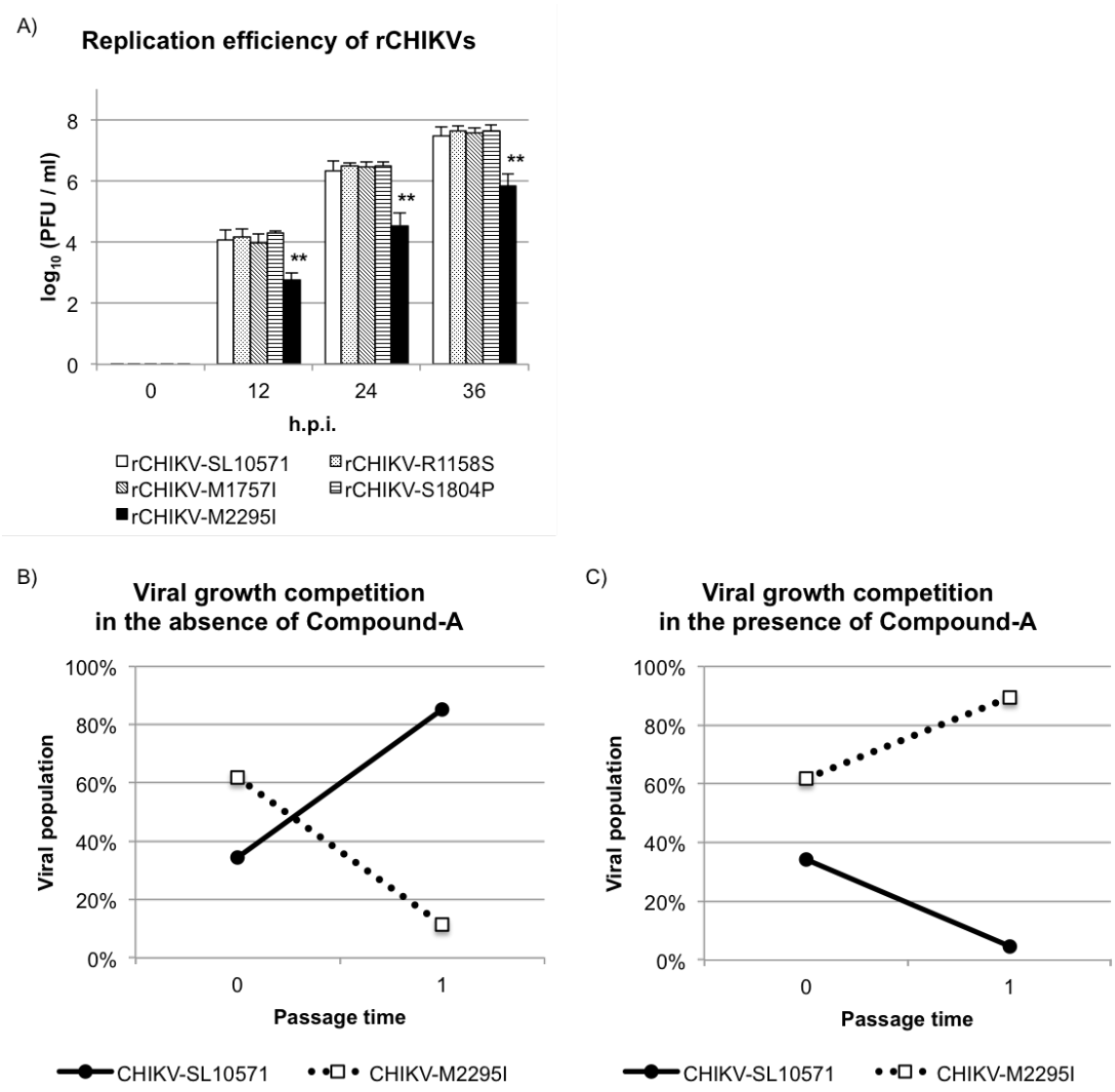


Figure 5. Selective pressure of Compound-A against rCHIKVs.

(A) rCHIKVs were inoculated at an MOI of 0.01 and cultivated for 12, 24 and 36 h.p.i. in the absence of Compound-A. The viral titers are shown (logarithmic scale) on the y-axis. Data represent mean values ± SD of at least three independent experiments. Significance was analyzed by the Tukey-Kramer test based on One-way ANOVA analysis and indicated by asterisks (** $p < 0.01$). (B and C) The mixture of rCHIKV-SL10571 (lines) and rCHIKV-M2295I (dotted lines) was inoculated into Vero cells in the presence or absence of Compound-A (1 μM).

After one time passage, the supernatants were collected and analysed change of proportions of inoculated rCHIKVs by Sanger sequencing. Data represent mean values of bidirectional sequence analysis from three independent experiments.

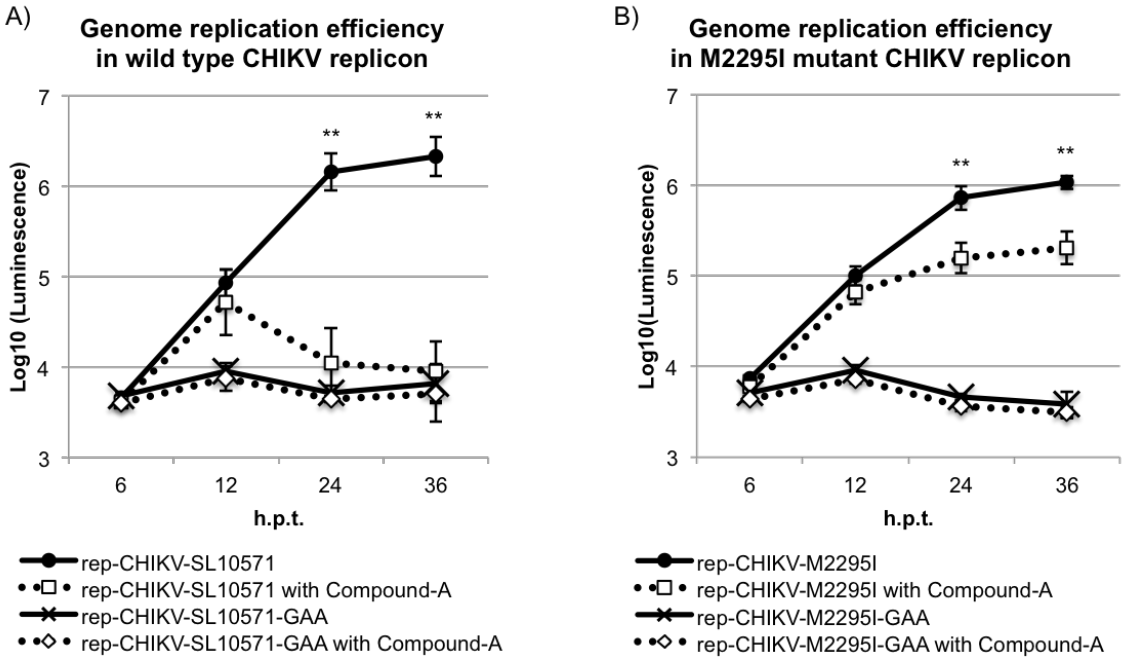


Figure 6. Genome replication efficiency of CHIKV replicon encoding NanoLuc luciferase in the presence or absence of Compound-A.

The CHIKV replicons were transfected into HEK-293T cells for 1 hour and cultivated for 6, 12, 24 and 36 h.p.t. in the presence or absence of Compound-A. Growth media containing 1 μM of Compound-A was added after RNA transfection and the activity of NanoLuc luciferase produced from CHIKV replicons was measured at each time point. (A and B) The RNAs of rep-CHIKV-SL10571, rep-CHIKV-SL10571-GAA, rep-CHIKV-M2295I and rep-CHIKV-M2295I-GAA were transfected and cultivated in the presence (lines) or absence (dotted lines) of Compound-A (1 μM). The RdRp function of rep-CHIKV-SL10571-GAA and rep-CHIKV-M2295I-GAA was inactivated by introducing mutations in the Motif C (GDD motif) from GDD to GAA. Data represent mean values ± SD of at least three independent experiments. Significance was analyed by the Student's *t*-test and indicated by asterisk (* $p < 0.05$, ** $p < 0.01$).

```
A)                                                                  Motif B (SGxxxT)
CHIKV-SL10571 2245 SLALTALMLLEDLGVDHSLL DLIEAAFGEISSCHLPTGTR FKFGAMMKSGMFLTLFVNTL 2304
CHIKV-S27     2245 ..................... ..................... ..................... 2304
CHIKV-BaH306  2245 ..................... ..................... ..................... 2304
ONNV          2284 ......M.........QPI. ..................... ..................... 2343
MAYV          2208 .....G..I.......NQ.. ........Q.T......... ................I..V 2267
SFV           2199 .....G..I.......QY.. ..................... ................I..V 2258
RRV           2251 ................QE.. ....E.....T.V....... ................I... 2310
GETV          2238 ................QE.. ..........T.V....... ................I... 2297
BFV           2181 .M......I.......QN.M N.........V.T....... ...................I 2240
SINV          2284 AM...G..I.......QP.. ....C............... ....................V 2343
WEEV          2242 AI.IS...I.......QP.. ........N.T.V....... ..................... 2301
EEEV          2254 AI..S...I.......QA.. ........D.T.V....... ................I..V 2313
VEEV          2267 AM......I.......AE.. T...........I....K.K ....................V 2326

                                                Motif C (GDD)
CHIKV-SL10571 2305 LNITIASRVLEDRLTKSACA AFIGDDNIIHGVVSDELMAA RCATWMNMEVKIIDAVVSLK 2364
CHIKV-S27     2305 ..................... ..................... ..................... 2364
CHIKV-BaH306  2305 ..................... ..................... ..................... 2364
ONNV          2344 ...........E...T.... ...............A.... ..................E. 2403
MAYV          2268 ...........A...N.... .......VV......K...D .....V..........MCI. 2327
SFV           2259 ...........Q...D.... ........V...I..K...E ...S.V..........MGE. 2318
RRV           2311 ...V..C...REK..N.I.. ........V...R..P...E ...S.V.........TMCE. 2370
GETV          2298 ...V..C...R.K.SS.... ........V...R..P...E ...S.V.........TMCE. 2357
BFV           2241 ..VV..C.....Q.AQ.PWP ...........II..K...D ............L.SI.GIR 2300
SINV          2344 ..VV.......E..KT.R.. ...............KE..E .....L..........IGER 2403
WEEV          2302 V..M......RE...T.... ........V......T...E .....L..........IGI. 2361
EEEV          2314 V..M......RE...N.P.. ........VK..K..A...D .....L...........G.. 2373
VEEV          2327 I..V......RE...G.P.. ........VK..K..K...D .....L...........GE. 2386
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B)                                                                  Motif B (SGxxxT)
CHIKV    2245 SLALTALMLLEDLGVDHSLL DLIEAAFGEISSCHLPTGTR FKFGAMMKSGMFLTLFVNTL 2304
RV       1885 TLATRDVELEISAALLGLPC AEDYRALRAGSYCTLRELGS TETGCERTSGEPATLLHNTT 1944
DENV     3045 ITDIMEPEHALLATSIFKLT YQNKVVRVQRPAKNGTVMDV ISRRDQRGSGQVGTYGLNTF 3104
HCV      2654 RTEEAIYQCCDLDPQARVAI KSLTERLYVGGPLTNSRGEN CGYRRCRASGVLTTSCGNTL 2713
BVDV     3626 DLQLIGEIQKYYYKKEWHKF IDTITDHMTEVPVITADGEV YIRNGQRGSGQPDTSAGNSM 3685
PV       1988 SLSPAWFEALKMVLEKIGFG DRVDYIDYLNHSHHLYKNKT YCVKGGMPSGCSGTSIFNSM 2047

                                                Motif C (GDD)
CHIKV    2305 LNITIASRVLEDRLTKSACA AFIGDDNIIHGVVSDELMAA RCATWMNMEVKIIDAVVSLK 2364
RV       1945 VAMCMAMRMVPKGVRWAGIF QGDDMVIFLPEGARSAALKW TPAEVGLFGFHIPVKHVSTP 2004
DENV     3105 TNMEAQLIRQMESEGIFSPS ELETPNLAERVLDWLKKHGT ERLKRMAISGDDCVVKPIDD 3164
HCV      2714 TCYIKARAACRAAGLQDCTM LVCGDDLVVICESAGVQEDA ASLRAFTEAMTRYSAPPGDP 2773
BVDV     3686 LNVLTMMYGFCESTGVPYKS FNRVARIHVCGDDGFLITEK GLGLKFANKGMQILHEAGKP 3745
PV       2048 INNLIIRTLLLKTYKGIDLD HLKMIAYGDDVIASYPHEVD ASLLAQSGKDYGLTMTPADK 2107
```
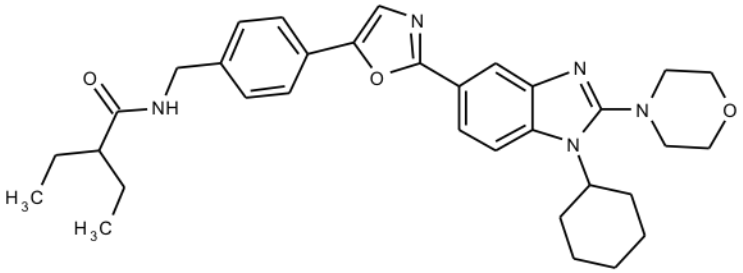

Figure 7. Sequence alignment analyses of viral nsP4.

(A) Multiple alignment analysis among the genus *Alphavirus*. The conserved sequences of motif B and C in the nsP4 of CHIKV strains and other representative alphavirus members are shown as bold and underlined characters. The M2295 in CHIKV and its corresponding amino acid residues in other alphaviruses are highlighted in gray. Each dot of other viruses represents identical amino acid

residues as those of CHIKV-SL10571. ONNV; o'nyong-nyong virus. MAYV; Mayaro virus. SFV; Semliki Forest virus. RRV; Ross River virus. GETV; Getah virus. BFV; Barmah Forest virus. SINV; Sindbis virus. WEEV; western equine encephalitis virus. EEEV; eastern equine encephalitis virus. VEEV; Venezuelan equine encephalitis virus. (B) Sequence alignment comparing residues adjacent to motif B in the nsP4 RdRp with other plus/positive-strand RNA viruses (rubivirus, flavivirus, hepacivirus, pestivirus and enterovirus). The conserved sequences of motif B and C are shown as bold and underlined characters. The M2295 in CHIKV and its corresponding amino acid residues of other positive-sense RNA viruses are highlighted in grey. RV; rubella virus. DENV; dengue virus (serotype 1). HCV; hepatitis C virus. BVDV; bovine viral diarrhea virus (genotype 1). PV; Poliovirus.

Compound-B



Compound-C

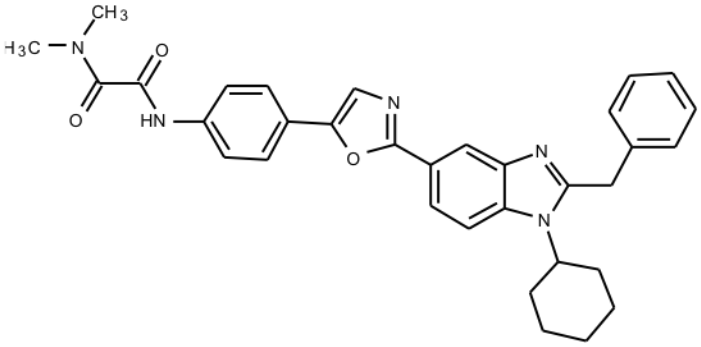


Figure 8. Chemical structure of Compound-B and -C.

Both of Compound-B and -C possess a benzimidazole structure.

Conclusion

CHIKF is associated with significant acute and chronic morbidity in endemic areas, and as such the development of CHIKV antiviral therapeutics is of great importance. In the present study, screening of chemical compound libraries was performed and a novel anti-CHIKV candidate designated Compound-A was discovered. This is a benzimidazole-related molecule and can inhibit CHIKV infection in cultured cells at nanomolar concentrations. It was elucidated that the target of Compound-A was the M2295 residue in the nsP4 by using Compound-A-resistant CHIKV clones and rCHIKVs synthesized by reverse engineering. Furthermore, the mechanism of action of Compound-A was also identified as a potent RdRp inhibitor by using CHIKV replicons.

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和文要旨

チクングニア熱（CHIKF）は、トガウイルス科アルファウイルス属に属するチクングニアウイルス（CHIKV）の感染により惹起される蚊媒介性の人獣共通感染症である。2004 年から現在まで続く CHIKF の流行は世界中の広範な地域（アフリカ、アジア、欧州、中南米等）で発生しており、甚大な数の感染例が報告されている。CHIKF の主症状として発熱、発疹、関節痛があげられる。特に、激しい痛みを伴い長期間（数ヶ月から数年以上）持続する関節痛は最も特徴的であり、重症例ではリウマチ様関節炎に移行する事が報告されている。この様に、CHIKF は世界中の罹患者の quality of life 及び経済活動に大きな被害を及ぼし、再興感染症として注目を集めている。

しかしながら、CHIKF に有効な予防法や治療法は依然として確立されていない。特に、治療薬の開発研究は途上段階にあり、抗 CHIKV 活性を有する化合物・成分等の報告は一定数存在するが、殆どの場合でその作用機序が解析されていない。そこで、本研究では、CHIKF 治療薬の開発に寄与する知見を獲得する事を目的として、CHIKF 治療薬の候補と成り得る新規抗 CHIKV 化合物の探索とその作用機序の解明を試みた。

第一章では、CHIKV 感染による細胞障害を指標とする細胞生存性試験により、化合物ライブラリーのスクリーニングを実施した。本スクリーニングにより、複数の CHIKV 株の感染を低濃度（EC_{50}：0.54 - 0.98 μM）で阻害するベンゾイミダゾール系化合物である Compound-A を見出した。更に、Compound-A は、CHIKV と同じトガウイルス科アルファウイルス属に属するシンドビスウイルスの感染を

阻害するが、フラビウイルス科フラビウイルス属に属するデングウイルスの感染は阻害しない事が明らかとなった。以上の結果から、Compound-A の抗ウイルス活性はアルファウイルス特異的である可能性が示唆された。また、Compound-A と非常に類似した化学構造を有する化合物を解析した結果、Compound-A が有する抗 CHIKV 活性には末端の 2-Pyrrolidone 構造が必須である事を明らかとした。

第二章では、Compound-A 耐性 CHIKV を用いて、Compound-A の抗 CHIKV 活性作用機序の解明を試みた。始めに、野生型 CHIKV を Compound-A 存在下で複数回継代培養する事により Compound-A 耐性 CHIKV クローン（res-CHIKV-1）を分離し、全ゲノム配列を解析した。続いて、res-CHIKV-1 が有するアミノ酸変異を単独、もしくは組み合わせで導入した遺伝子組換え CHIKV を作出し、Compound-A が有する抗 CHIKV 活性に対する感受性の変化を解析した。その結果、Compound-A は非構造タンパク質（nsP）4 に位置する M2295 を標的として抗 CHIKV 活性を示す事が明らかとなった。一般的に、アルファウイルスの nsP4 は RNA 依存性 RNA ポリメラーゼ（RdRp）として機能する事が知られている。Compound-A が RdRp の機能を阻害する可能性を調査する為に、CHIKV レプリコンを作出し、Compound-A 存在下における CHIKV のゲノム複製効率を評価した。その結果、Compound-A は M2295 を標的として CHIKV のゲノム複製を阻害する事が明らかとなり、Compound-A は RdRp 阻害剤として作用する可能性が示唆された。更に、種々の RNA ウイルスのアミノ酸配列を解析した結果、M2295 は RdRp の機能的モチーフである Motif B 内に位置し、アルファウイルスで広範に保存されている事が明らかとなった。本解析により得られた結果は、Compound-A がア

ルファウイルス特異的に抗ウイルス活性を示す可能性、及び RdRp 阻害剤として作用する可能性を支持するものである。また、Compound-A と同様のベンゾイミダゾール骨格を有する他の化合物の抗 CHIKV 活性を解析した結果、M2295 を標的とする抗 CHIKV 活性は Compound-A に特異的な性質である事が明らかとなった。これより、ベンゾイミダゾール骨格は Compound-A の抗 CHIKV 活性に必須ではない可能性が示唆された。