

Antiviral Strategies Against Chikungunya Virus

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Abstract

In the last few decades the Chikungunya virus (CHIKV) has evolved from a geographically isolated pathogen to a virus that is widespread in many parts of Africa, Asia and recently also in Central- and South-America. Although CHIKV infections are rarely fatal, the disease can evolve into a chronic stage, which is characterized by persisting polyarthralgia and joint stiffness. This chronic CHIKV infection can severely incapacitate patients for weeks up to several years after the initial infection. Despite the burden of CHIKV infections, no vaccine or antivirals are available yet. The current therapy is therefore only symptomatic and consists of the administration of analgesics, antipyretics, and anti-inflammatory agents. Recently several molecules with various viral or host targets have been identified as CHIKV inhibitors. In this chapter, we summarize the current status of the development of antiviral strategies against CHIKV infections.

Key words Chikungunya virus, Antivirals, Chloroquine, Arbidol, Ribavirin, Favipiravir, Immune modulators

1 Introduction

Chikungunya virus (CHIKV), belonging to the *Alphavirus* genus of the *Togaviridae* family is an arthropod-borne virus transmitted by female mosquitoes of the *Aedes* species [1]. CHIKV infections cause Chikungunya fever which is characterized by abrupt fever, rash and bilateral symmetric arthralgia. In most of the CHIKV-infected patients the acute phase is followed by persistent disabling polyarthrititis that can severely incapacitate the patient for weeks up to several months [1]. Despite the widespread emergence of CHIKV and the high morbidity rate associated with it, there is no approved vaccine or antiviral treatment available at the moment. The current therapy is therefore purely based on the relief of the patient's symptoms and consists of the administration of analgesics, antipyretics, anti-inflammatory agents such as paracetamol, and nonsteroidal anti-inflammatory drugs, and of bed rest and fluids intake [2]. The use of aspirin during CHIKV infection is to be avoided because of the risk of bleeding and the potential risk of

developing Reye's syndrome [3]. In addition, the use of systemic corticosteroids is not recommended due to the strong rebound effect after cessation of treatment [1]. In severe cases, where patients have limited response to nonsteroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs (DMARDs) such as methotrexate, hydroxychloroquine, or sulphasalazine can be administered to relieve the symptoms [3, 4]. The development of novel, potent antiviral drugs against CHIKV is thus urgently needed.

2 Antiviral Strategies for the Treatment of CHIKV Infection

2.1 *Inhibitors of Viral Entry*

Antiviral agents targeting the entry of enveloped viruses are of major interest since they inhibit an early step in the viral life cycle which minimizes the cell damage caused by intracellular viral replication. In addition, viral entry inhibitors may target extracellular components, which are more accessible; therefore, they could be effective in lower dosages with limited toxicity [5].

2.1.1 *Chloroquine*

Chloroquine is an antimalarial drug that has also been shown to inhibit the *in vitro* replication of several viruses, including HIV, severe acute respiratory syndrome (SARS) coronavirus, and alphaviruses [6]. Chloroquine was reported to inhibit CHIKV entry into cells, possibly by raising the endosomal pH and thus preventing the fusion of the CHIKV E1 protein with the endosomal membrane [7, 8]. The potential effect of chloroquine treatment was assessed in two clinical trials. In a first clinical trial improvement of symptoms in the chronic phase of CHIKV infection were reported following chloroquine treatment [7], whereas another study failed to prove the efficacy of chloroquine as a treatment for the acute phase of CHIKV infection [8]. Therefore, the use of chloroquine as anti-CHIKV antiviral requires further study to prove its effectiveness and to determine the appropriate dosage and length of treatment.

2.1.2 *Arbidol and Its Derivatives*

Arbidol is a broad-spectrum antiviral that has been licensed in Russia and China for the treatment and prophylaxis of influenza and other respiratory infections [9]. Arbidol was also reported as an inhibitor of CHIKV infection in MRC-5 cells [10]. The mechanism of its anti-CHIKV activity has not been totally elucidated. An arbidol-resistant CHIKV strain could be selected and was shown to have acquired a mutation at amino acid 407 (G407R) in the CHIKV E2 glycoprotein, which may be involved in binding to host receptors [10]. Recently, a series of arbidol analogues have been synthesized and evaluated for their anti-CHIKV activity [11]. Two analogues in this series (IIIe and IIIf) inhibited the CHIKV-induced cytopathic effect (CPE) with selectivity indices higher than that of the parent compound arbidol [11].

2.1.3 Phenothiazines

Phenothiazines are clinically approved antipsychotics. In a screening assay using Semliki Forest virus (SFV) as a bio-safe surrogate for CHIKV, six compounds containing a 10H-phenothiazine core, including chlorpromazine, perphenazine, ethopropazine, thiethylperazine, thioridazine, and methdilazine were identified as possible SFV entry inhibitors. The antiviral activity of these molecules was confirmed using a recombinant CHIKV strain carrying a luciferase reporter gene (CHIKV-Rluc). When compared to the SFV-based screening results, the molecules showed similar potencies against the CHIKV-Rluc; however, the EC₅₀ values determined using the CHIKV-Rluc were higher. The antiviral target of these inhibitors still needs to be identified [12].

2.1.4 Epigallocatechin Gallate

Epigallocatechin gallate (EGCG) is the major constituent of green tea. Recently, EGCG has been reported to have a modest but significant antiviral activity against CHIKV [13]. The inhibition of CHIKV entry and attachment to the target cells by EGCG was confirmed using pseudo-particles carrying the CHIKV envelope proteins [13].

2.2 Inhibitors of Viral Protein Translation

2.2.1 RNA Interference

RNA interference is induced by small interfering RNAs (siRNA) that are homologous in sequence to the gene that needs to be silenced. Small interfering RNAs are 21-23 nucleotides long dsRNA molecules having 3'-overhangs of two nucleotides. Treatment of cells with exogenous siRNAs results in the assembly of a RNA-induced silencing complex (RISC) which degrades specific complementary mRNA molecules. Consequently, protein expression of the targeted gene is markedly reduced.

Small interfering RNA (siRNA) sequences targeting CHIKV nsP3 and E1 genes were reported to significantly reduce CHIKV titers at 24 h post-infection in transfected Vero cells [14]. However, the inhibitory effect of these siRNAs was transient and diminished after 3 days of infection. These siRNAs could thus be used in combination with other antivirals for more effective treatment. In a more recent study, nsP1 and E2 siRNAs were generated and their potential activity was evaluated in cell culture and in animal models [15]. SiRNAs directed against nsP1 and E2 as well as their combinations, reduced *in vitro* CHIKV replication in Vero cells with more than 90 %. Interestingly, when CHIKV-infected mice were injected 3 days post-infection with these siRNAs, CHIKV replication was completely inhibited at the highest dose of siRNA tested (1 mg/kg body weight; [15]).

Plasmid-based small hairpin RNAs (shRNAs) were also designed and evaluated as strategy to inhibit CHIKV replication. ShRNAs produced from the shRNA-plasmid construct resulted in their intracellular processing to siRNAs causing specific knockdown of viral RNA and subsequent inhibition of viral protein expression. Stable cell clones expressing shRNA against CHIKV E1 and nsP1

showed significant and sustained inhibition of CHIKV infection [16]. In addition, mice pretreated with E1 targeting shRNA were completely protected against CHIKV induced disease and their survival was observed up to 15 days post-infection (untreated animals died between 6 and 10 days post-infection; [16]).

2.2.2 *Harringtonine and Homoharringtonine*

Harringtonine, a cephalotaxine alkaloid derived from *Cephalotaxus harringtonia*, has been reported as a potent inhibitor of CHIKV with minimal cytotoxicity [17]. In addition, homoharringtonine, a more stable analogue of harringtonine, also showed anti-CHIKV activity. Homoharringtonine has been recently approved by the FDA for the treatment of chronic myeloid leukemia in 2012. Harringtonine and homoharringtonine were found to suppress the production of viral nsP3 and E2 proteins, most likely through the inhibition of the host cell protein translation machinery [17]. In addition, the decrease in nsP3 production resulted in a reduction of viral replicase complexes formation. Consequently, the level of negative-sense RNAs was decreased leading to the reduced synthesis of the viral positive-sense RNA genome [17].

2.3 *Inhibitors of Viral Genome Replication*

2.3.1 *Ribavirin*

Ribavirin, a structural analogue of guanosine, is a broad-spectrum antiviral drug that has been approved by the FDA for the treatment of respiratory syncytial virus in infants [18], and in combination with pegylated interferon alpha (IFN- α) for treatment of chronic hepatitis C virus infection [19]. Ribavirin was shown to inhibit the replication of CHIKV *in vitro* [20]. In addition, the combination of ribavirin and IFN- α 2b was reported to result in synergistic inhibitory effect against CHIKV replication in Vero cells [20]. The mechanism of the antiviral action of ribavirin is likely different for different viruses. The 5'-monophosphate metabolite of ribavirin acts as a competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) resulting in a depletion of intracellular GTP (and dGTP) pools [21]. The predominant mechanism by which ribavirin inhibits the replication of other RNA viruses such as flaviviruses and paramyxoviruses has been shown to be mediated by depletion of GTP pools [22]. Other suggested mechanisms by which ribavirin inhibits RNA virus replication include the inhibition of viral RNA capping, inhibition of the viral polymerase, and lethal mutagenesis of the RNA genome [23].

2.3.2 *6-Azauridine*

6-Azauridine is a broad-spectrum anti-metabolite that inhibits the replication of both DNA and RNA viruses [24]. It is a uridine analogue that competitively inhibits the orotidine monophosphate decarboxylase enzyme, which is involved in the *de novo* synthesis of pyrimidines [24]. 6-Azauridine showed strong inhibitory effect against CHIKV replication in Vero cells with an EC₅₀ value of 0.82 μ M [20].

2.3.3 *Mycophenolic Acid*

Mycophenolic acid (MPA) is a non-competitive inhibitor of IMPDH which has been used clinically as an immunosuppressant to prevent the rejection of transplant organs. MPA inhibits *in vitro* CHIKV replication [21]. The inhibition of CHIKV replication appears to be due to the depletion of intracellular GTP pools [21].

2.3.4 *Favipiravir (T-705)*

Favipiravir (T-705) is a broad-spectrum antiviral agent that was originally discovered as an inhibitor of influenza A virus replication. In the cell, T-705 is metabolized to its ribofuranosyl 5'-triphosphate form, which was shown to be a competitive inhibitor for the incorporation of ATP and GTP by the viral RNA-dependent RNA polymerase (RdRp; [25]). However, the exact mechanism of action of T-705 has not been totally clarified yet. Recently, it has been reported that T-705 and its defluorinated analogue, T-1105 inhibit the *in vitro* replication of CHIKV [26]. In addition, the oral treatment of CHIKV-infected AG129 mice with T-705 protected these mice from severe neurological disease and reduced the mortality rate by more than 50 %. Low-level T-705-resistant CHIKV variants have been selected. These variants carried a K291R mutation in the F1 motif of the RdRp that was shown to be responsible for the observed resistance to T-705. This position is highly conserved in the polymerase of +ssRNA viruses [26].

2.4 *Inhibitors of CHIKV nsP2*

The CHIKV nsP2 protein exhibits RNA triphosphatase/nucleoside triphosphatase activity, as well as helicase activity within the N-terminal half while the C-terminal half encodes the viral cysteine protease required for processing of the non-structural viral polyprotein [27, 28]. In addition, nsP2 plays an important role in shutting down host cell mRNA transcription via degradation of a subunit of the DNA-directed RNA polymerase II. It also inhibits the host antiviral response by suppressing transcription and type I/II interferon-stimulated JAK/STAT signaling [28]. In a high-throughput screening for CHIKV nsP2 inhibitors that target the nsP2-mediated transcriptional shut off, a natural compound derivative (ID1452-2) was shown to partially block the nsP2 activity resulting in inhibition of CHIKV replication in cell culture [29]. In another study, a number of nsP2 inhibitors were identified using a computer-aided screening procedure of which one lead compound (compound 1) showed a significant antiviral activity against CHIKV [30]. This compound was predicted to bind to the central portion of the nsP2 protease active site.

Recently, a number of arylalkylidene derivatives of 1,3-thiazolidin-4-one have been shown to inhibit the *in vitro* replication of CHIKV. The inhibition of the CHIKV protease was suggested to be the mechanism of action of these compounds [31].

2.5 Inhibitors of Host Targets

2.5.1 Furin Inhibitors

Cellular furins and furin-like proteases are involved in the cleavage of viral pE2 into mature E2 and E3 proteins. The inhibition of cellular furin may therefore be expected to inhibit the formation of mature viral particles. Decanoyl-RVKR-chloromethyl ketone (dec-RVKR-cmk) is an irreversible furin inhibitor that was shown to inhibit CHIKV infection *in vitro* via inhibition of viral glycoprotein maturation [32]. The combination of dec-RVKR-cmk and chloroquine resulted in an additive inhibitory effect on CHIKV replication. Surprisingly, pretreatment of cells with dec-RVKR-cmk revealed a significant inhibition of viral entry, indicating that dec-RVKR-cmk treatment could alter the cleavage of proteins involved in CHIKV endocytosis or early replication steps or that this molecule could even inhibit CHIKV receptor maturation [32].

2.5.2 Modulators of Cellular Kinases

Protein Kinase C(PKC) Activators

Prostratin and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) are well-known triterpene diterpenoids with a basic phorbol carbon skeleton esterified at position 13 [33]. Due to their chemical structure, they act as natural analogues of diacylglycerol that induce the activation of protein kinases C. Previously, prostratin and TPA were reported to have antiviral activity against HIV [34]. Prostratin and TPA were also identified as potent and selective CHIKV inhibitors *in vitro* [33]. Further studies are required to determine their mode of action against CHIKV.

Kinase Inhibitors

In a cell-based screening of a kinase inhibitor library, six kinase inhibitors were found to inhibit CHIKV-associated cell death in a dose-dependent manner [35]. Of these molecules, four compounds have a benzofuran core scaffold, one a pyrrolopyridine and one a thiazol-carboxamide scaffold. Using image analysis, it was shown that CHIKV-infected cells treated with these molecules had less prominent apoptotic blebs, which are typical for the CHIKV cytopathic effect. Moreover, these compounds reduced viral titers up to 100-fold. It was suggested that the inhibition of the virus-induced CPE by these compounds was the result of inhibition of kinases involved in apoptosis [35].

2.5.3 HSP-90 Inhibitors

HSP-90 is a family of highly conserved molecular chaperones which includes two cytoplasmic isoforms: stress-induced HSP-90 α and constitutively expressed HSP-90 β . In general, HSP-90 is involved in maturation, localization, and turnover of its client proteins in a cell. HSP-90 has been reported to play an important role in the replication of many DNA and RNA viruses such as hepatitis B virus, hepatitis C virus, human cytomegalovirus and influenza virus. Consequently, HSP-90 inhibitors may have a role as broad(er)-spectrum antiviral agents. Two HSP-90 inhibitors, HS-10 and SNX-2112, were reported as CHIKV replication inhibitors. The treatment of CHIKV-infected mice (SvA129) with HS-10 and SNX-2112 significantly reduced the serum viral load at

48 h post-infection and protected against the CHIKV-induced inflammation in the limb of infected mice [36]. In co-immunoprecipitation studies CHIKV nsP3 and nsP4 were shown to interact with HSP-90. Interestingly, the knockdown of the HSP-90 α subunit resulted in a more pronounced inhibition of viral replication than targeting the HSP-90 β subunit. HSP-90 α is thought to be involved in the stabilization of CHIKV nsP4 and the formation of the CHIKV replication complex [36]. Further mechanistic studies are required to unravel the role of HSP-90 in the replication cycle of CHIKV.

2.5.4 Modulators of Host Immune Response

The innate immune system plays an important role in the acute phase of CHIKV infection. Detection of CHIKV RNA by Toll-like receptors (TLRs) 3, 7, and 8, as well as RIG-I like receptors during the acute phase of infection is believed to trigger the production of type I IFNs. Consequently, type I IFNs activate the transcription of interferon-stimulated genes (ISGs), which encode proteins involved in the host antiviral defense leading to clearance of the infection [37]. Therefore, activation of the innate immune response could be interesting for the treatment of CHIKV infections.

IFN- α

Treatment with IFN- α 2a and IFN- α 2b inhibited CHIKV replication in Vero cells in a dose-dependent manner [20]. The combination of IFN- α 2b and ribavirin resulted in a synergistic antiviral effect on in vitro CHIKV replication. A CHIKV strain carrying the E1 A226V mutation was reported to be more sensitive to the antiviral activity of recombinant IFN- α than wild-type virus [38].

2',5'-Oligoadenylate Synthetase 3 (OAS3)

The role of OAS3 in the innate immunity towards CHIKV was investigated using a stable HeLa cell line expressing OAS3 [39]. The expression of OAS3 by this cell line efficiently inhibited CHIKV infection by blocking the early stages of virus replication. A CHIKV variant with a glutamine-to-lysine mutation at position 166 of the envelope E2 glycoprotein proved resistance to the antiviral activity of OAS3 [40].

Polyinosinic Acid–Polycytidylic Acid

Polyinosinic acid–polycytidylic acid (poly (I:C)), a synthetic analogue of dsRNA, is a potent stimulant of IFN that interacts with TLR3. Treatment of human bronchial epithelial cells with poly (I:C) before CHIKV infection suppressed virus-induced CPE up to 72 h post-infection. Poly (I:C) resulted in a significant up regulation of IFN- α , IFN- β , OAS, and MxA in uninfected cells [41].

RIG-I Agonists

RIG-I (retinoic acid-inducible gene I) is a member of the RIG-I like receptor family which recognizes viral dsRNA leading to activation of multiple antiviral factors that block viral infection at different stages. Interestingly, chemically or enzymatically synthesized dsRNA molecules with an exposed 5'-triphosphate end (5' ppp)

were reported to induce RIG-I [42, 43]. It has been recently shown that pretreatment of MRC-5 cells with an optimized 5' triphosphorylated RNA molecule triggered RIG-I stimulation resulting in protection against CHIKV infection [44]. Moreover, the protective response against CHIKV induced by this 5' ppp RNA was largely independent of the type I IFN response. These results suggest the potential efficacy of RIG-I agonists as an antiviral treatment for CHIKV infection.

2.6 Inhibitors with an Unknown Target

2.6.1 *Trigocherrierin A*

Trigocherrierin A is a new daphnane diterpenoid orthoester isolated from the leaves of *Trigonostemon cherrieri* [45]. Trigocherrierin A inhibits CHIKV in cell culture but the mechanism of its action remains elusive.

2.6.2 *Aplysiatoxin Derivatives*

Debromoaplysiatoxin and 3-methoxydebromoaplysiatoxin are marine toxins isolated from the marine cyanobacterium, *Trichodesmium erythraeum* [46]. Both compounds had significant antiviral activity against CHIKV at non-toxic concentrations. The compound was reported to block a post-entry step in the CHIKV lifecycle.

2.6.3 *5,7-Dihydroxy- flavones*

A number of 5,7-dihydroxyflavones (apigenin, chrysin, naringenin, and silybin) were identified as inhibitors of the CHIKV subgenomic replicon [12]. The molecular target of these compounds is still unknown.

3 Conclusion

The global re-emergence of CHIKV and the high morbidity rate associated with its infection emphasizes the need to develop potent antiviral agents against CHIKV. So far, a number of classes of compounds that inhibit viral replication by targeting either a viral or a host factor have been reported. Most of the compounds have relatively modest activity and for most of them, activity in infection models (in mice) was not assessed. Some of these classes may serve as a starting point for the design of more specific and selective inhibitors of CHIKV replication. Also, to the best of our knowledge, no information is available yet on the effect of antivirals on the chronic stage of CHIKV infection. Recently, mouse models for CHIKV-induced arthritis and chronic joint disease have been developed which will help the evaluation of CHIKV antiviral agents in different stages of CHIKV infection [47, 48]. Several other viruses belonging to the *Alphavirus* genus, in particular the equine encephalitis viruses are considered to be a potential bioterroristic threat. When designing/developing antivirals against the Chikungunya virus it may be important to develop classes of compounds that have pan-alphavirus activity and that could thus also be used for the treatment of alphaviruses other than CHIKV.

Among the reported CHIKV inhibitors, favipravir, ribavirin, arbidol, and IFN- α have been approved previously for the treatment of other viral infections. This could markedly facilitate their evaluation for clinical use in CHIKV-infected patients. Favipravir, a drug with a broad-spectrum antiviral activity, has been approved in Japan for the treatment of influenza virus infections. It is currently also being evaluated in Western Africa for the treatment of Ebola virus infection. If its activity is demonstrated against this infection, this compound may be considered for treatment of other infections such as those caused by CHIKV. However, given the growing number of patients suffering from CHIKV infections, it may be justified to develop specific CHIKV/alphavirus drugs. Highly potent drugs are today available for the treatment of infections with herpes viruses, HIV, the hepatitis B and C virus. Without a doubt, it should also be possible, when investing sufficient effort, to develop highly effective and safe drugs for the treatment (and perhaps even prophylaxis) of infections with alphaviruses.

Acknowledgments

The original work of the authors is supported by EU FP7 SILVER (contract no. HEALTH-F3-2010-260644) and the BELSPO IUAP consortium BELVIR (Belgium). Leen Delang is funded by the Scientific Fund for Research of Flanders (FWO).

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