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Chikungunya virus nonstructural protein 1 as an antiviral target

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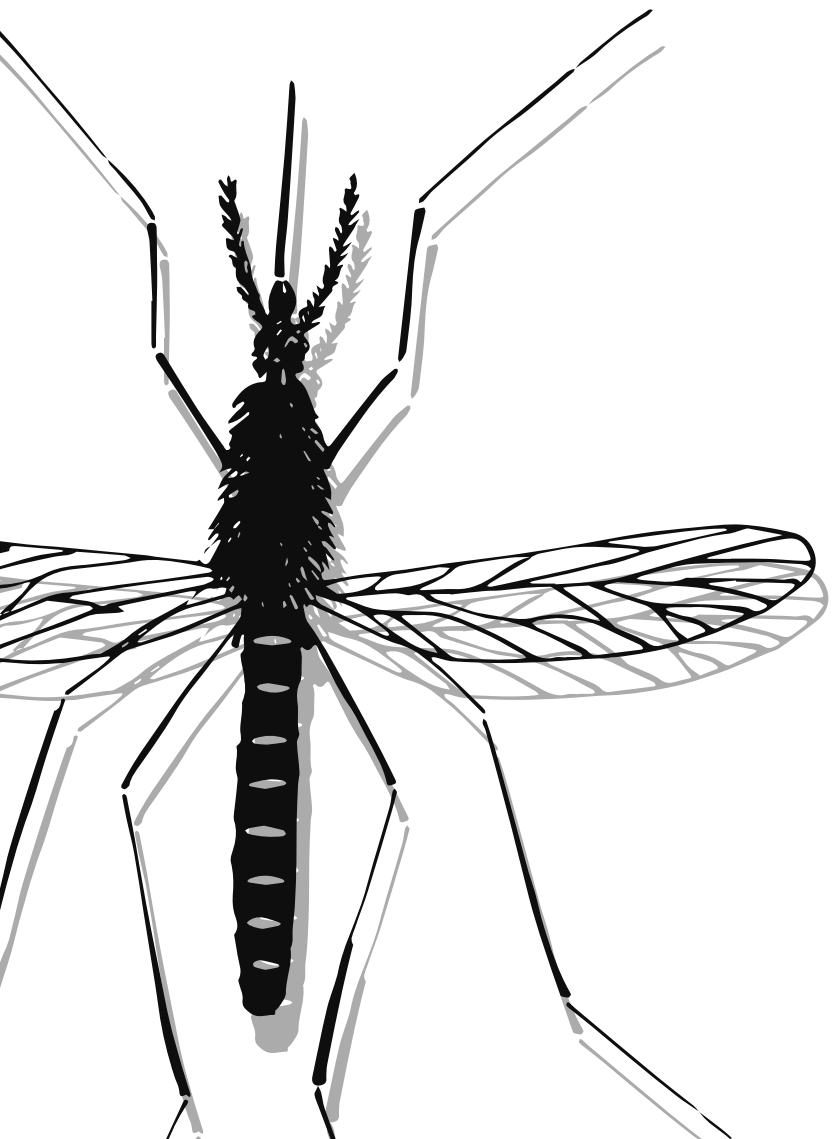

CHAPTER 5

Novel class of Chikungunya virus small molecule inhibitors that targets the viral capping machinery

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Abstract

Despite the worldwide reemergence of the chikungunya virus (CHIKV) and the high morbidity associated with CHIKV infections, there is no approved vaccine or antiviral treatment available. Here, we aimed to identify the target of a novel class of CHIKV inhibitors i.e. CHVB series. CHVB compounds inhibit the *in vitro* replication of CHIKV isolates with 50% effective concentrations in the low-micromolar range. A CHVB-resistant variant ($CHVB^{res}$) was selected that carried two mutations in the gene encoding nonstructural protein 1 (nsP1) (responsible for viral RNA capping), one mutation in nsP2 and one mutation in nsP3. Reverse genetics studies demonstrated that both nsP1 mutations were necessary and sufficient to achieve ~18-fold resistance, suggesting that CHVB targets viral mRNA capping. Interestingly, $CHVB^{res}$ was cross-resistant to the previously described CHIKV capping inhibitors from the MADTP series, suggesting they share a similar mechanism of action. In enzymatic assays, CHVB inhibited the methyltransferase and guanylyltransferase activities of alphavirus nsP1 proteins. To conclude, we identified a class of CHIKV inhibitors that targets the viral capping machinery. The potent anti-CHIKV activity makes this chemical scaffold a potential candidate for CHIKV drug development.

Introduction

Chikungunya virus (CHIKV) is an arthropod-borne virus that belongs to the genus *Alphavirus* of the *Togaviridae* family (1, 2). After two decades of sporadic outbreaks in Africa and Asia, CHIKV reemerged in Kenya in 2004, after which large epidemics of CHIKV infections were reported in several Indian Ocean islands and Southeast Asian countries (3). At the end of 2013, the first local transmission of CHIKV occurred in the Americas. Since then, millions of CHIKV cases have been reported in the Caribbean region as well as in several countries of Central and South America (4). Most recently, in 2018, CHIKV caused outbreaks in Brazil, India, Sudan and Thailand (5). Due to the high socioeconomic impact of CHIKV infections, the virus is now considered an imminent health threat to tropical and temperate areas colonized by *Aedes* mosquitoes.

Acute CHIKV infections are characterized by fever, arthralgia, and, in many cases, maculopapular rash (6). Although a CHIKV infection is rarely fatal, 15 to 60% of patients suffer from chronic debilitating polyarthritis that can last for weeks up to years after the acute infection (1, 6). Recently, severe neurological complications such as Guillain-Barré syndrome and meningoencephalitis, have also been reported during CHIKV infections (1, 7). Other complications associated with CHIKV infections include myocarditis, pancreatitis, and kidney failure (8).

Despite the worldwide spread and the high morbidity rate of CHIKV infections, there is no licensed vaccine or antiviral treatment available. The current treatment is only symptomatic (2). A number of classes of antiviral compounds targeting either a viral or a host factor have been reported to inhibit CHIKV replication in cell-based assays (9, 10), but none have progressed to clinical trials. We previously reported on the first class of inhibitors ([1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones, the MADTP series, that block the capping of CHIKV RNA genomes. The MADTP compounds mainly target the guanylyltransferase (GTase) activity of Venezuelan Equine Encephalitis virus (VEEV) nsP1 (11). This class of compounds has a low barrier to resistance, since only a single mutation in CHIKV nsP1 (proline-34-serine) resulted in high resistance to the compounds (11). Ideally, antiviral drugs should have a high barrier to resistance to avoid rapid emergence of drug-resistant variants.

In a large-scale cytopathic effect (CPE)-based anti-CHIKV screening campaign (33,000 molecules), we identified CHVB-00 (N-ethyl-2-(4-((4-fluorophenyl) sulfonyl) piperazin-1-yl)-6-methylpyrimidin-4-amine) as a molecule that was able to completely inhibit CHIKV replication at nontoxic concentrations (50% effective concentration [EC_{50}] value of 8.7 ± 0.5 μM, 50% cytotoxic concentration [CC_{50}] of 122 ± 24 μM) (Fig. 1).

Initial hit optimization resulted in the synthesis and biological evaluation of 67 analogs. Of these analogs, 24 molecules were more potent than the original hit compound. The synthesis of this class of molecules and the structure-activity relationship will be published elsewhere (12). Here, we report on the particular characteristics of the anti-CHIKV activity of this class of compounds and the molecular mechanism underlying the antiviral activity.

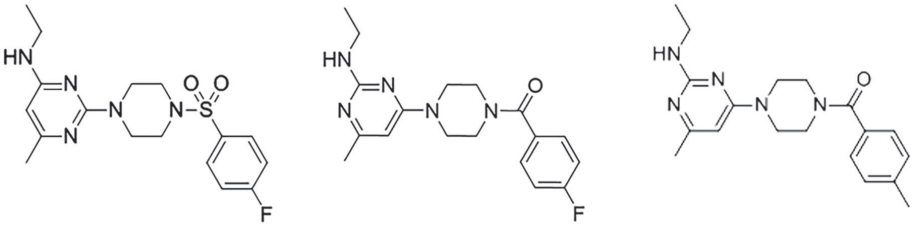

Figure 1. Chemical structures of the initial hit and selected analogs of the CHVB series.

Materials and Methods

Cells, viruses and compounds

African green monkey kidney cells (Vero cells [ATCC CCL-81], VeroE6 cells [ATCC CRL-1586]), Baby hamster kidney [BHK-21] cells were maintained as described before (11). CHIKV and other alphaviruses strains are as mentioned before (11). CHIKV LS3 (GenBank accession no. KC149888), which was used for reverse genetics studies, is derived from a full-length cDNA clone belonging to the collection of the Leiden University Medical Center, The Netherlands (13).

CHVB-032 and CHVB-066 (Fig. 1) were synthesized in the laboratory of G. Pürstinger (University of Innsbruck, Austria) and T. Langer (University of Vienna, Austria). MADTP-0372 was synthesized by M.J. Pérez-Pérez (Spanish National Research Council, CSIC) (11). T-705 (favipiravir) was purchased as a custom synthesis product from BOC Sciences. Chloroquine was purchased from Sigma.

CPE reduction assay

Vero cells were seeded at a density of 2.5×10^4 in 96-well plates (BD Falcon) and were allowed to adhere overnight. The next day, cells were treated with a dilution series of the compounds, after which the cultures were infected with CHIKV-899 (multiplicity of infection [MOI] of 0.01). On day 5 postinfection, the inhibition of CPE was quantified using MTS/PMS as described by the manufacturer (Promega). The cells were checked by microscope for minor signs of virus-induced CPE or compound-induced adverse effects on the cell monolayer morphology. The 50% effective concentration (EC_{50}), which is defined as the concentration of compound that is required to inhibit virus-induced cell death by 50%, was determined using logarithmic interpolation.

Virus yield assay

Vero cells were seeded in 96-well plates at a density of 5×10^4 cells/well. The next day, cells were treated with serial dilutions of the compound and then infected with CHIKV-899 (MOI of 0.01). Two hours postinfection, the cells were washed to remove nonadsorbed virus, followed by incubation with the same serial dilutions of compounds. After 48 h of incubation, supernatants were collected and viral RNA was quantified by real-time quantitative RT-PCR (qRT-PCR), while the amount of infectious progeny virus was determined by titration assay (50% cell culture infectious dose [$CCID_{50}$]/ml) as described before (14).

Delay of treatment assay

Vero cells were seeded in 96-well plates at a density of 5×10^4 cells/well. The following day, cells were infected with CHIKV 899 (MOI of 1) for 1 h at 37°C, after which the viral inoculum was removed, and cells were washed 3 times with the assay medium. The selected compounds (20 µM CHVB-032, 5 µM CHVB-066, 50 µM MADTP-0372, 200 µM T-705 or 50 µM of chloroquine) were added to cells at 0, 2, 4, 6, 8 and 10 h after infection. At 12 h postincubation (i.e. one viral replication cycle), culture supernatants were collected for quantification of extracellular viral RNA load (by qRT-PCR) and infectious virus progeny (by endpoint titration).

Selection of compound-resistant virus isolates

A 5-step clonal resistance selection protocol was used to isolate CHVB-resistant virus variants as previously described (15). In the first step, Vero cells were seeded in 96-well plates at a density of 2.5×10^4 cells/well and were allowed to adhere overnight. Subsequently, antiviral assays were performed in a checkerboard format using serial dilutions of CHVB-032 and different amounts of CHIKV-899 (ranging from 10 to 1,000 $CCID_{50}$). After 5 days of incubation, all assay wells were checked microscopically. Based on these data, the lowest concentration of compound and the highest virus input at which complete and reproducible inhibition of virus-induced CPE was observed were selected. In the second step, three 96-well plates containing adherent Vero cells were treated with the selected CHVB-032 concentration of 29 μM and then were infected with the optimal amount of virus (50 $CCID_{50}$). After 5 days, 7 assay wells (out of 162) showed signs of virus-induced CPE, with only 4 of them showing full CPE. The supernatants of these 4 wells were collected and semipurified 6-fold by titration (1:5 dilution series) in the presence of 29 μM of CHVB-032. Four virus isolates (one from each original sample) were selected that produced the most pronounced signs of CPE in the presence of CHVB-032 at the lowest virus input possible. Subsequently, the resistant phenotype of the selected virus isolates was determined in comparison with the wild-type virus in CPE reduction assays. In parallel, the genotype was determined by full genome sequencing.

Assays for alphavirus nsP1 enzymatic activity

The DNA sequence encoding SFV nsP1 (amino acid 1 to 537) with C-terminal hexahistidine (6xHis) tag was cloned into the pET34 vector. Recombinant wild-type and active site mutant D64A were expressed in *E. coli* Rosetta cells (Novagen) following overnight induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 19˚C. The recombinant proteins were batch purified by immobilized metal affinity chromatography (IMAC) using Talon (Cobalt) beads (TaKaRa), and the eluted protein was concentrated and used in the covalent m^7GMP-nsP1 complex formation assay (16). This assay was performed in a 30-μl mixture containing 25 mM HEPES pH 7.5, 5 mM dithiothreitol, 10 mM KCl, 2 mM $MgCl_2$, 10 μM S-adenosylmethionine (SAM), 0.75 mCi of [α^{32}P] GTP (3,000 Ci/mmol) and 0.5 μM SFV nsP1 wt or D64A mutant. The reaction mixture was incubated at 30˚C for 30 min and stopped by adding 3 μl of 10% SDS. The inactivated reaction mixture was mixed with 4xLSB (Laemmli Sample Buffer)

and 10 µl samples were resolved on a 10% SDS-PAGE gel. The gel was dried, and a PhosphorImager screen was placed on top. After overnight exposure, the ^{32}P-labelled covalent m^{7}GMP-nsP1 intermediate products were visualized with a Typhoon Imager (Amersham). The protocols for VEEV nsP1 enzymatic activity assays were previously described (11).

Statistical analysis

Graphing and statistical analysis were performed using Prism8 software (GraphPad).

Results

Molecules of the CHVB series inhibit the replication of various CHIKV isolates in cell culture

The antiviral efficacy of CHVB-032 and CHVB-066 against the CHIKV-899 strain was assessed in a CPE reduction assay on Vero cells. Both molecules exerted potent inhibition of virus-induced CPE, with EC_{50} values of 2.7 µM and 0.45 µM, respectively (Fig. 2A). When assessing the potential adverse effects of the compounds on Vero cells (by colorimetric readout using MTS/PMS), CHVB-032 was less cytotoxic than CHVB-066 (CC_{50} values of >75 µM and 15 µM, respectively) (Fig. 2A). The anti-CHIKV

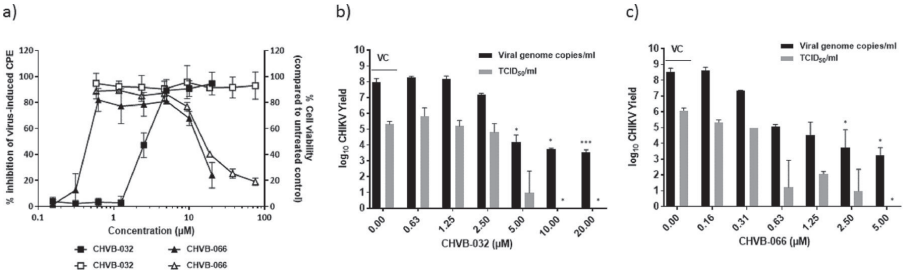

Figure 2. CHVB compounds are selective inhibitors of CHIKV replication. (A) Dose-response effect of CHVB-032 and CHVB-066 on CHIKV899-induced cytopathic effect (CPE) [filled squares and filled triangles, respectively] (MOI 0.01) and cell viability (open squares and open triangles, respectively) as quantified in Vero cells by the MTS/PMS method. Data are mean values ± the standard deviations (SD) from at least three independent experiments. The effect of different concentrations of CHVB-032 (B) and CHVB-066 (C) on the release of CHIKV particles by CHIV899-infected Vero cells (MOI 0.01) was quantified by real-time qRT-PCR (for viral RNA; black columns) and endpoint titration assay (for infectious progeny virus particles; grey columns) at 48h p.i. Data shown are mean values ± SD from at least two independent experiments. Significant differences from untreated virus control were analyzed by two-tailed Student's *t* test (*, $P < 0.05$; ***, $P < 0.005$). TCID, tissue culture infectious dose; VC, virus control (untreated).

activity was further confirmed in a virus yield assay in which treatment with both CHVB compounds resulted in a marked dose-dependent reduction of viral RNA replication and production of infectious virus progeny, with a pronounced > 4 $\log_{10}$ reduction at the highest tested concentrations (Fig. 2B and C). The antiviral activity of CHVB-032 was further evaluated against various CHIKV isolates (Venturini [Italy 2008], St Martin 20235 P2, St Martin 20236 P3, EFS-1 P3 Martinique and Congo 95 [2011]). CHVB-032 reduced the viral RNA yield of all the tested CHIKV isolates, with EC_{50} values in the range of 1.2 to 7.3 µM (Table 1). However, the compound was devoid of antiviral activity against other alphaviruses, i.e. O'Nyong Nyong virus, Mayaro virus strain TC625, Barmah Forest virus strain BH2193 and Ross River virus 5281v (data not shown).

Table 1. Antiviral activity of CHVB-032 compound against different chikungunya virus isolates.

	CHVB-032	
CHIKV strain	**EC_{50} (µM)**	**EC_{90} (µM)**
899 (lab)	2.7 ± 0.4[a]	5.5 ± 0.2[a]
OPY1 (La Reunion)	1.24 ± 0.3[b]	6.7 ± 0.5[b]
Venturi (Italy)	2.1 ± 0.4[b]	2.6 ± 0.4[b]
Congo (Congo)	1 ± 0.15[b]	1.2 ± 0.1[b]
St Martin 20235 P2	1.7 ± 0.4[b]	5 ± 0.3[b]
St Martin 20236 P3	4.1 ± 0.6[b]	17 ± 8[b]
EFS-1 P3 Martinique	7.3 ± 2.3[b]	13 ± 3[b]

[a] CPE reduction
[b] Viral RNA reduction

CHVB compounds inhibit a postentry step in the CHIKV replication cycle

A delay-of-treatment assay was performed to estimate the stage of CHIKV replication cycle inhibited by the CHVB series. Chloroquine was used in this assay as a reference compound for early-stage inhibition, as this molecule is known to interfere with virus entry (17). MADTP-0372 (inhibits viral RNA capping) and T-705 (inhibits viral RNA synthesis) were used as reference molecules for replication stage inhibition (11, 15). Similar to the inhibition profile of the MADTP-0372 compound, CHVB-032 and CHVB-066 reduced viral RNA (Fig. 3A) and infectious virus loads (Fig. 3B) when added to the infected cells up to 4h postinfection (p.i.), suggesting that compounds

in the CHVB series target a postentry step in the CHIKV replication cycle. To confirm the absence of an effect on virus entry, a CHIKV entry assay was performed using CHIKV pseudoparticles (CHIKVpp) as described before (18). In contrast to chloroquine, the CHVB compounds did not inhibit the entry of CHIKVpp in BGM cells (Fig. 3C), demonstrating that CHVB compounds act at a postentry stage of the CHIKV replication cycle.

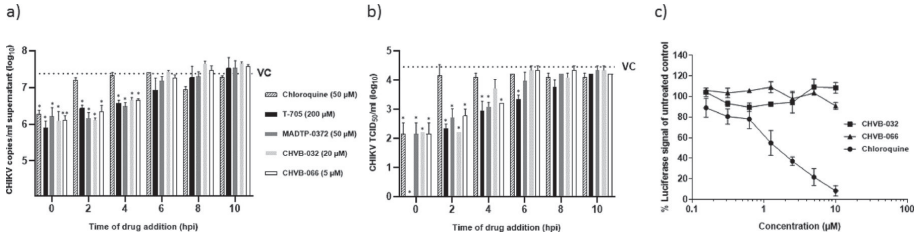

Figure 3. CHVB compounds inhibit a postentry step in the CHIKV replication cycle. The delay of treatment effect of CHVB-032 (20 µM; light grey columns) and CHVB-066 (5 µM; white columns) on viral replication in CHIKV899-infected Vero cells (MOI of 1) was determined at 12h p.i. by real-time qRT-PCR (A) and end-point titration assay (B). Chloroquine (50 µM; dashed columns) served as a reference compound for early-stage inhibition, whereas MADTP-0372 (50 µM; dark grey columns) and T-705 (200 µM; black columns) served as references for postentry-stage inhibition. Data are mean values ± SD from at least two independent experiments. Significant differences from untreated virus control were analyzed by two-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). (C) The effect of CHVB-032 (filled squares) and CHVB-066 (filled triangles) on CHIKV entry using CHIKV pseudoparticles (CHIKVpp). Chloroquine (filled circles) served as a reference compound for early-stage inhibition. BGM cells were treated with serial dilutions of each compound and then were infected with CHIKVpp. On day 3 p.i., cells were washed and then lysed to determine the luciferase activity. Data presented as percentage relative light units (RLU) of the untreated control, and the values plotted are mean values ± SD from at least three independent experiments. TCID, tissue culture infectious dose; VC, virus control (untreated).

CHVB-resistant variants carry mutations in CHIKV nsP1, nsP2 and nsP3 proteins

To identify the viral target of the CHVB series, a resistance selection procedure (11) was performed using CHVB-032 to generate compound-resistant virus variants. In total, four independent, putative CHVB-resistant virus variants were obtained. CPE reduction assays showed that only resistant virus strain 4 was markedly less susceptible to CHVB compounds, displaying a 19-fold resistance to CHVB-066 (Table 2). Resistant virus 4 was also resistant to other CHVB analogs (Table 3). Whole-genome sequencing of the resistant viruses revealed that all the variants carried an S454G mutation in nsP1 and an M703T mutation in the methyltransferase-like (MTase-like) domain of nsP2 (Table 4). Resistant virus 4 acquired two additional mutations, W456R in nsP1

and L494P in the highly variable domain of nsP3, whereas resistant virus 3 had an additional mutation, H280Q, in the alphavirus unique domain (AUD) of nsP3 (Table 4). Deep sequencing of the original virus stock used for resistance selection revealed that only the nsP3-L494P mutation was preexisting in the virus population with a frequency of 23.5%.

Table 2. Phenotype of putative CHVB-resistant clones

CHIKV strain	CHVB-066	
	EC_{50} (μM)	Fold resistance[a]
Wt	0.55 ± 0.01	1
Resistant virus 1	1.6 ± 0.3	2.9
Resistant virus 2	0.94 ± 0.05	1.7
Resistant virus 3	0.63 ± 0.4	1.1
Resistant virus 4	13 ± 0.4	19

[a] Fold resistance is EC_{50} variant/EC_{50} wt.

Table 3. Phenotype of resistant virus 4 against different CHVB analogs

Compound	EC_{50} (μM)		Fold resistance[a]
	wt	Resistant virus 4	
CHVB-023	1.2	7.5	6.5
CHVB-057	0.86	17.3	20
CHVB-032	2.7	> 100	> 37

[a] Fold resistance is EC_{50} variant/EC_{50} wt.

Table 4. CHVB-resistant variants carry mutations in CHIKV nsP1 to nsP3 genes

Protein	wt	Resistant virus 1	Resistant virus 2	Resistant virus 3	Resistant virus 4	Conserved in CHIKV
nsP1	**S454**	S454G	S454G	S454G	S454G	82%
	W456				W456R	97.9%
nsP2	**M703**	M703T	M703T	M703T	M703T	99.3%
nsP3	**H280**			H280Q		99.8%
	L494				L494P	99.3%

To determine which of the identified mutations contributed to phenotypic resistance against the compound, these mutations were introduced into the CHIKV LS3 cDNA clone using site-directed mutagenesis as described before (11). The

susceptibility of the recombinant CHIKV variants to the antiviral activity of the CHVB compounds was determined in a CPE reduction assay (Fig 4). Despite the presence of S454G and M703T mutations in all resistant clones, the single mutants rCHIKV+S454G and rCHIKV+M703T were not resistant to CHVB-032 or CHVB-066 (Fig. 4).With EC_{50} values of 0.38 µM and 0.31 µM, respectively, these variants were even more sensitive to the antiviral effect of CHVB-066 than wt virus (Table 5). A more sensitive phenotype was also found for CHVB-032. The combination of the S454G and M703T mutations in rCHIKV+S454G+M703T also did not yield a compound-resistant phenotype but rather one with increased susceptibility, as an EC_{50} value of 0.32 µM was determined (Table 5). The other mutations present in resistant virus 4 were then introduced into the CHIKV infectious clone in various combinations. Recombinant rCHIKV+W456R was as

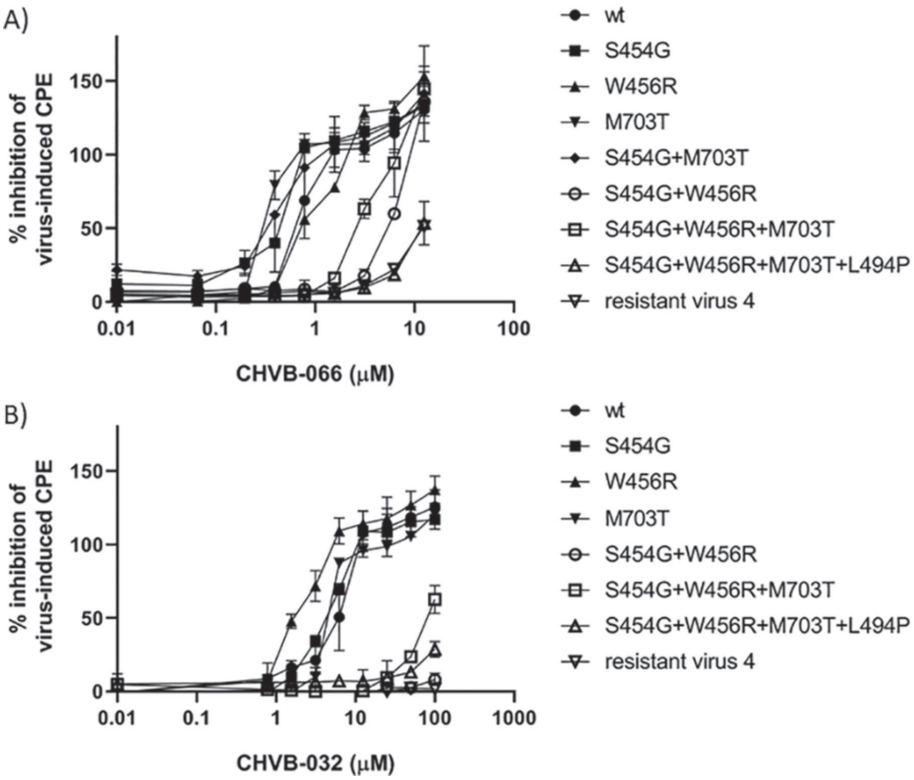

Figure 4. Phenotypic resistance of reverse-engineered CHVB-resistant mutants compared to resistant virus 4 and rCHIKV wt**.** Wild-type (filled circles) and recombinant CHIKV strains with individual or combined mutations that were identified in the CHVB-resistant isolate 4 (open down triangles) were assessed in a CPE reduction assay with increasing doses of CHVB-066 (A) and CHVB-032 (B). VeroE6 cells were treated with 0 to 12.5 µM CHVB-066 or 0 to 100 µM CHVB-032 and infected with rCHIKV LS3 wt or the recombinant CHIKV mutants at an MOI of 0.05. Four days postinfection, the cell viability was determined by the MTS/PMS method. Data represent

the means ± SD from at least two independent experiments performed in quadruplicate (*n*=8). CPE, cytopathic effect; wt, wild-type.

susceptible to CHVB-066 (EC_{50} of 0.76 μM) as wt virus, and it was even more susceptible to CHVB-032 (EC_{50} of 1.76 μM) compared to wt virus. Interestingly, it was specifically the combination of the two nsP1 mutations S454G and W456R that increased the resistance to CHVB-066 8-fold (EC_{50} of 5.4 μM) and to CHVB-032 more than 18-fold (EC_{50} of > 100 μM) (Table 5). The S454G and W456R mutations in nsP1 provided the virus the same level of resistance against CHVB-032 as the originally isolated resistant variant 4, and, thus, were sufficient to result in the CHVB-032-resistant phenotype. Introduction of the nsP2-M703T mutation in the variant with the nsP1-S454G and -W456R mutations (rCHIKV+S454G+W456R+M703T) did not further increase the resistance to both compounds (EC_{50} of 2.6 μM for CHVB-066 and EC_{50} of 81 μM for CHVB-032) (Table 5). This amino acid at position 703 is located in the MTase-like domain of nsP2, suggesting an interaction of this domain with nsP1 during the viral RNA capping. Importantly, the presence of all four mutations that were identified in resistant virus 4 in the reverse-engineered virus resulted in a CHVB-066-resistant phenotype comparable to that of the originally isolated virus 4 (EC_{50} of 12 μM) (Fig. 4). This reverse-engineered virus, rCHIKV+S454G+W456+M703T+L494P, was also

Table 5. Phenotypic resistance of CHVB-resistant mutants compared to those of original resistant virus 4 and rCHIKV wt

	CHVB-066		CHVB-032	
CHIKV strain	**EC_{50} (μM)**	**Fold resistance[a]**	**EC_{50} (μM)**	**Fold resistance**
wt	0.65 ± 0.002	1	5.45 ± 1.64	1
nsP1-S454G	0.38 ± 0.09	< 1	4.10 ± 0.16	< 1
nsP1-W456R	0.76 ± 0.1	1.2	1.76 ± 0.27	< 1
nsP2-M703T	0.31 ± 0.01	< 1	4.53 ± 0.02	< 1
nsP1-S454G + nsP2-M703T	0.32 ± 0.01	< 1	ND[b]	ND
nsP1-S454G + nsP1-W456R	5.4 ± 0.2	8.3	> 100	> 18
nsP1-S454G + nsP1-W456R + nsP2-M703T	2.6 ± 0.3	4.1	81 ± 10	15
nsP1-S454G + nsP1-W456R + nsP2-M703T + nsP3-L494P	12 ± 3.7	18.5	> 100	> 18
Resistant virus 4	13 ± 04	20	> 100	> 18

[a] Fold resistance is EC_{50} variant/EC_{50} wt.
[b] ND, not determined.

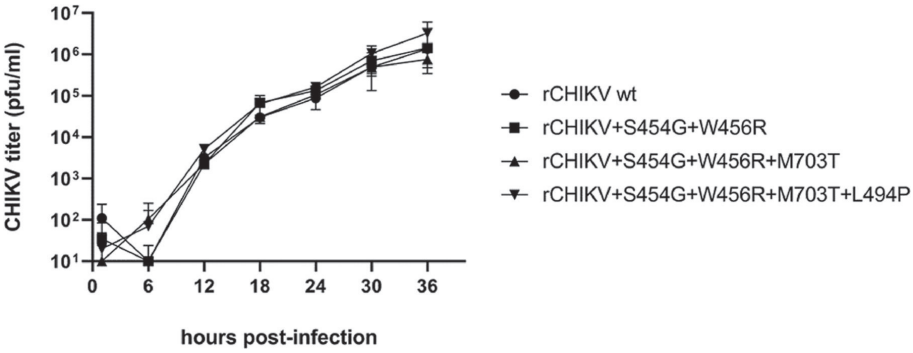

Figure 5. Growth kinetics of CHVB-066-resistant mutants in comparison with rCHIKV wt. VeroE6 cells were infected with CHIKV LS3 wt (filled circles) and the reverse-engineered double (filled squares), triple (filled up triangles) and quadruple (filled down triangles) mutants at an MOI of 0.1 for 1h at 37 °C. Following infection, the cells were washed twice with warm phosphate-buffered saline (PBS) and the medium was replaced with Eagle's minimal essential medium-2% fetal calf serum. At 6-h intervals, samples from the medium were harvested and stored at -80 °C. At 1 h postinfection, a sample was taken to determine how much virus of the inoculum remained after washing. The virus titers in the samples were determined by plaque assay. The experiment represents means ± SD from two independent infections titrated in duplicate (*n*=4). PFU, plaque-forming units; wt, wild-type; rCHIKV, recombinant CHIKV.

fully resistant to CHVB-032 (EC_{50} of > 100 µM). In summary, the S454G and W456R mutations in CHIKV nsP1 are sufficient and required for CHVB-032 resistance and a high level of resistance to CHVB-066. Maximum resistance to CHVB-066 required two additional mutations in nsP2 and nsP3, suggesting CHVB-066 has a higher barrier to resistance than CHVB-032. To exclude that the observed resistance is due to increased replication, the growth kinetics of the three variants with the highest fold resistance values were investigated. All the recombinant variants replicated with kinetics very similar to those of rCHIKV wt. Noticeably, at 6h postinfection, the reverse-engineered viruses with the M703T substitution in nsP2 produced higher titers than the other viruses, but at later time points there were no differences (Fig. 5).

The CHVB-resistant variants are cross-resistant to other CHIKV nsP1 inhibitors

Cross-resistance studies were performed with favipiravir and MADTP-0372. Favipiravir was able to protect cells against CHIKV-induced CPE for all the recombinant viruses tested, with EC_{50} values in the range of 90 to 142 µM, comparable to the wt (Table 6) Interestingly, the CHVB-resistant mutant with two mutations in nsP1 and the CHVB-resistant mutant with four mutations proved fully cross-resistant to MADTP-0372 (EC_{50}

of > 25 µM). Likewise, the MADTP-resistant mutant (carrying the nsP1-P34S mutation) was fully cross-resistant to CHVB-066 (EC_{50} of > 12.5 µM), suggesting that the modes of action of the CHVB and MADTP series are similar (Table 6).

Table 6. Cross-resistance of CHVB-resistant mutants against favipiravir and MADTP-0372

CHIKV strain	Favipiravir		MADTP-0372	
	EC_{50} (µM)	Fold resistance[a]	EC_{50} (µM)	Fold resistance
wt	115 ± 5.4	1	1.2 ± 0.06	1
nsP1-S454G + nsP1-W456R	136 ± 7.5	1.2	> 25	20
nsP1-S454G + nsP1-W456R + nsP2-M703T	90 ± 8.6	< 1	ND	ND
nsP1-S454G + nsP1-W456R + nsP2-M703T + nsP3-L494P	142 ± 25	1.2	> 25	20

[a] Fold resistance is EC_{50} variant/EC_{50} wt.

CHVB compounds inhibit the enzymatic functions of VEEV and SFV nsP1

The alphavirus nsP1 possesses both MTase and GTase activities, which are required for the capping of viral RNA (19). Because CHVB-resistant mutants displayed clear cross-resistance to the MADTP series, we assessed the effect of CHVB-032 and CHVB-066 on the enzymatic activities of purified recombinant nsP1. Since attempts to purify recombinant CHIKV nsP1 from *Escherichia coli* failed to obtain enzymatically active protein, *in vitro* assays with purified nsP1 of VEEV (19) and Semliki Forest virus (SFV) (16) were used. The *in vitro* GTase activity of VEEV nsP1 was quantified by measuring the formation of the m^7GMP-nsP1 complex by Western blotting, using m^7GTP as a substrate and an anti-m^3G/m^7Gp antibody for detection. The MTase activity of VEEV nsP1 was quantified by a filter-based assay using ^{3}H-*S*-adenosylmethionine (SAM). CHVB-066 inhibited the GTase activity of VEEV nsP1 in a concentration-dependent manner with a 50% inhibitory concentration (IC_{50}) of < 0.5 µM, more potently than the known inhibitor sinefungin (Fig. 6A). The MTase activity of VEEV nsP1 was also inhibited, but the effect was less pronounced (IC_{50} of 1.9 µM) (Fig. 6B). Since VEEV belongs to a different serocomplex than CHIKV, we wanted to confirm the inhibition of the m^7GMP-nsP1 complex formation using the nsP1 protein of SFV, which is

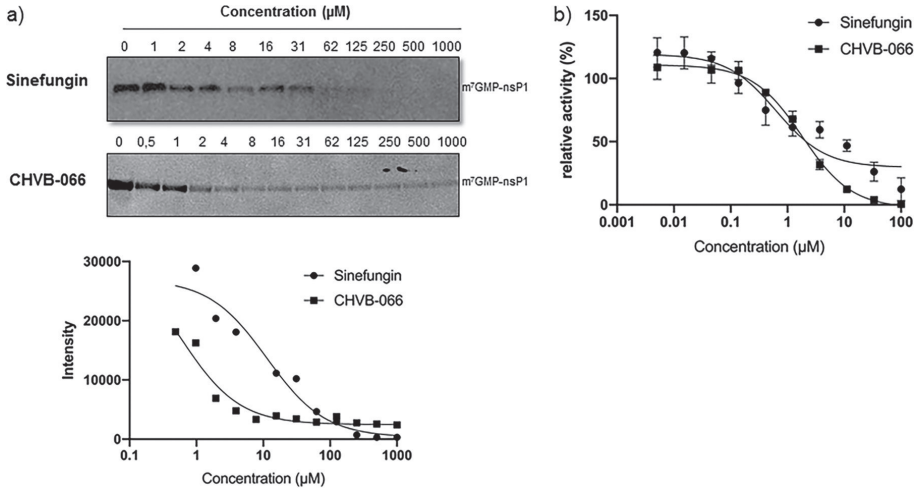

Figure 6. Effect of CHVB-066 on the enzymatic activities of VEEV nsP1. (A) Dose-response effect of CHVB-066 (filled squares) on the *in vitro* guanylylation of VEEV nsP1. Sinefungin (filled circles) was included in the assay as a reference compound. The formation of m^7GMP-nsP1 complex was detected by Western blotting using an anti-methyl^3/^{7}Gp antibody. (B) Dose-response effect of CHVB-066 (filled squares) on the MTase activity of VEEV nsP1. The nsP1-MTase product (^{3}H-methyl) GIDP was measured by a scintillation counter. Data presented are the average values ± SD from two independent experiments.

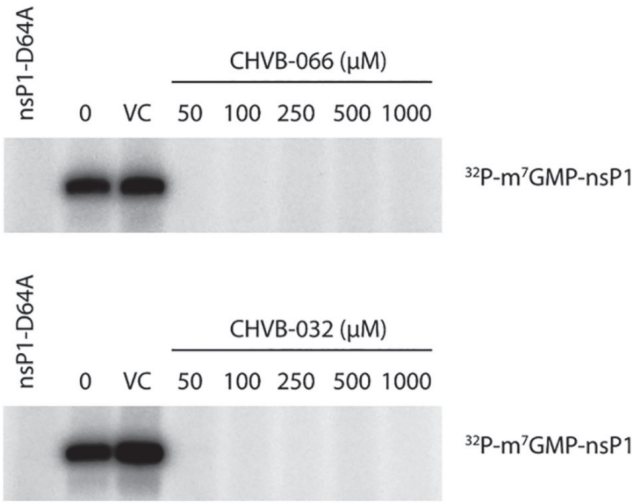

Figure 7. The effect of CHVB-032 and CHVB-066 on the enzymatic activities of SFV nsP1. Purified wt SFV nsP1 was incubated for 30 min at 30°C with increasing doses (50 μM to 1 mM) of CHVB-032 or CHVB-066, the concentration of solvent present at the highest dose of compound, i.e. 5% of dimethyl sulfoxide (DMSO) (VC) or left untreated (0). The D64A mutant was used as a negative control (left lane). The samples were resolved on a 10% SDS-PAGE gel, and the covalent ^{32}P-labelled m^7GMP-nsP1 reaction product was detected by autoradiography after overnight exposure. VC, virus control (untreated).

genetically more closely related to CHIKV (20). The readout of the SFV nsP1 assay is the formation of the covalent ^{32}P-labelled m^{7}GMP-nsP1 complex by using [α^{32}P]GTP and SAM as reaction substrates, thereby measuring the combined activities of the MTase and GTase. An active-site mutant with the D64A substitution was included as a negative control, since this mutant lacks MTase and GTase activity (21). CHVB-066 and CHVB-032 completely inhibited the activity of the wt SFV nsP1 protein at all tested doses (50 µM to 1 mM) (Fig. 7). These data demonstrate that the CHVB compounds directly inhibit the enzymatic activity of nsP1, particularly the MTase activity.

Discussion

Capping of viral mRNA is an essential posttranscriptional modification that influences subsequent downstream processing, translation, nuclear export, and stability of mRNA. Viruses have developed different strategies to cap their genomes and thereby mimic the cap structure of the host cell. Viral RNA caps can be acquired by a cap-snatching mechanism (orthomyxoviruses), or viruses can covalently attach a peptide (picornaviruses) or a cap moiety to their 5' ends (such as alphaviruses) (22, 23). The alphavirus nsP1 and nsP2 proteins contain the MTase, GTase and RNA triphosphatase (RTPase) functions that are needed for the viral RNA capping pathway. This alphavirus capping mechanism is unique and proceeds in a sequence that differs from the capping mechanism in the host cell. First, a GTP molecule is methylated using SAM as a methyl donor and producing SAH as a by-product of the reaction. In the second step, the methylated GTP (m^{7}GTP) is transferred onto nsP1, releasing pyrophosphate and forming the m^{7}GMP-nsP1 covalent intermediate (24). In the third step, the m^{7}GMP is attached to the first nucleotide of the viral RNA, which had been modified by the RTPase activity of nsP2 to remove the γ-phosphate (25). Inhibitors targeting this unique capping pathway may have a limited effect on cellular capping and host MTases (26).

We previously demonstrated, for the first time, that small molecule inhibitors of the alphavirus capping machinery could efficiently and completely inhibit CHIKV replication in cell culture (11). This compound series with a triazolopyrimidinone scaffold, known as the MADTP series, inhibits the GTase activity of VEEV nsP1 in an *in vitro* assay. Recently, 6'-β-fluoro-homoaristeromycin and 6'-fluoro-homoneplanocin A also have been shown to potently inhibit CHIKV replication by targeting nsP1 (16). These compounds affected the MTase activity of SFV nsP1 in enzymatic assays.

Other reports on CHIKV nsP1 as an antiviral target have followed, using target-based screening methods. A nsP1-GTP competition screen resulted in the identification of several small molecules able to compete with GTP for the CHIKV nsP1-GTP binding site (27). In contrast, an enzyme-linked immunosorbent assay to identify inhibitors of CHIKV nsP1 by measuring the formation of the m^7GMP-nsP1 complex did not result in the identification of new inhibitors (28). A similar assay using VEEV nsP1, on the other hand, resulted in the identification of a number of molecules that inhibited the GTase activity (by >80%), and cross-resistance studies indicated that their mechanism differed from that of the MADTP series (26). We now describe a third series of CHIKV nsP1-targeting compounds with yet another chemical scaffold, but the same target as the MADTP series, since CHVB-resistant viruses were cross-resistant to MADTP-0372 and vice versa. The described MADTP-resistant virus carried the P34S mutation in nsP1, whereas the CHVB-resistant viruses described here require at least two mutations, S454G and W456R, in nsP1 to confer resistance. Interestingly, during our initial attempts to produce virus stocks of recombinant CHIKV mutants, we noticed the emergence of the P34S mutation in viruses with the W456R mutation. Because several mutants, in particular the ones containing the W456R mutation, exhibited the tendency to revert back to wt, we started culturing our virus stocks in the presence of compound to retain mutations. Under this selection pressure, the P34S mutation was no longer identified by Sanger sequencing. This might explain why the mutation did not appear during the resistance selection procedure for the CHVB compounds, as these conditions might have favored the presence of the W456R mutation over the P34S mutation. Although a crystal structure of CHIKV nsP1 is currently not available to support our hypothesis, we suggest that the P34 residue in the N-terminal region of the protein might be in close proximity to the W456 in the C-terminal region of the protein.

Similar to the MADTP series, the CHVB series is also CHIKV specific, with no or modest antiviral activity against other alphaviruses in cell-based assays. Because the enzymatically active region of the alphavirus nsP1 is well conserved, it is puzzling that neither series is inactive against other alphaviruses. It was recently shown that the Sindbis virus generates a high amount of noncapped viral genomes, more specifically during the early stage of the replication cycle (29). It is not known whether the same holds true for CHIKV. A possible explanation for the differential susceptibility of alphaviruses to the two series of compounds is that CHIKV is very susceptible to capping inhibitors because it generates more capped viral genomes than other

alphaviruses. The crystal structures of nsP1 proteins of CHIKV and other alphaviruses could provide answers, but such structures have not yet been resolved.

In contrast to the MADTP series, which required only one amino acid substitution at position 34 (P34S) in nsP1 to develop full resistance, resistance to CHVB-032 required the presence of two mutations in nsP1, and full resistance to CHVB-066 even required two additional mutations in nsP2 and nsP3. Moreover, in the second step of the resistance selection protocol, 17% of culture wells showed full CHIKV-induced CPE for the MADTP series (24 wells out of 144) versus only 2.5% (4 wells out of 162) in case of the CHVB series. These data suggest that the barrier to resistance is higher for the CHVB series and that the development of resistance in the clinical settings is less likely to occur, as two mutations are needed. Furthermore, in the reverse-engineered variants mutations causing resistance to CHVB compounds tended to revert back to wt in the absence of selective pressure.

In conclusion, we report on a novel class of small molecule inhibitors of the CHIKV capping machinery. Several other CHIKV inhibitors that target the capping have been reported, indicating that the capping machinery of the virus is an interesting target for antiviral drug development.

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