Review

Antivirals against the Chikungunya Virus

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Abstract: Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus that has re-emerged in recent decades, causing large-scale epidemics in many parts of the world. CHIKV infection leads to a febrile disease known as chikungunya fever (CHIKF), which is characterised by severe joint pain and myalgia. As many patients develop a painful chronic stage and neither antiviral drugs nor vaccines are available, the development of a potent CHIKV inhibiting drug is crucial for CHIKF treatment. A comprehensive summary of current antiviral research and development of small-molecule inhibitor against CHIKV is presented in this review. We highlight different approaches used for the identification of such compounds and further discuss the identification and application of promising viral and host targets.

Keywords: Chikungunya virus; alphavirus; antiviral therapy; direct-acting antivirals; host-directed antivirals; in silico screening; in vivo validation, antiviral drug development

1. Introduction

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus and belongs to the *Togaviridae* family. The virus was first isolated from a febrile patient in 1952/53 in the Makonde plateau (Tanzania) and has been named after the Makonde word for "that which bends you up", describing the characteristic posture of patients suffering severe joint pains due to the CHIKV infection [1]. In the following years, only local and periodic outbreaks have been documented. However, in 2004 the CHIKV re-emerged at the coast of Kenya, spreading to La Reunion Island and surrounding island in the Ocean and South Asia [2]. A new CHIKV mutation with the A226V amino acid variant in the envelope glycoprotein E1 (CHIKV 06.21) was reported during that outbreak. This mutation, along with specific mutations in the E2 protein, allowed the virus to expand its vector potential from primarily *Aedes aegypti* to the more global *Ae. Albopictus* and thus permitting the virus to spread in different temperature zones [3].

Consequently, in 2013 a CHIKV outbreak was reported on the Caribbean island St. Martin, following Brazil in 2014 and afterwards the rest of the American continent – causing more than 1.2 million cases of CHIKV infection in one year [4, 5]. Furthermore, the first autochthonous outbreaks started to occur also in Europe (Italy and France) [6–9]. Due to globalisation, climate change, and lack of immunity in the worldwide population, this global spreading of the CHIKV is still ongoing – reaching new territories and higher numbers of infections [10]–[12].

Infection with the Chikungunya virus causes a high onset of fever for 3 to 10 days, followed by rash, myalgia, nausea, and severe joint pain [12]. Although this Chikungunya Fever (CHIKF) is rarely fatal, in approximately 50% of infected patients, the severe arthralgia and myalgia can last for months to years even after clearance of the viral infection [13]. However, the precise mechanism of these chronic CHIKF symptoms is still unclear. Furthermore, complications in patients with comorbidities and the elderly have been reported [14]. Up today there are no licensed drugs or vaccines available, and the alleviation of symptoms by, e.g. NSAIDs, is the only possible treatment for CHIKF patients [15].

2. CHIKV Replication Cycle

Like other alphaviruses, the entry of the CHIKV in cells involves the initial interaction of viral proteins with attachment factors and specific receptors from the host cell [16]. The CHIKV virion's surface contains 80 trimeric spikes of E1 and E2 glycoproteins [17]. E2 facilitates the viral attachment to the host cell by interacting with surface host receptors followed by clathrin-mediated endocytosis (CME). The fusion of the viral membrane with the membrane of the host is triggered by a low pH environment leading to conformational changes of the viral envelop glycoprotein E1 [17]. Subsequently, the viral nucleocapsid is released into the cytoplasm, where it is disassembled to release the viral genome. The viral genome is then translated by the host cell translation machinery creating the non-structural-polyprotein P1234, which is cleaved into the precursor P123 and the viral non-structural-protein nsP4 [18]. P123 and nsP4 form the early replication complexes (RCs). They are responsible for synthesising the negative-strand RNA as a template to synthesise the desired positive-strand genomic RNA and sub-genomic RNA (26S RNA). Eventually, the formation of the P123 and nsP4 reaches a concentration threshold and the cleavage of the precursor P123 into fully processed nsPs is triggered. The 26S RNA, on the other hand, serves as the mRNA encoding the structural viral proteins C-pE2-6K-E1 [19–21].

Once formed, the capsid protein (C) is released by its autocleavage activity, while the remaining pE2-6K-E1 precursor is processed in the endoplasmic reticulum (ER) into pE2, 6K, and E1. While pE2 and E1 form heterodimer complexes, the cleaved capsid protein binds newly synthesised viral RNA, initiating to form the nucleocapsid core. This complex migrates towards the cell membrane through the Golgi secretory pathway, where pE2 is cleaved by the host enzyme furin or furin-like proteinases into the mature E2 and E3. E2 and E3 interact with the already formed nucleocapsid core and encapsidate the remaining viral RNA genome. Finally, the nucleocapsid core is recruited to the cell membrane where the particle buds from the cell where a new replication cycle begins [21–23].

3. Strategies for Identification of Antiviral Compounds

Various approaches have been utilised to identify antiviral compounds, like cell-based high-throughput screening (HTS) and computational methods, including rational structure-based drug design on known crystal structures or homology models of viral and proviral host proteins. The most conventional method is the cell-based HTS with the virus-induced cytopathic effect (CPE) as readout. This method has the advantage to provide two simultaneous information – the antiviral activity of the screened compounds and their cytotoxicity. Different compound libraries were used for the *in silico* and *in vitro* screening, ranging from libraries containing only FDA-approved drugs and libraries with compounds showing special chemical features to fragment-based libraries. The fast and economic computer-aided drug design has also been widely used for the identification of novel lead compounds by virtual screening. The identified hits were often further optimised by structure-activity relationship (SAR) assays. In addition, resistance selection in the presence of a compound was often performed in cell-based assays to identify the target protein of such compounds and give valuable insight into the viral pathways.

Theoretically, all involved factors of the viral replication cycle could be potential targets for antiviral compounds. Like other viruses, the CHIKV uses a variety of interactions of viral proteins and host factors for its replication. The known and utilised proviral host factors are discussed below. Targeting such a host factor could provide broad-spectrum antiviral compounds as many viruses use the same replication strategies. On the other hand, unwanted side effects are more often seen in compounds with such an approach. Therefore, a combination of antiviral compounds with a different mechanism of action could provide a synergistic effect, leading to the reduction of antiviral drug concentration and thus may help decrease serious side effects. Moreover, such a combination could prevent the formation of drug resistance.

4. Virus Targeting Inhibitors

A comprehensive overview of small molecules targeting viral proteins is given in **Table 1**. Studies without any *in vivo* or *in vitro* data were excluded. It is worth mentioning that the values given in Table 1 are not directly comparable to each other as they performed experiments, and setups differed between the discussed studies. Different virus strains, readouts (e.g. CPE and virus titer reduction) and cell lines were, for example, used and influence the assay results.

Table 1. Virus targeting compounds.[a]

		In vitro				*In vivo*		
Compound[b]	**Viral target**[c]	**EC_{50} (μM)**[d]	**CC_{50} (μM)**	**SI**	**Cell line**	**Efficacy**	**Mouse model**	
Arbidol*	E2	12.2 ± 2.2	376	36	MRC5	—	—	[24]
IIc	E2	6.5 ± 1	156	22	Vero	—	—	[25]
Suramin*	E2	8.8 ± 0.5	>700	>39.1	BHK-21	Reduced viral burden and decreased foot swelling	C57BL/6	[26–28]
Bis(benzofuran-thiazolidone)s (3g)	—	1.5	>200	<133	Vero	—	—	[29]
LQM334	—	81.1 ± 6.4% viral inhibition	n.s.	n.s.	Vero	—	—	[30]
Micafungin*	—	17.2 ± 1.08	>100	>5.81	U2OS	—	—	[31]
Picolinic acid	C	60.63% inhibition with 2 mM	n.s.	n.s.	Vero	—	—	[32]
AP4	—	10.66 ± 2.25	2172 ± 104	n.s.	Vero	—	—	[33]
EAC	—	4.01 ± 1.96	1657 ± 1109	n.s.	Vero	—	—	[33]
PSU	—	22.91 ± 3.83	2505 ± 0683	n.s.	Vero	—	—	[33]
Amantadine*	6K	29.51	n.s.	n.s.	Vero	—	—	[34]
MADTP (9b)	nsP1	1.2 ± 0.009	84 ± 19	70	Vero	—	—	[35–38]
CHVB-032	nsP1	2.7	>75	n.s.	Vero	—	—	[39, 40]
Lobaric acid	nsP1	5.3 ± 0.4	50 ± 1.3	7	Huh-7	—	—	[41]
FHA	nsP1	0.12 ± 0.04	>250	>1000	Vero	—	—	[42, 43]
FHNA	nsP1	0.18 ± 0.11	>250	>1000	Vero	—	—	[43]
5-IT	nsP1	0.409	>50	n.s.	Vero	—	—	[44]
Compound 25	nsP2	3.2 ± 1.8	101 ± 50	32	Vero	—	—	[45–47]
Compound 8	nsP2	1.5	>200	>133.3	BHK-21	—	—	[48]
MBZM-N-IBT	—	38.68	>800	>21	Vero	—	—	[49]
1,3-thiazolidin-4-one (compound 7)	—	0.42	>100	n.s.	Vero	—	—	[50]
peptidomimetic 3a	—	8.76	n.s.	n.s.	Vero	—	—	[51]
PEP-I	—	34	Maximum nontoxic dose is 50 μM	n.s.	BHK-21	—	—	[52]
ID1452-2	—	31	>31	n.s.	HEK293T	—	—	[53]
Novobiocin*	—	20	n.s.	n.s.	Vero	—	—	[54]
Telmisartan*	—	45	n.s.	n.s.	Vero	—	—	[54]
Nelfinavir*	—	14 ± 1	22 ± 6	1.6	Vero	—	—	[55]
SRI-43750	nsP3	23	>40	n.s.	NHDF	—	—	[56]
Favipiravir*	nsP4	25 ± 3	>636	n.s.	Vero	decreased mortality by >50% and	AG129	[57]

						improved disease out-come		
						Reduced vi-ral replica-tion in joints	C57BL/6J	[58]
NHC	nsP4	0.2 ± 0.1	7.7	n.s.	Vero	—	—	[59, 60]
Sofosbuvir*	nsP4	2.7 ± 0.5	402 ± 3 2	149	Huh-7	Reduced viremia and joint pain	Swiss Webster	[61]
Compound A	nsP4	0.54 ± 0.08	3.70 ± 0.32	n.s.	Vero	—	—	[62]

[a]EC_{50}, 50% effective concentration (if no EC_{50} value was reported another readout is presented); CC_{50}, 50% cytotoxic concentration; SI, selectivity index; n.s., not specified; —, not determined; *, repurposed drug.
[b]If the study reported a compound series/class with anti-CHIKV activity, the antiviral data of the most potent or most representative compound is reported. Only compounds with *in vitro* or *in vivo* data are included.
[c]The viral target is only reported if there is enough data about the mode of action.
[d]If a compound was reported in multiple studies, cell lines, and CHIKV strains, the best activity value with the corresponding cell line is listed.

a. Viral Entry and Membrane Fusion

Many different factors and, therefore, potential targets are involved in the viral entry and fusion of the CHIKV, making it a widely used target for many antiviral compounds. The broad-spectrum antiviral drug **arbidol** (Figure 1), also known as umifenovir, and its metabolites, have shown to be early-stage inhibitors of CHIKV replication in different cell lines. The mode of action was confirmed by selecting an arbidol-resistant variant carrying an arginine (G407R) mutation localised in the viral E2 glycoprotein – a type I transmembrane protein involved in the virus binding to the host membrane [24]. Indole-based arbidol analogues with sulfoxides and tert-butyl esters have demonstrated an increased potency and selectivity index. However, docking studies, an entry assay, and a time of addition assay indicate a different mode of action for the most promising analogue **IIc** (Figure 1) [25, 63].

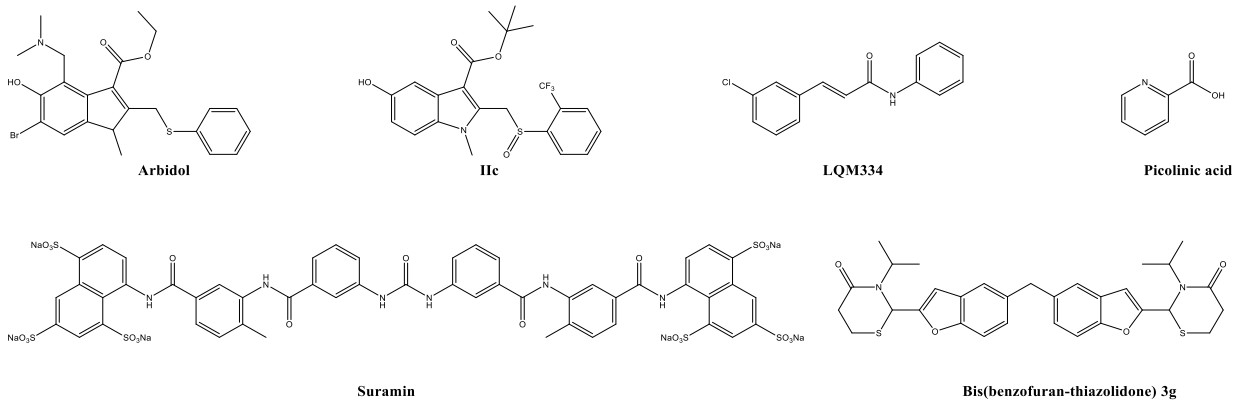

Figure 1. Chemical structure of selected virus-targeting compounds. Inhibitors of viral entry and membrane fusion: arbidol, IIc, suramin, bis(benzofuran-thiazolidone) 3g, and LQM334. Inhibitor of the viral capsid protease: picolinic acid.

Suramin (Figure 1), a symmetrical sulfonated naphthylurea compound and an FDA approved drug against trypanosomiasis, has also demonstrated to inhibit the early-stage of the CHIKV replication cycle in different independent studies – using not only time of addition assays but also *in silico* methods [26, 28, 64]. A more detailed investigation of the mechanism of action was performed by Albulescu et al., where suramin was found to interact directly with the viral particles of CHIKV by inhibiting the viral attachment to the host membrane and interfering with the fusion step by suppressing the conformational changes of viral envelope glycoproteins [65]. Moreover, suramin showed a synergistic effect with the green tea catechin **epigallocatechin gallate** (**EGCG**) – a known *in vitro* early-stage inhibitor of CHIKV infection [66, 67]. Although an *in vivo* study with CHIKV-infected C57BL/6 mice demonstrated amelioration of CHIKV-induced foot swelling, inflammation, and cartilage damage, clinical trials have shown severe

side effects during long term treatment of suramin [27, 68, 69]. Also, the suramin-based analogues with the chemical structures of **bis(benzofuran-thiazolidinone)s** (Figure 1) and **bis(benzofuran-thiazinanone)s** demonstrated a 29 - 42 fold more potent anti-CHIKV activity than suramin, but their high toxicity remains an unsolved issue [29].

An *in silico* screening experiment based on a molecular docking approach utilising a variety of biological targets of CHIKV and the following *in vitro* evaluation by an MTT assay of the obtained hits identified **LQM334** (Figure 1) as a promising inhibitor of CHIKV. The mode of action of the newly found lead compound remains unclear, but a molecular docking study indicates a possible interaction between LQM334 and the E2 domain A from the mature E3-E2-E1 glycoprotein complex [30]. In addition, Agarwal et al. performed an *in silico* docking study using the structure of the envelope glycoprotein of CHIKV to identify promising new lead compounds, but the *in vitro* confirmation of their antiviral activity has still to be shown [70].

Micafungin, an FDA approved drug to treat candidiasis, showed a broad spectrum of inhibitory effects against different alphaviruses – including SINV, SFV and CHIKV. Although a molecular docking study indicated a possible interaction with the CHIKV envelope glycoprotein, a time of addition assay also pointed to a late-stage inhibition of the CHIKV infection, indicating an inhibitory effect against viral replication and intracellular and extracellular transmission of CHIKV [31].

b. Capsid Protease

Picolinic acid (**PCA**, Figure 1), a pyridine containing compound with known antiviral effects against various viruses, including the alphavirus SINV, was discovered to bind to the hydrophobic region of CHIKV capsid protein, which could interfere with the cd-E2-capsid interaction [32, 71]. A significant reduction in vRNA levels and infectious virus was observed when treated with PCA [18]. The same research group used these findings in combination with other studies about proposed capsid protease inhibitors like **dioxane** and **piperazine** to perform an *in silico* screening for potential new lead compounds targeting the capsid protease [72–75]. Their most promising hits **(S)-(+)-mandelic acid** (**MDA**) and **ethyl 3-aminobenzoate** (**EAB**) showed better binding tendencies than dioxane and *PCA in silico*, but the *in vitro* evaluation of their antiviral activity is still pending [75]. More recently, a structure-assisted drug-repositioning study based on in silico screening and ranking of the hits by their docking score identified three compounds targeting the auto-proteolytic activity of the capsid protease: **P1, P4-Di(adenosine-5′) tetraphosphate** (**AP4**), **Eptifibatide acetate** (**EAC**) and **Paromomycin sulphate** (**PSU**) [33].

c. 6K Protein

The practicability of 6K as a possible target for antiviral drug development demonstrates the anti-influenza drug **amantadine** mode of action by targeting the ion channel-forming M2 viroporin of the influenza virus [76]. Electrophysiology experiments indicated that amantadine hinders the ion channel activity of CHIKV 6K and alters the morphology of CHIKV virus-like particles. The anti-CHIKV potential of amantadine was shown in infected Vero cells [34].

d. Non-Structural Protein

i. nsP1

The first class of small molecules reporting the nsP1 of CHIKV as the potential target is the **MADTP** series (Figure 2), with a triazolopyrimidinone scaffold and MADTP-314 as the initial lead compound [35–38]. Overall, three consecutive structure-activity-relationship studies were performed - aggregating detailed information about the influence of various structural changes and demonstrating potent inhibitory effects on various CHIKV strains and VEEV nsP1 in an enzymatic assay [36–38]. The selection of a MADTP-resistant CHIKV strain in cell culture and the following reverse genetics identified the single-amino-acid substitution P34S in the GTase functional domain of nsP1 as responsible for the MADTP-resistance [35]. Recently, 2-(4-(Phenylsulfonyl)piperazine-1-yl)pyrimidine analogues, i.e. the **CHVB** series (Figure 2), were identified as potent and selective anti-CHIKV compounds and analysed based on their structure-activity relationship [39]. In addition, CHVB compounds showed potent inhibitory effects of the MTase and GTase activities of nsP1 of Semiliki Forest virus (SFV) and VEEV [40]. Interestingly, a CHVB-resistant virus demonstrated cross-resistant to the MADTP series, suggesting that both compound families utilise a similar mode of action. However, the CHVB series required the presence of at least two mutations in nsP1, namely, S454G and W456R, indicating that the barrier of resistance is higher for the CHVB series and the occurrence of resistance in clinical settings is less likely [40].

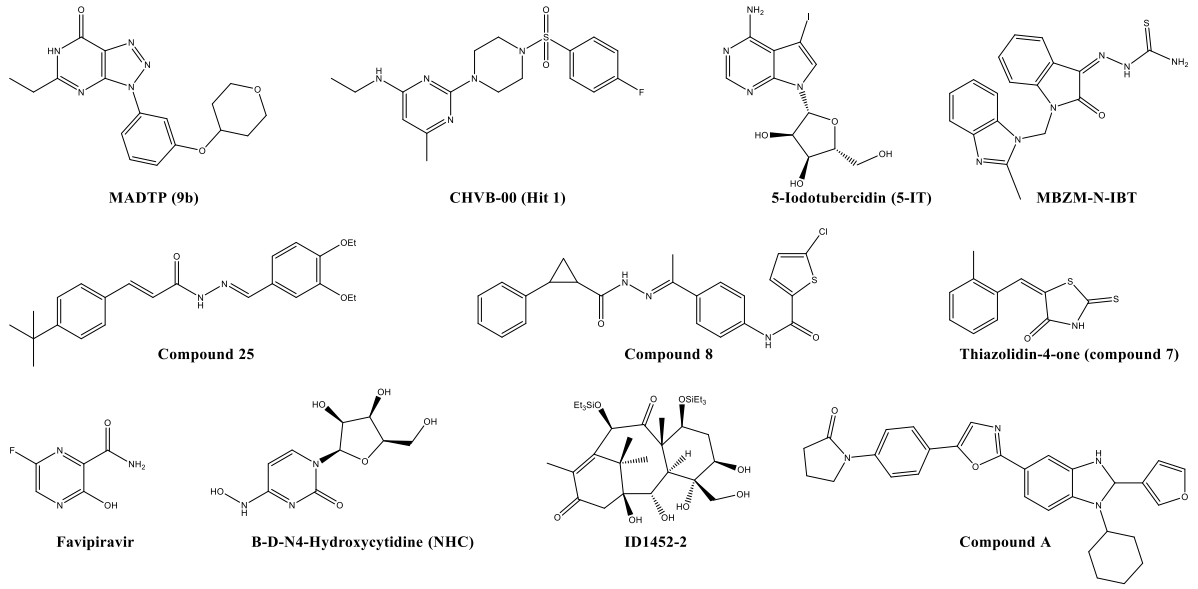

Figure 2. Chemical strucutre of selected virus-targeting compounds. Inhibitors of nsP1: MADTP, CHVB, and 5-iodotubercidine. Inhibitor of nsP2: MBZM-N-IBT, compound 25, compound 8, thiazolidin-4-one, and ID1452-2. Inhibitor of nsP4: favipiravir, β-D-N4-Hydroxycytidine, and compound A.

A fluorescence polarisation-based assay measuring the GTP binding site was used to perform an HTS and led to the identification of a series of hits [41]. The cherrypicked hits were subsequently not only evaluated with an orthogonal assay to measured their ability to interfere with the guanylation step of the capping reaction of nsP1 but also tested for their antiviral activity [41]. These findings led to the identification of the naturally-derived compound, **lobaric acid**, as a potent CHIKV nsP1 inhibitor [41].

More recently, two carbocyclic adenosine analogues, namely, **6`-β-fluoro-homoaristeromycin** (**FHA**) and **6`-fluoro-homoneplanocin A** (**FHNA**), have been identified as inhibitors of the MTase activity of nsP1 by screening a library designed to inhibit the host target SAH hydrolase (see 5.e.i.1 *Hydrolases*) [42, 43]. Resistance selection unveiled two mutations G230R and K299E in nsP1 to develop resistance against both compounds [43]. Additionally, **5-iodotubercidin** (**5-IT**, Figure 2) showed also an inhibitor of the MTase activity of nsP1 in a capillary electrophoresis-based assay [44]. This derivate of tubercidine and a known adenosine kinase inhibitor demonstrated potent anti-CHIKV activity in a plaque-reduction assay [44].

ii. nsP2

The nsP2 has become a significant target of interest in the development of anti-CHIKV drugs primarily because of its essential role in the CHIKV replication but also because of its relatively early publication of its 3D structure (PDB: 3RTK) [77]. This led to a significant number of publications focussing on the *in silico* approach to identify potential new nsP2 inhibitors [78]–[86]. However, for all of those hits predicted in these studies, no *in vitro* evaluation was made. This vital evaluation step was made by Bassetto et al. by the combination of virtual screening with an optimised homology model of nsP2 and the following evaluation by virus-cell-based CPE reduction assay [45]. This approach led to discovering the first lead compound, **compound 25** (Figure 2), with potential nsP2 protease inhibition activity. Two following structure-activity-relationship studies with altogether 100 analogues were additionally performed by this research group to determine the compound series's critical components and investigate the impact of some chemical-structure changes on antiviral activity and water solubility [46, 47]. Interestingly, molecular docking on nsP2 showed no significant differences in binding mode between the most active compound (compound 25 with EC_{50} = 3.2 μM) and some of its poorly active analogues. In their virtual screening study based on pharmacophoric features of the initial hit of Bassetto et al., Das et al. describe a set of 12 compounds with anti-CHIKV activity with the most potent inhibitor **compound 8** (EC_{50} = 1.5 μM, Figure 2) [45, 48]. A cell-free protease assay was performed to verify their effects on nsP2, in which the majority of the compounds demonstrated inhibitory effects against nsP2 [48]. Surprisingly, the initial hit of Bassetto et al. did not show any inhibition [45, 48]. However, three analogues showed significant inhibitory effects, pointing to two different sites of actions in the compound series [46], [48]. This example highlights the significance of biological evaluations of *in silico* predictions as they may sometimes not correlate with the *in vitro* results. On the other

hand, molecular docking and molecular dynamic simulations may be an economical and fast starting point to investigate a possible mode of action of potential new antiviral drugs. Mishra et al. used such a computational approach to examine the target of their antiviral compounds **MBZM-N-IBT** (Figure 2) and **MIBT** [49]. Although their docking result points to an nsP2 inhibition, a time-of-addition assay revealed an early stage and a late-stage inhibition of the CHIKV – indicating multiple modes of actions. Another study described five arylalkylidene derivates of **1,3-thiazolidin-4-one** (Figure 2) with low micromolar antiviral concentrations and interactions with the nsP2 protease domain in MD-simulations [50].

Small peptidomimetics were discovered using quantum mechanics-based ligand descriptors and biologically evaluated in virus-cell-based assay [51]. Docking of the most potent analogues, **peptidomimetic 3a/4b**, with the crystal structure of nsP2 exposed the advantage of lower molecular weight in this compound series, likely due to their more accessibility to the target pocket [51]. Other peptidomimetics, **PEP-I** and **PEP-II**, were identified by screening based on pharmacophoric features derived from nsP2-nsP3/4 (protein-peptide) interaction and evaluated by their anti-CHIKV activity using plaque reduction assay [52, 87]. Their inhibitory effect on chikungunya nsP2 protease and the proteolytic activity of nsP2 was investigated by a FRET-based protease assay [52]. Both compounds inhibited their target protein in the micromolar range [52].

The natural compound, **ID1452-2** (Figure 2), was discovered by high-throughput screening to identify small molecules targeting the chikungunya nsP2 protease [53]. ID1452-2 showed moderate anti-CHIKIV activity (IC_{50} = 31 μM) and inhibited nsP2 effects in dose-dependent manner [53]. More recently, a target-based drug screening with 30,000 FDA approved molecules and SPR experiments identified **telmisartan**, an antihypertensive drug, and the antibiotic **novobiocin** as a strong inhibitor of the chikungunya nsP2 protease [54]. Both drugs inhibited the nsP2 protease activity in low micromolar concentrations [54]. This strategy of repurposing of FDA approved drugs were also used by Bhakat et al. to accelerate the identification and development of future anti-CHIKV drugs [55]. MM/GBSA-based binding free energy results and molecular docking on nsP2 determined **nelfinavir**, an HIV/HCV inhibitor, as a potential new anti-CHIKV drug [55]. This compound showed a moderate antiviral effect in the CPE-reduction assay [55].

iii. nsP3

No small molecules targeting CHIKV nsP3 are reported up to date. Recently, a fragment library and x-ray crystallography screening led to the discovery of 40 fragments binding to the distal ribose binding site of ADP-ribose in the nsP3 macrodomain crystal structure (PDB-code: 6VUQ) [56]. As most of the fragments share a similar pyrimidine-based scaffold, it could be an interesting starting point for further development of a CHIKV nsP3 inhibitor [56]. A similar approach to identify small molecules with anti-nsP3 activity was conducted by Nguyen et al. [88]. With virtual screening and molecular docking, various hits were detected, but the ability of the compounds to inhibit the CHIKV *in vitro* has not yet been published [88].

iv. nsP4

The nsP4 protease functions as an RNA-dependent polymerase (RdRp) and is the most highly conserved protein in the alphavirus family [89]. Consequently, many compounds targeting this protease have been reported to inhibit the CHIKV and other alphavirus replication cycles. **Favipiravir** (**T-705**, Figure 2) and its defluorinated analogue **T-1105,** for example, were reported to inhibit *in vitro* replication of different CHIKV strains as well as other (arthritogenic) alphaviruses [57]. All favipiravir resistant CHIKV strains carried a unique K291R mutation in a highly conserved F1 motif of the RNA-dependent RNA polymerase of +ssRNA viruses in nsP4 [57]. This highly conserved lysine is crucial for the anti-CHIKV activity and responsible for the broad-spectrum antiviral activity of T-705 [58]. In addition, a CHIKV-mouse model of lethal infection in AG129 mice, favipiravir treatment (300 mg/kg/day for seven days) prevented the development of severe neurological disease and increased the survival rate [57]. Further, favipiravir treatment (300 mg/kg/day for four days) in C57BL/6J mice reduced the viral replication in the joints when administered in the acute phase and prevented systemic viral spread [90]. Recent findings highlight the influence of different cell lines on the antiviral outcome in biological assays of T-705 and the impact of various CHIKV strains on the disease severity in mouse strains, and the efficacy of favipiravir treatment [91, 92].

Another nucleoside analogue, **β-D-N^4-hydroxycytidine** (**NHC**, Figure 2), showed more potent anti-CHIKV effects in Vero Cells than the control nucleoside analogues favipiravir and ribavirin [59]. The alphavirus VEEV needs for the development of even low-level resistance against NHC multiple cooperative mutations within the RdRp of nsP4 [60]. Additionally, NHC has shown in a time-of-addition assay to have no effect on viral entry but in the early stage of

the CHIKV replication circle [59]. Both findings indicate the RdRp domain of nsP4 as the potential target of NHC, but the precise mode of action remains unclear.

Sofosbuvir, the FDA approved drug against hepatitis C virus, and uridine analogue also showed interesting activity against CHIKV in different cell lines [61]. Additionally, treatment with sofosbuvir in the CHIKV-mouse model of adult Swiss mice (20 mg/kg/day) protected against CHIKV-induced disease and increased the survival rate of neonate mice (40 and 80 mg/kg/day) [61].

Although the nsP4-targeting compounds have shown to be primarily nucleoside analogues, a high throughput screening of chemical compound libraries identified **compound A** (Figure 2) as a CHIKV infection inhibitor [62]. This benzimidazole-related compound inhibited several different CHIKV strains and SINV strains in Vero cells [62]. A key mutation for development compound A resistance was identified in a mechanism of action study – the M2295I residue located in the functional domain of RdRp of nsP4 [62]. Kumar et al. conducted an *in silico* study on a CHIKV nsP4 homology model to identify potential new hits. However, the biological evaluation of their collected data has yet to be done [93].

5. Targeting Host Factors

As discussed above, a variety of proviral host factors have been shown to influence the viral replication cycle. Therefore, many small molecules modulating such factors have been reported to possess antiviral activity. A comprehensive overview of anti-CHIKV compounds with proviral host targets is given in **Table 2**. The same criteria for inclusion and issues with data comparison as in Table 1 (see chapter *4 Virus Targeting Inhibitors*) are also applied here.

Table 2. Host factor targeting compounds.[a]

		In vitro				*In vivo*		
Compound[b]	**Host target**[c]	**EC_{50} (μM)**[d]	**CC_{50} (μM)**	**SI**	**Cell line**	**Efficacy**	**Mouse model**	
Viral Entry and Membrane Fusion								
Chloroquine*	pH	7.0 ± 1.15	>260	37.14	Vero	—	—	[94]
Obatoclax*	Bcl-2/E1	0.03 ± 0.01	20.1 ± 4.8	670	BHK-21	—	—	[95]
Niclosamide*	pH	0.36 ± 0.08	>20	>55.55	U2OS	—	—	[96]
Nitazoxanide*	pH	2.96 ± 0.18	25	8.45	BHK-21	—	—	[96]
EIPA	—	Detectable inhibition observed at 0.03 μM	n.s.	n.s.	HSMM	—	—	[97]
Lipid Pathway Inhibitors								
Orlistat*	FASN	0.82	8.67	10.57	HEK293T	—	—	[98–100]
Cerulenin*	FASN	3	7.57	2.53	HEK293T	—	—	[98, 100]
CAY10566	SCD1				HEK293T	—	—	[100]
TOFA	FASN	0.15	>60	n.s.	HEK293T	Reduction of the viral replication and joint swelling	C57BL/6	[98]
Tivozanib*	FLT4	0.8	8.34	n.s.	HEK293T	—	—	[98]
Pimozide*	calmodulin	0.28	19.18	69.75	HEK293T	Reduction of the viral replication and joint swelling	C57BL/6	[98]
Imipramine*	—	Detectable inhibition	n.s.	n.s.	HFF1	—	—	[101]

		observed at 10 μM						
U18666A	—	Detectable inhibition observed at 0.63 μM	n.s.	n.s.	HFF1	—	—	[101]
LXR-623	LXRβ	2.50	63.30	25.3	HFF	—	—	[102]
Pyrimidine and Purine Synthesis Inhibitors								
Ribavirin*	IMPDH	341.1	>30,000	24	Vero	+ doxycycline: Reduction of pathological signs and virus titre	Adult ICR	[103, 104]
Doxycycline*	—	15.51 ± 1.62	n.s.	n.s.	Vero	+ ribavirin: Reduction of pathological signs and virus titre	Adult ICR	[104]
Mefenamic acid*	—	13	>100	n.s.	Vero	+ ribavirin: Reduction of viral titre and hypertrophic effects in liver and spleen	Adult ICR	[105]
Meclofenamic acid	—	18	>100	n.s.	Vero	—	—	[105]
Merimepodib*	IMPDH	1.8 ± 1.0	27 ± 3	n.s.	Vero	—	—	[106]
6-Azauridine	OMP	0.816	208	204	Vero	—	—	[103]
DD363	DHODH	3.6 ± 0.6	87 ± 7	n.s.	HEK293T	—	—	[107, 108]
RYL-634	DHODH	0.26	>2.5	>10	Vero	—	—	[109]
Atovaquone*	—	<0.75	>11.25	n.s.	Vero	—	—	[110]
Protein Synthesis Inhibitors								
Halofuginone	EPRS	3 $\log_{10}$ viral titer reduction at 100 nM	n.s.	n.s.	HFF	—	—	[111]
Harringtonine		0.24	n.s	n.s.	BHK21	—	—	[112]
Sorafenib*	FLT4	0.16	n.s.	n.s.	Vero	—	—	[98, 113]
Bortezomib*	—	0.023	0.47	20.6	HeLA	—	—	[114]
SR9009*	—	100-fold reduction in viral titer at 10 μM	n.s.	n.s.	Huh7	—	—	[115]
Cellular Protein Inhibitors								
Sirtinol	SIRT	>2 $\log_{10}$ viral titer reduction at 200 μM	n.s.	n.s.	U2OS	—	—	[116]

Geldanamycin*	HSP90	2.5 log_{10} viral titer reduction at 1.4 μM	>100	n.s.	HEK293T	—	—	[117]
HS-10	HSP90	>2 log_{10} viral reduction in titre with 6.25 μM	>100	n.s.	HEK293T	Reduced viral titer, inflammation, and swelling	SVA129	[117]
SNX-2112	HSP90	>2 log_{10} viral reduction in titre with 6.25 μM	>100	n.s.	HEK293T	Reduced viral titer, inflammation, and swelling	SVA129	[117]
16F16	PDI	6.6 ± 0.45	8.9 ± 9.2	1.35	HEK293T	—	—	[118]
PACMA31	PDI	12.1 ± 0.3	12.2 ± 9.7	1.00	HEK293T	Less reduction in footbed swelling and viremia than in auranofin group	C57BL/6	[118]
Auranofin*	TRX	1.0 ± 0.13	1.6 ± 8.6	1.6	HEK293T	reduced footbed swelling and viremia	C57BL/6	[118]
Cellular Enzyme Inhibitors - Hydrolases								
Amodiaquine*	Cathepsin B	18.3	>50	>2	HFF	—	—	[119]
DEAQ	Cathepsin B	17.3	>50	>2.9	HFF	—	—	[119]
Aristeromycin	SAH	0.8	6.3	7.9	Vero	—	—	[120]
6,6`-Difluoroaristeromycin (2c)	SAH	0.13	1.25	>9.6	Vero	—	—	[120]
Cellular Enzyme Inhibitors - Kinases								
Dasatinib*	SFK	>10-fold reduction in viral titer at 20 μM	>50	n.s.	NHDF	—	—	[121]
Torin 1	mTORC1/2	>10-fold reduction in viral titer at 1 μM	>1	n.s.	NHDF	—	—	[121]
CND3514	—	2.2	>50	>22.7	HuH-7	—	—	[122]
Berberine	MAPK	1.8 ± 0.5	>100	>55.6	BHK21	Reduced viremia and disease symptoms	C57BL6/J	[123, 124]
Ivermectin*	—	0.6 ± 0.1	37.9 ± 7.6	62.4	BHK-21	—	—	[123]
Abamectin*	—	1.5 ± 0.6	28.2 ± 1.1	19.2	BHK-21	—	—	[123]
Miltefosine*	Pi3-Akt	antiviral activity was observed at 20–40 μM	n.s.	n.s.	hPDF	—	—	[125]

Prostatin	PCK	0.2 ± 0.05	50	n.s.	CRL-2522	—	—	[126]
Bryostatin analogue (4)	PCK	0.8 ± 0.1	>50	n.s.	BGM			[127, 128]
Isothiazolo[4,3-b]pyridine (12r)	GAK	antiviral activity was observed <10 μM	n.s.	n.s.	Vero	—	—	[129]
Cellular Enzyme Inhibitors – Lyases/Transferases								
DFMO*	ODC1	200-fold reduction in viral titer at 500 μM	n.s.	n.s.	BHK-21	Low reduction in viral titer	C57BL6/J	[130]
Cellular Receptor Inhibitors – Channel-linked Receptors								
Digoxin*	Na^+/K^+ ATPase	0.049	>10	n.s.	U2OS	—	—	[20]
Lanatoside C*	Na^+/K^+ ATPase	38.99% reduction of viral titer with 1 μM	>1	n.s.	BHK-21	—	—	[131]
DIDS	CLIC1/4	8-fold reduction in viral titer	n.s.	n.s.	HuH-7	—	—	[132]
9-ACA	CLIC1/4	8-fold reduction in viral titer	n.s.	n.s.	HuH-7	—	—	[132]
NPPB	CLIC1/4	18-fold reduction in viral titer	n.s.	n.s.	HuH-7	—	—	[132]
Cellular Receptor Inhibitors – Enzyme-linked Receptors								
5-NT	5-HT	2.8	>5	n.s.	U2OS	—	—	[133, 134]
MM	5-HT	97 ± 1.0% viral reduction 10 μM	>10	n.s.	U2OS	—	—	[133]
Immunomodulatory Agents								
Tilorone*	IFN-inducer	4.2	32	7.6	Vero76	—	—	[135]
C11	STING	EC_{90}: 16.44 μM	>50	n.s.	THF	—	—	[136]
G10	STING	IC_{90}: 8.01 μM	n.s.	n.s.	THF	—	—	[137]
AV-10	TRIF	IC_{90}: 3.54 μM	n.s.	n.s.	THF	—	—	[138]
Pentosan polysulfate*	—	—	—	—	—	Reduced disease symptoms	C57BL/6	[139]
Pixatimod*	—	0.51 ± 0.50	n.s.	n.s.	Vero	Reduced disease symptoms	C57BL/6	[140]

[a]EC_{50}, 50% effective concentration (if no EC_{50} value was reported another readout is presented); CC_{50}, 50% cytotoxic concentration; SI, selectivity index; n.s., not specified; —, not determined; *, repurposed drug.
[b]If the study reported a compound series/class with anti-CHIKV activity, the antiviral data of the most potent or most representative compound is reported. Only compounds with *in vitro* or *in vivo* data are included.
[c]The host target is only reported if there is enough data about the mode of action.
[d]If a compound was reported in multiple studies, cell lines, and CHIKV strains, the best activity value with the corresponding cell line is reported.

a. *Viral Entry and Membrane Fusion*

Alphaviruses use a receptor-mediated endocytotic entry and pH-dependent fusion to release their viral RNA genome into the host cell cytoplasm. Recent findings in biochemistry and structural identification of involved proteins have given a valuable insight into this process, where many different host factors could be used as potential antiviral targets [16].

One of such antiviral compounds is **chloroquine** (Figure 3), a 9-aminoquinoline known since 1934 as an antimalarial drug [141]. It has been used in clinical trials against CHIKV even long before its anti-CHIKV effect was reported *in vitro* assays. The reasoning behind this unique approach lies in the lack of other treatment options and promising research results published before. Chloroquine and other NSAIDs like mefenamic acid (see c *Pyrimidine and Purine Synthesis Inhibitor*) have been reported to inhibit the multiplication of various viruses (e.g. Sindbis, influenza A2, herpes simplex,…) in chick and mouse embryo cells [142, 143]. Additionally, it has been shown to influence the pH-dependent fusion of the Sindbis virus and Semiliki Forest virus with the endosomal membrane by raising the endosomal pH in BHK-21 cells [144, 145]. Other weak bases like NH_4Cl, amantadine, methylamine and tributylamine showed similar lysosomotropic effects [144], [145]. Moreover, chloroquine has been reported to lessen the joint inflammation of patient with rheumatoid arthritis in several trials in the 1950s [146]. The first clinical trial 1984 with chloroquine phosphate on 10 CHIKF patients was conducted after empirical observations that one of the patients joint pains improved while taking antimalarial drugs prophylactically [147]. Treatment with chloroquine led to alleviating patients' symptoms and opened the door for further clinical trials [147]. However, a randomised, double-blind, placebo-controlled, prospective trial (*CuraChik trail*) with 54 adult patients diagnosed with CHIKV showed no significant difference between the placebo and the chloroquine groups in terms of fever clearance time or viremia clearance time [148, 149]. Moreover, patients treated with chloroquine were more likely to complain about persistent arthralgia ($p < 0.01$) and suffered moderate adverse effects of the treatment [148, 149]. Another clinical trial showed no advantage of chloroquine treatment over the NSAID **meloxicam** in patients with early musculoskeletal pain and arthritis following acute chikungunya virus infection [150]. While the *in vivo* performance of chloroquine in humans and non-human primate models is limited, the *in vitro* effects of chloroquine are remarkable better [94, 151–153]. These contradictory findings may be due to the immunomodulatory effects of chloroquine *in vivo*: it inhibits, i.e. IFN-I responses, which may influence the immune response to the viral replication negatively and may have been missed in the used Vero-E6 cells, which do not produce IFN-I [152].

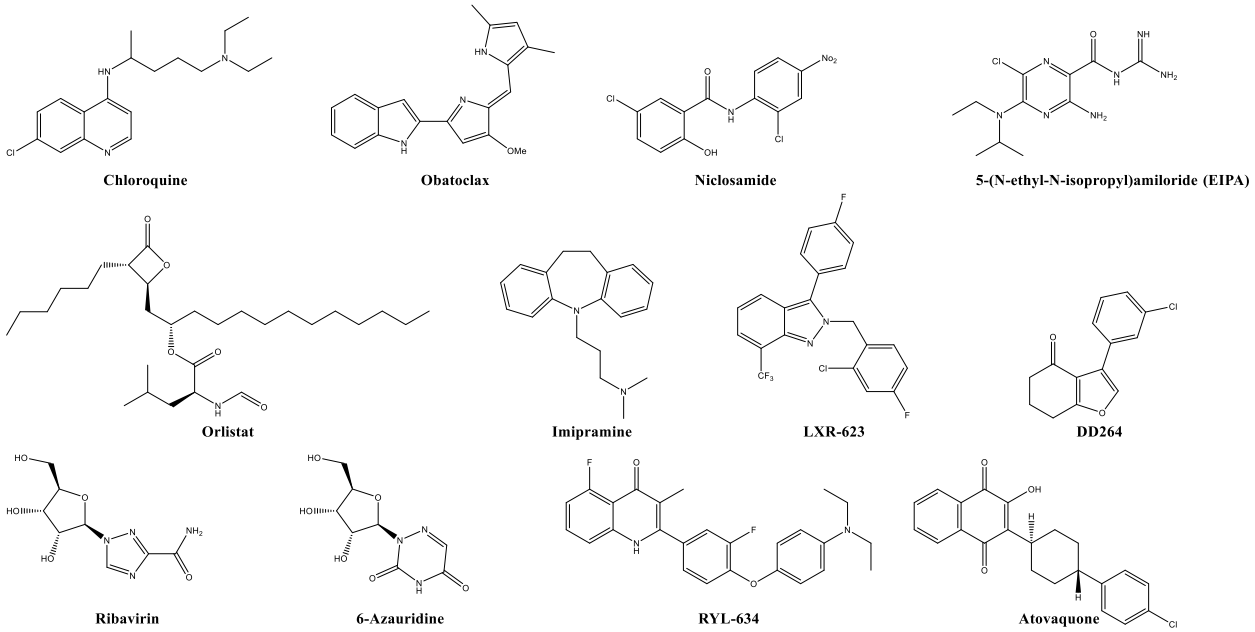

Figure 3. Chemical structure of selected host-targeting compounds. Inhibitor of the viral entry and membrane fusion: chloroquine, obatoclax, niclosamide, and 5-(N-ethyl-N-isopropyl)amiloride. Inhibitor of the lipid pathway: orlistat, imipramine, and LXR-623. Inhibitor of the pyrimidine and purine synthesis: ribavirin, 6-azauridine, DD264, RYL-634, and atovaquone.

Several clinical trials were conducted to investigate **hydroxychloroquine** (**HCQ**) on Chikungunya virus infection [154]. HCG seems to have no beneficial impact in the early stage of CHIKV infection or reduction of joint pain when administered alone or in combination with aceclofenac [155]. However, the combination of HCQ with methotrexate

improved the disease activity and reduced disability and pain in patients [156, 157]. In contrast, HCQ treatment n the RHUMATOCHIK study had to interrupted in 4 out of 39 patients because of adverse effects such as nausea, rash, stomatitis, and headache [158]. A 50% reduction of synovitis and 19.2% complete remission was reported in the remaining sample [158].

The pH-dependent endocytosis and the high sensitivity of CHIKV to the antiviral activity of type I and type II interferons was also shown by Sourisseau et al. [159]. Their study demonstrated the anti-CHIKV effect of chloroquine and the vacuolar proton ATPase inhibitor **bafilomycin-A.** Furthermore, the high sensitivity of CHIKV to the antiviral effect of IFNs (see g *Immunomodulatory*) has been reported [159]. **Concamycin A**, another vacuolar proton ATPase inhibitor, and the Bcl-2 inhibitor **obatoclax** (Figure 3) inhibited the viral fusion of Semiliki Forest virus and, in the case of obatoclax, also of CHIKV due to their lysosomotropic characteristics [95, 160].

A broad range of antiviral effect on pH-dependent viruses demonstrated the anithelmic drugs **niclosamide** (Figure 3) and **nitazoxanide** [161]. Radiometric imaging allowed the precise measurement of endosomal pH and revealed their neutralising effect of acidic endosomes [161]. An additional structure-activity relationship study exposed the importance of the hydroxy and chloride group in position R1 and R4 for the antiviral effect of this compound series [161]. An HTS for CHIKV fusion inhibitors with FDA approved drugs found not only niclosamide and nitazoxanide as potential anti-CHIKV candidates but also suramin (see 2.1 *Viral Membrane Fusion and Entry*) [96]. All of them showed an additional inhibitory effect on the cell-to-cell transmission of CHIKV. Additionally, the antiviral effect of the hits was evaluated, measuring the CHIKV-induced CPE in BHK-21 cells, and no toxicity in the used micromolar range was observed in zebrafish embryos [96].

Pre-infection treatment with the macropinocytosis inhibitor **5-(N-ethyl-N-isopropyl)amiloride** (**EIPA**, Figure 3) results in a dose-dependent inhibition of CHIKV infection [97]. Amiloride and an amiloride analogue HOE-694 have been reported to block the activity of Na(+)/H(+) exchanger, lowering the submembranous pH and consequently preventing the necessary macropinocytosis [162]. Macropinocytosis has been identified as a significant pathway of CHIKV into muscle cells and is, therefore, an attractive target for new anti-CHIKV compounds [97].

b. *Lipid Pathway Inhibitors*

Alphavirus fusion is dependent on the cholesterol and sphingolipid in the host cell membrane [16]. Consequently, inhibition of the fatty acid synthase by the anti-obesity drug **orlistat** (Figure 3), the antibiotic FASN inhibitor **cerulenin**, and the SCD1 inhibitor **CAY10566** resulted in decreased CHIKV and MAYV genome replication [98, 100]. Both enzymes play a central role in the *de novo* synthesis of long-chain fatty acids and are crucial in the replication of various viruses [95]. Furthermore, orlistat was reported as a potential broad-spectrum agent against mosquito-transmitted viruses like DENV, JEV, ZIKV and CHIKV [99].

Additionally, the fatty acid synthase was identified by Bakhache et al. as an important proviral host factor [98]. A genome-wide CHIKV/HEK-293 loss-of-function siRNA screen led to identifying 156 proviral and 41 host targets influencing the CHIKV replication cycle [98]. The validated host proviral factors were used to screen a drug repurposing database [98]. This approach led to the identification of 20 compounds interacting with six unique host targets, including enzymes of fatty acid synthesis (fatty acid synthase, ATP citrate lyase, and acetyl CoA carboxylase), calmodulin signalling, the vacuolar-type H^+ ATPase (vATPase), CLK1, fms-related tyrosine kinase 4 (FLT4 or VEGFR3), and K (lysine) acetyltransferase 5 (KAT5 or TIP60). All 20 compounds showed strong antiviral activity when tested in HEK293-T cells, but, as expected, some of them exhibited a narrow Therapeutic Index. The *in vitro* outcome was further validated in a CHIKV-C57BL/6 mouse model, in which **tivozanib** (targeting FLT4), **pimozide** (calmodulin inhibitor) and **5-tetradecyloxy-2-furoic acid** (**TOFA**, fatty acid synthesis inhibitor) reduced the viral replication in the footbed significantly. The combination of TOFA and pimozide had a synergistic effect in reducing the viral replication and joint swelling [98].

The host membrane cholesterol is a key component in the unmasking of the fusion peptide in class II envelope glycoproteins [101]. Compounds interfering with the cholesterol transport like the tricyclic antidepressant **imipramine** (Figure 3) and the class II cationic amphiphilic compound **U18666A** have shown to affect the fusion and replication step from not only the CHIKV but also several Flaviviridae like ZIKV, West Nile virus, and DENV [101].

The ubiquitously expressed liver X receptors (LXRs) are essential in the regulation of cholesterol homeostasis and a potential proviral host target for antiviral compounds [102]. The selective, synthetic agonist of LXRβ **LXR-623** (Figure 3) inhibited the CHIKV replication in human foreskin fibroblasts in a dose-dependent manner. This effect was partially reversed when the cells were incubated with cholesterol [102].

c. *Pyrimidine and Purine Synthesis Inhibitors*

The inosine monophosphate dehydrogenase (IMPDH) is a key enzyme in the *de novo* guanine nucleotide biosynthesis by converting inosine-5`-monophosphate to xanthine 5`-monophosphate and is, therefore, an interesting target for antibacterial, anticancer and antiviral drugs [163]. **Ribavirin** (Figure 3), an FDA-approved drug against respiratory syncytial virus infection in infants and chronic hepatitis C infection, is a guanosine analogue with multiple postulated biomechanisms [164, 165]. The inhibition of IMPDH leading to depletion of GTP pools as well as the inhibition of the RNA-dependent RNA polymerase (RdRp) is considered the major causes of the broad-spectrum antiviral activity of ribavirin [165]. The scientific research about the precise mode of action is still ongoing, but a time-of-addition study unveiled that ribavirin is primarily active at the early stage of the CHIKV replication cycle [49]. Ribavirin showed antiviral activity against CHIKV *in vitro* and *in vivo* studies and exhibited a synergistic effect with the tetracycline **doxycycline**, **IFN α2a,** and the NSAID **mefenamic acid** (**MEFE**) [103–105, 166]. Moreover, a 7-day treatment with 200 mg ribavirin twice daily significantly improved joint pains in human patients [167].

Another potent and selective IMPDH inhibitor, **merimepodib**, inhibited the CHIKV and the ZIKV in a dose-dependent manner [106]. On the other hand, the immunosuppressive agent and non-competitive inhibitor of IMPDH, **mycophenolic acid** (**MPA**), has been reassessed regarding its anti-CHIKV inhibitory effect [168]. Although it has demonstrated high antiviral effects in a virus-induced cytopathic effect assay, 19 synthesised analogues of MPA did not show any CHIKV inhibition [168, 169]. The following retest of MPA revealed the unexpected characteristic of MPA: after reduction of GTP and downregulation of CHIKV replication, the antiviral effect of MPA diminishes, and the virus regains its full replication potential [168].

6-azauridine (Figure 3), on the other hand, inhibits the orotidylic acid decarboxylase (OMP) – resulting in depletion of UTP pools in cells [170]. It is used in the treatment of psoriasis and showed broad-spectrum antiviral activity [170–172]. Additionally, 6-azauridine has been reported to reduce the viral titer of CHIKV and SFV [103].

Another proviral host target is the dihydroorotate dehydrogenase (DHODH) – the fourth enzyme in the pyrimidine biosynthetic pathway bound to the inner mitochondria membrane [108]. DHODH is the suggested target of the **DD264** series (Figure 3) – a small compound series found through an HTS with broad-spectrum antiviral activity (DNA and RNA viruses, including CHIKV) [107, 108]. Interestingly, the antiviral effect of DD264 was suppressed when added exogenous uridine but not with added guanosine, which supports the theory that the pyrimidine level is essential for the CHIKV replication [108]. **Antimycin A1a** also inhibits the cellular mitochondrial electron transport, suppressing the *de novo* pyrimidine synthesis and resulting in a broad spectrum of antiviral activity [173].

Phenotypic screening of around 200 biaryl-substituted quinolones and the following *in vitro* validation and SAR study led to the identification of the broad-spectrum antiviral compound **RYL-634** (Figure 3) [109]. Via activity-based protein profiling (ABPP), 78 potential human proteins were identified as possible targets of RYL-634. Further *in silico* investigations and enzymatic activity assays validated the DHODH as the target enzyme [109]. Although it has been already shown that the antimalaria drug **atovaquone** (Figure 3) has an inhibitory effect on DHODH, its biomechanism regarding the antiviral effect on CHIKV remains unclear [110, 174]. A mechanism of action study showed an early stage inhibition of ZIKV infection and a possible inhibitory effect on the pyrimidine biosynthesis pathway [110].

d. *Protein Synthesis Inhibitors*

Halofuginone (Figure 4) is an antagonist of the host prolyl-tRNA synthetase enzyme (EPRS), causing the accumulation of uncharged $tRNA^{pro}$ and forcing the cell to suppress the translation even when proline levels are sufficient [175]. This orally available synthetic derivative of the plant compound febrifugine has been reported to inhibit the viral progeny production of CHIKV, ONNV, ZIKV and DENV [111]. **Harringtonine**, another naturally derived compound, has been identified to inhibit CHIKV RNA production and viral protein expression in a dose-dependent manner [112]. This cephalotaxine alkaloid is known to inhibit eukaryotic translation [176].

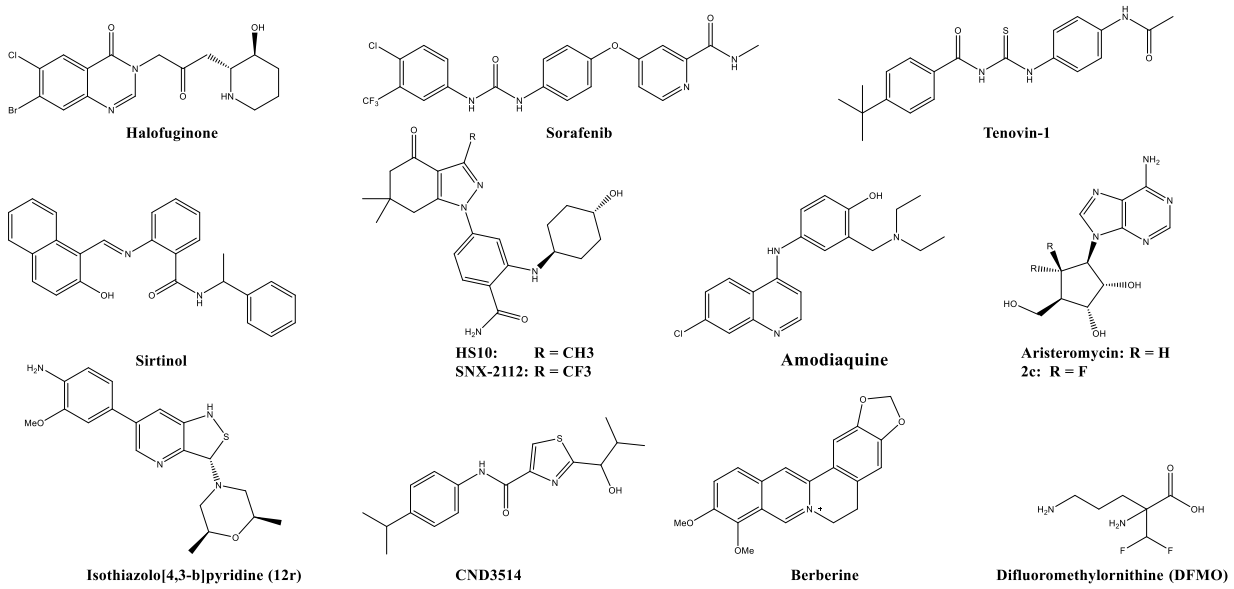

Figure 4. Chemical structure of selected host-targeting compounds. Inhibitor of the protein synthesis: halofuginone and sorafenib. Inhibitor of cellular proteins: tenovin-1, sirtinol, HS10, and SNX-2112. Inhibitor of cellular enzymes: amodiaquine, aristeromycin, isothiazolo[4,3-b]pyridine, CND3514, berberine, and difluoromethylornithine.

The anticancer drug **sorafenib tosylate** (Figure 4) inhibited CHIKV replication at 8 and 16 h post-infection through dephosphorylation of several key enzymes for the viral translation - including the cap-binding protein eIF4E (eukaryotic translation factor 4E) and p70S6K [98, 113]. As the research from McKendrick et al. suggest that the phosphorylation of eIF4E is not essential for the global cellular protein synthesis, this enzyme could be an attractive target for further antiviral drug development [113, 177]. Moreover, **sylvestrol**, a specific inhibitor of the RNA helicase eIF4A (eukaryotic translation factor 4A), inhibited the CHIKV replication cycle at an early stage [178]. The DEAD-box helicase eIF4A unwinds the RNA secondary structure in the 5`-untranslated regions (5`-UTRs) of mRNA and allows translation. Silvestrol holds the eIF4A helicase to its mRNA substrate and inhibits thereby the following translation [178]. More recently, Blum et al. reported the influence of sylvestrol on the inflammatory status of immune cells [179].

The ubiquitin-proteasome system (UPS) is central for ensuring protein quality control and maintaining a critical level of important regulatory proteins [180]. As many viruses have evolved to manipulate this cellular machinery in their favour, it is not surprising that the FDA-approved proteasome inhibitor **bortezomib** was reported to inhibit different CHIKV strains in various cell lines [114, 180]. Investigation of the CHIKV protein level by western plot analysis revealed a 50 to 80% reduction of E2, E1 and capsid protein [114]. The synthetic agonist of the nuclear receptors Rev-erb α/β **SR9009** showed inhibitory effects against the CHIKV and O`nyong`nyong virus [115]. Although the precise mechanism of action is still unclear, a subgenomic RNA translation inhibition was observed [115].

e. Cellular Protein Inhibitors

Sirtuins (SIRTs) are an evolutionarily conserved family of seven lysine deacetylases (KDACs) and are present in nuclear and cytoplasmic compartments [116]. Their precise functions in human cells are not fully elucidated, and their impact on viral replications varies not only on the viral pathogen but also on the subgroup of SIRTs itself [116]. Different sirtuin inhibitors like **tenovin-1** (Figure 4), **sodium phenylbutyrate** (a pan-KDAC inhibitor), and **sirtinol** (a specific SIRT1 and SIRT2 inhibitor, Figure 4) have been reported to inhibit a set of flaviviruses, bunyaviruses, and alphaviruses – including the CHIKV. Interestingly, the inhibition of only SIRT1/2 was not enough to block the viral infection [116].

Like cellular proteins, viral proteins need chaperones for proper folding and assembling for precise and stable function. Two different chaperon families, namely the heat shock protein 90 (HSP90) and the protein disulfide isomerase (PDI), were reported as proviral host factors in the case of CHIKV infection [117, 118]. HSP90 has a critical role in the proper folding, maturation, localisation, and turn-over of cellular and viral proteins. Its essential function for RNA and DNA viruses makes it a desirable host target for broad-spectrum antivirals [181]. Interestingly, the known HSP90 inhibitor **geldanamycin** showed antiviral effects in CHIKV infected HEK-293T cells. Further, the antiviral effects of two specific HSP90 inhibitors, **HS-10** and **SNX-2112** (Figure 4), ruled out any off-target effects of geldanamycin during the

CHIKV replication cycle. They even prevented joint swelling and inflammation in a CHIKV-SVA129 mouse model [117]. Further, geldanamycin treatment led to reducing the nsP2 concentration and the for viral replication essential interaction between HSP90 and nsP2 [182].

Alphaviruses require specific disulfide bonding in their envelope proteins E1 and E2 for proper folding and assembling and, therefore, likely depend on the host protein disulfide isomerase (PDI) [118]. Consistent with these findings, the *in vitro* assays with the PDI inhibitor **16F16** led to a significant reduction of cell-cell fusion events. Although 16F16 and **PACMA31** (another PDI inhibitor) showed interesting antiviral effects, their toxicity profile was remarkably poor. However, the FDA approved TRX-inhibitor **auranofin** had a much better therapeutic index of 104.5 at 12 hours post-infection and even reduced food swelling in a CHIKV mouse model [118].

i. Cellular Enzyme Inhibitors

1. Hydrolases

So far, three different hydrolase families have been reported as proviral host targets for the inhibition of the CHIKV infection, namely furin, cathepsin B, and the SAH-hydrolase [153, 183]. The membranous furin hydrolase is vital for the cleavage of the alphavirus envelope glycoproteins E1 and E2 precursor P62 [153], [184]. As this process is a crucial moment in replicating the CHIKV and many other alphaviruses, furin is a desirable target for antiviral compounds [184–186]. Accordingly, the inhibition of the P62 cleavage by the irreversible furin inhibitor **decanoyl-RVKR-chloromethyl ketone** (**dec-RVKR-chmk**) leads to a significant viral reduction envelope glycoproteins E1 and E2 in infected myoblast cultures. Furthermore, given chloroquine, almost total viral spread and yield were observed [153]. The furin inhibitor **phenylacetyl-Arg-Val-Arg-4-amidinobenzylamide** and a set of its analogues significantly reduced the viral titer in BHK-21 cells [187].

The endosomal cathepsin B protease was recently confirmed as a proviral host target not only for MLV, Ebola virus, and SARS-CoV infections but also for CHIKV. Cathepsin B mediates the lysosomes-endosomes-fusion and is used by pathogens to enter the cell [119, 183]. The antimalaria drug **amodiaquine** (Figure 4) and its primary active metabolite **desethyl-amodiaquine** (**DEAQ**) have shown to act as an anti-pathogen against various bacteria toxins and as an inhibitor of multiple viruses (e.g. the CHIKV) by inhibiting the host cathepsin B protease [119].

The naturally occurring carboxylic nucleotide **aristeromycin** (Figure 4) showed potent anti-CHIKV effects, but further usage was limited by its high cytotoxicity [120, 188]. This type I S-adenosyl-L-homocysteine hydrolase (SAH) inhibitor was the starting point of a series of 6`-fluorinated aristeromycin analogues (Figure 4). Since both the viral RdRp and the host SAH hydrolase are crucial for the viral RNA capping and replication, the design of dual-target antiviral compounds was performed by Yoon et al. [120]. Surprisingly, the inhibition of the viral RdRp was found to be less important in this new compound series than the SAH inhibition [120]. As already discussed in *4.d.i nsP1*, based on these results, 6`-fluorinated-5`-homoaristeromycin (FHA) and 6`-fluoro-homoneplanocin (FHNA) were synthesised and showed potent anti-CHIKV activity [42]. Interestingly, they seem to target the viral nonstructural protein nsP1 rather than the proviral host factor SAH [43].

2. Kinases

Targeting the proviral host kinases has led to the identification of multiple compounds with interesting antiviral activity. Many viruses modify the host kinases signalling pathways to regulate the cellular environment and to stimulate their replication [189]. The Src family kinases (SFKs), for example, have been identified through kinome profiling as essential proteins for the replication of several viruses, including the CHIKV. Accordingly, the chemical inhibitors of these membrane-associated kinases, **dasatinib** and the mTORC1/2 inhibitor **Torin 1,** were able to reduce the viral yields in human fibroblasts [121]. Other kinase inhibitor compounds, the **CND series** (Figure 4), were found through HTS utilising a kinase inhibitory chemical library (BioFocus). A mechanism of action study with this compound series suggested that the inhibition of virus-induced CPE was likely performed by targeting kinases involved in apoptosis [122]. However, their precise target kinase requires further investigation [122].

Another potential proviral host kinase target is the mitogen-activated protein kinase (MAPK) signalling pathway. **Berberine** (Figure 4), a plant-derived alkaloid, was found with **ivermectin** and **abamectin** through a high throughput screening. All three compounds showed good activity against different alphaviruses [123]. Furthermore, the activation of the MAPK during a CHIKV infection and the resulting changes in phosphorylation levels were detected by a human phosphokinase array detected [124]. Berberine treatment decreased the viral titer in HEK-293T cells by reducing this CHIKV induced MAPK activity. Additionally, berberine treatment in CHIKV-infected C57BL6/J led to reduced

inflammation in the joint footbed [124]. Recently, berberine was reported to interfere with the virus ability to form a stable cytoplasmic nucleocapsid core (NC) – inhibiting the formation of infectious virus particles [190].

Depending on the host cell, the CHIKV entry occurs via endocytosis or macropinocytosis [97, 183]. Macropinocytosis activation occurs when the virus activates signal transduction in the host cell via different cellular proteins like the phosphatidylinositol-3kinase (PI3K) and the protein kinase C [97]. The inhibition of the AKT-phosphorylation through the interaction with the Pi3-Akt signalling pathway by the anti-leishmaniosis drug **miltefosine** led to a reduction of the CHIKV replication in human dermal fibroblasts [125].

The serin/threonine-protein kinase C (PKC) regulates several cellular processes, including cell proliferation and apoptosis [126]. PCK modulators have been reported to inhibit CHIKV replication *in vitro*. The phorbol ester **prostatin** is a potent activator of PCK and showed antiviral activity on different CHIKV strains [126, 191]. The antiviral activity of prostatin, however, was strongly dependent on the used cell type. Potent antiviral activity were reported in BGM and Vero A cells, but the PCK inhibitor showed no antiviral activity in HEL cells [126]. Furthermore, prostatin has been reported to have tumour promoting effects [126]. Analogues of the pan-PCK modulator **bryostatin**, also potently inhibited the CHIKV replication. Interestingly, when the hydroxyl group on C26, which is vital for the PCK interaction, was capped, the antiviral activity was still found. This suggests an additional PCK-independent mode of action [127, 128, 192].

Additionally, the cyclin G-associated kinase (GAK) inhibitors with an **isothiazolo[4,3-b]pyridine** (Figure 4) scaffold showed moderate antiviral activity against the Dengue virus, Ebola virus, and the Chikungunya virus [129].

3. Lyases/transferases

Reducing the polyamine concentration in host cells has negative effects on the viral replication of various RNA viruses as they need it for viral translation and transcription [193]. Intracellular polyamine synthesis relies on a set of different proteins like the ornithine decarboxylase 1 (ODC1) and the spermidine/spermine N1-acetyltransferase 1 (SAT1). Depletion of spermidine and spermine by induction of SAT1 led to decreased CHIKV replication [193]. Interestingly, the resistance of this polyamine dependency was found in a CHIKV variant with mutations in the viral nsP1 and nsP4 [194]. The irreversible inhibitor of ODC1, **difluoromethylornithine** (**DFMO**, Figure 4), and the SAT1 upregulator, **diethylnorspermine** (**DENSpm**), reduced the viral titer in different cell lines. Despite these good *in vitro* results, DFMO showed a low reduction in viral titer in CHIKV-infected C57BL/6 mice [130].

f. *Cellular Receptor Inhibitors*

i. Inhibitors of Channel-linked Receptors

Critical steps of the viral replication cycle have been reported to depend on the virus's ability to manipulate the host cell ionic environment. Consistent with these findings, several viral proteins have been shown to influence cellular ion channel activity [195]. So far, two different ion channel families have been exploited as anti-CHIKV targets – the sodium-potassium ATPase and the chloride channel 1 and 4 (CLIC1, CLIC4) [20, 131, 132].

A high throughput screening has identified the known sodium-potassium ATPase inhibitor **digoxin** and its related cardiac glycoside **ouabain** as a potent CHIKV inhibitor in human cell lines [20]. Furthermore, mechanistic studies revealed that digoxin is acting in a post-entry step of the viral replication. Its antiviral effect was reversed when exogenous potassium was added during digoxin treatment, which led to the hypothesis that the CHIKV may require a specific ion balance for its replication. Digoxin-resistant CHIKV mutations carried the V209I mutation in the viral nonstructural protein nsP4, indicating that digoxin may also interact with the viral replication [20]. In addition, another FDA approved cardiac glycoside, **lanatoside C,** was reported to inhibit a broad spectrum of viruses, including the dengue virus, the Sindbis virus, and the CHIKV [131].

The discovery of CLIC1 and CLIC4 as proviral factors in human cells was performed using the siRNA silencing technique. This resulted in the identification of three chloride channel inhibitors, **4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid** (**DIDS**), **9-Anthracenecarboxylic acid** (**9-ACA**), and **5-Nitro-2-(3-phenylpropylamino)benzoic acid** (**NPPB**, Figure 5). All three compounds showed a significant reduction in CHIKV replication not only in human cell lines (Huh-7) but also in mosquito cells (C6/C3) [132].

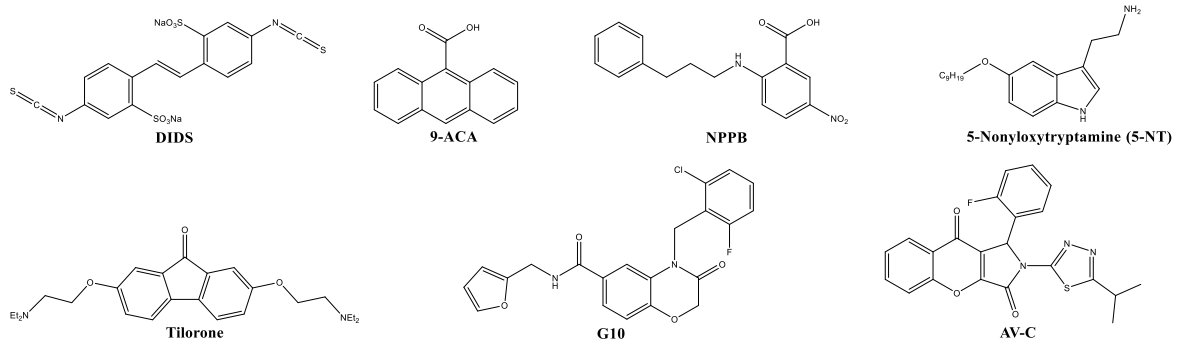

Figure 5. Chemical structure of selected host-targeting compounds. Inhibitors of cellular receptors: DIDS, 9-ACA, NPPB, and 5-nonyloxytryptamine. Immunomodulatory agents: tilorone, G10, and AV-C.

ii. Inhibitors of Enzyme-linked Receptors

The serotonin or 5-hydroxytryptamine (5-HT) receptors are primarily G-protein coupled and regulate essential physiological functions and various signalling pathways [134]. The serotonin receptor agonist **5-nonyloxytryptamine** (**5-NT**, Figure 5) has been shown to inhibit CHIKV replication in U2OS cells [134]. Interestingly, also the 5-HT antagonist **methiothepin mesylate** (**MM**) was able to inhibit 97 ± 1.0% of the CHIKV replication at 10 μM. A time-of-addition study suggested two different modes of actions: for 5-NT, the inhibition of the uncoating and for MM, the internalisation and membrane hemifusion step [133].

g. *Immunomodulatory Agents*

The host immune system and responses, primarily the type I interferon (IFN) signalling, are crucial for controlling and preventing CHIKV infections [196]. Accordingly, many antiviral compounds have shown synergistic effects when given with IFNs [91, 103, 108, 130, 166]. Furthermore, the orally available IFN-inducer **tilorone dihydrochloride** (Figure 5) is known since 1970 for its antiviral activity against SFV in mice [197]. More recently, tilorone was reported to inhibit CHIKV infections in Vero cells [135].

Polyinosinic acid: polycytidylic acid (**poly(I:C)**), a double-stranded RNA, is another potent IFN inducer and interacts with the toll-like receptor 3 (TLR3) which are expressed in the membrane of B-cells, macrophages, and dendritic cells. The TLR3 receptors are part of the innate immune response and recognize proteins, lipids, carbohydrates and others from invading microorganism. When activated by poly(I:C), they suppress the CHIKV infection by inducing IFNs and other antiviral genes [198]. Pre-treatment of mice with poly(I:C) reduced viral titer in the brain and achieved 100% survivability of the mice [199]. The double-stranded RNA **5´pppRNA** is like poly(I:C), a well-studied adjuvant for enhancing the efficacy of influenza virus vaccines [200, 201]. In addition, 5`pppRNA and its analogue **M8** inhibited the *in vitro* and *in vivo* replication of different viruses (including the CHIKV) by interacting with the retinoic acid-inducible gene I (RIG-I) [202–204].

The IFN-inducible protein viperin was also reported to play an essential role in the *in vivo* infection of the CHIKV and could be an interesting target for antiviral drugs. Mice lacking viperin were reported to develop higher viremia and more severe joint inflammation than infected wild-type mice [205, 206]. A novel small molecule (**C11**) was recently shown to induce IFN secretion from human cells and transcription/translation of interferon-dependent antiviral genes like viperin. Reverse genetics and a loss-of-function assay suggested the adaptor protein STING for its IFN activation ability. C11 had antiviral effects against the Ross River virus, VEEV, Mayaro virus, O´nyong-nyong virus, and CHIKV [136]. **G10** (Figure 5) is another small molecule preventing the replication of various alphaviruses (e.g. VEEV, Sindbis virus, and CHIKV) by indirectly activating the STING protein, which supports the hypothesis of STING as a possible antiviral target [137]. The same research group reported **AV-C** (Figure 5) as a novel interferon-activating small molecule. AV-C showed inhibitory effects against ZIKV, Dengue virus, and CHIKV infection in THF cells [138].

Further, heparan sulfate mimetics like **pentosan polysulfate** and **PG545** (**pixatimod**) have been reported as interesting compounds for alleviating alphavirus-induced disease *in vivo*. Pentosan polysulfate is an FDA-approved drug against cystitis, whereas pixatimod is currently in a clinical trial to treat advanced cancer and pancreatic adenocarcinoma [139, 207, 208]. Although pentosan polysulfate treatment did not influence the kinetics of virus infection, it alleviated virus-induced arthritis in C57BL/6 mice [139]. Furthermore, it was recently successfully evaluated in phase II clinical trials for the treatment of RRV-induced arthritic disease (PARA_004, Paradigm BioPharmaceuticals)

[209]. Pixatimod treatment also reduced the severity of alphavirus-induced arthritis and showed good antiviral effects against different CHIKV strains [140].

6. Undefined Targets

Quinolones, like ciprofloxacin and N-acyl hydrazones, were reported as compounds with antibacterial and antiviral properties. Therefore a series of **quinolone-N-acylhydrazone hybrids** (Figure 6) were synthesized in a 4 step synthesis route. Accordingly, they demonstrated antiviral effects against the ZIKV and CHIKV in Vero cells. A mechanism of action study suggests that the compounds act in the early stages and in some post-infection stages of the CHIKV replication cycle. Nevertheless, the precise mode of action remains unknown [210].

The **thieno[3,2-b]pyrrole** (Figure 6) scaffold was initially found by screening and has been shown to be a potential lead compound. Two structure-activity-relationship studies were made to improve its antiviral activity and its metabolic stability and yielded in the design of the most promising analogue: compound 20. This compound series has been reported to inhibit the expression of viral nsP1, nsP3, capsid, and E2 proteins and to affect the CHIKV life cycle. Additionally, they have shown to inhibit other alphaviruses like the O`nyong-nyong and Sindbis virus [211, 212].

Recently, Fares et al. reported a new scaffold of a CHIKV inhibitor based on the fusion of uracil and rhodanine pharmacophoric features, which were previously identified as antiviral active (Figure 6) [38, 50, 213, 214]. The best performing analogue, **compound 15**, had a p-methyl biphenyl tail functionality and may be an interesting lead compound for further drug development [213]. An overview of compounds without a known target is given in **Table 3**.

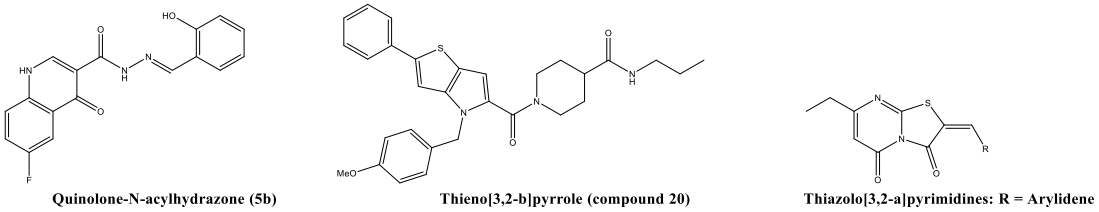

Figure 6. Chemical structure of anti-CHIKV compounds without known target.

Table 3. Antiviral compounds without a known target.[a]

	In vitro				*In vivo*		
Compound[b]	**EC_{50} (μM)**[d]	**CC_{50} (μM)**	**SI**	**Cell line**	**Efficacy**	**Mouse model**	
Quinolone-N-acylhydrazone (5b)	1.06 ± 0.08	669 ± 4.33	631.7	Vero	—	—	[210]
Thieno[3,2-b]pyrrole (compound 20)	3-4	>100	n.s.	HEK293T	Good in vivo pharma-cokinet-ics	C57BL/6	[211, 212]
Compound 15	42	n.s.	n.s.	MCF-7	—	—	[213]

[a]EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration; n.s., not specified; —, not determined.
[b]If the study reported a compound series/class with anti-CHIKV activity, the antiviral data of the most potent or most representative compound is reported. Only compounds with *in vitro* or *in vivo* data are shown.
[c]If a compound was reported in multiple studies, cell lines, or if different CHIKV strains have been used, the best activity value with the corresponding cell line is reported.

5. Conclusions

Due to the severity and chronicity of the Chikungunya fever and the rapid worldwide spread, the CHIKV remains a clinically relevant pathogen. Moreover, the lack of approved antiviral compounds and vaccines against this alphavirus further lightens the crucial development of inhibitors against the Chikungunya virus. A comprehensive overview of several antiviral compounds is given in this review, but most of them are still in the early stage of drug development as their activity against the CHIKV is only tested in *in vitro* assays. This issue could be overcome by repurposing already

approved drugs for the anti-CHIKV treatment. As they already have been intensively investigated for their safety in humans, the clinical evaluation of such drugs could be a fast and safe option for emergency treatment in CHIKV infection outbreaks. On the other hand, a much more effective and stronger antiviral activity can be expected from compounds directly designed to inhibit the alphavirus.

Another challenge in the development of CHIKV antiviral drugs is its already shown ability to mutate and to develop resistance to antiviral therapy. A combinatory approach of compounds with synergistic effect or the design of an antiviral with multiple targets could diminish this escape mechanism of the virus. Furthermore, recent studies have identified new promising host factors as possible targets for CHIKV inhibitors. Such inhibitors could demonstrate panviral inhibitory effects as many viruses use the same replication strategies. However, targeting a crucial host cell factor could also lead to more (serious) side effects due to the manipulation of important biomechanism of the host. More research is required to identify new and safe targets by collecting more detailed information about the CHIKV life cycle. Furthermore, the current absence of a vaccine makes the development of a potent and safe CHIKV inhibitor crucial for the treatment of this severe disease.

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