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The protein disulfide isomerase inhibitor 3-methyltoxoflavin inhibits Chikungunya virus

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A B S T R A C T

Chikungunya virus (CHIKV) is the etiological agent of chikungunya fever, a (re)emerging arbovirus infection, that causes severe and often persistent arthritis, as well as representing a serious health concern worldwide for which no antivirals are currently available. Despite efforts over the last decade to identify and optimize new inhibitors or to reposition existing drugs, no compound has progressed to clinical trials for CHIKV and current prophylaxis is based on vector control, which has shown limited success in containing the virus. Our efforts to rectify this situation were initiated by screening 36 compounds using a replicon system and ultimately identified the natural product derivative 3-methyltoxoflavin with activity against CHIKV using a cell-based assay (EC_{50} 200 nM, SI = 17 in Huh-7 cells). We have additionally screened 3-methyltoxoflavin against a panel of 17 viruses and showed that it only additionally demonstrated inhibition of the yellow fever virus (EC_{50} 370 nM, SI = 3.2 in Huh-7 cells). We have also showed that 3-methyltoxoflavin has excellent *in vitro* human and mouse microsomal metabolic stability, good solubility and high Caco-2 permeability and it is not likely to be a P-glycoprotein substrate. In summary, we demonstrate that 3-methyltoxoflavin has activity against CHIKV, good *in vitro* absorption, distribution, metabolism and excretion (ADME) properties as well as good calculated physicochemical properties and may represent a valuable starting point for future optimization to develop inhibitors for this and other related viruses.

1. Introduction

Chikungunya virus (CHIKV) is a member of the *Alphavirus* genus (*Togaviridae* family) which is the etiologic agent of Chikungunya fever, a globally spreading mosquito-borne disease. CHIKV was first isolated in 1952 and causes periodic and explosive outbreaks of a severe and often persistent arthritis[1]. CHIKV has reemerged recently as a significant public health threat since the 2005 outbreak in La Réunion. This major outbreak occurred on these islands of the Indian Ocean, followed by many CHIKV infection cases reported in Europe and India in 2006 and 2007, respectively.[2–3] In December 2013, CHIKV emerged on the American continent, on the island of St. Martin, Caribbean, and by the end of December 2015, nearly 1 million cases had been reported in the Americas[4,5] The reemergence of the virus in many parts of the world represents a serious public health concern[6]. Since 2013, hundreds of thousands of cases of the disease have been confirmed in the north and northwest parts of Brazil.[7] This outbreak is still not contained, and the risk of expansion to the southern part of Brazil and other countries still exists.

Driven by the medical importance of this virus, as well as the lack of approved therapeutics, research into the field of CHIKV antivirals has recently intensified. CHIKV is transmitted to humans by *Aedes* sp. mosquitoes, mainly A. aegypti and A. albopictus, both of which are widely disseminated in tropical and subtropical regions.[8] Humans are the primary host of CHIKV during epidemic periods. Rare *in utero* transmission has also been documented mostly during the second trimester. Intrapartum transmission has also been reported when the mother was viremic around the time of delivery.[9] The incubation period of the virus is typically 3–7 days (range, 1–12 days) and most people infected with CHIKV show symptoms.[10]

CHIKV infection in humans is distinguished by a very high viremia and debilitating symptoms such as joint pain and inflammation, fever, rash and even morbidity that may persist for years.[11–12] Without treatments or vaccines available to contain the infection prophylaxis is based on vector control, which has shown limited success in containing the virus.[11,13] Treatment for CHIKV symptoms can include rest, fluids, and use of non-steroidal anti-inflammatory drugs (NSAIDs) to relieve acute pain and fever. Therefore, availability of effective antiviral treatments

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against CHIKV would mitigate viral spread and limit the morbidity associated with the disease.[12] Owing to mechanisms that are poorly understood, recurrent and persistent myalgia and arthralgia have been reported to last for years after the infection clears in some patients. A recent study with a macaque model suggested that the chronic phase could be caused by inflammatory responses toward persistent CHIKV in certain tissues, rather than an autoimmune-mediated response, as was initially believed.[14–15]

Some of the well-known broad-spectrum antivirals like ribavirin and interferon, may prove to be promising against CHIKV[16] although there is no evidence supporting their clinical efficacy, they could be subject to clinical trials in future.[16–17] Recent drug discovery efforts have included computer-aided design to identify an inhibitor of the E2-E1 envelope glycoprotein complex leading to an optimal compound with an EC_{50} of 1.6 μM.[18] Several classes of CHIKV inhibitors have been described such as the benzoannulene replication inhibitors,[19] pyrimidones[20] (inhibitors of NSP3 macrodomain), indoles[21] (inhibitors of virus replication), quinolone-*N*-acylhydrazone hybrids[22] (inhibitors of virus replication), Itraconazole[23] (inhibitor of viral replication), tilorone (broad spectrum antiviral)[24] and others[25–26] derived by fragment-based screens or high throughput screening targeting nsP1 and viral capping machinery and inhibitors targeting nsP2[27] (Table 1). Other classes of inhibitors and their mechanism of action for CHIKV is covered elsewhere.[2829] Despite the efforts performed in the last decade to identify and optimize new inhibitors or to reposition existing drugs, no compound has progressed towards clinical trials for this debilitating infectious disease to date. This unmet clinical need stimulated our interest to search for potential novel inhibitors of CHIKV and we now describe 3-methyltoxoflavin as an antiviral.

2. Results

2.1. BHK-21-Gluc-nSP-CHIKV-99659 replicon cell line screening

We initially screened 36 compounds (Table 2) that were available in the laboratory (having being initially selected and screened against the Zika virus,[30] using the BHK-21-GLuc-nSP-CHIKV-99659 replicon cell line. Each compound was first assayed at 20 μM concentration in a 96-well format plate (Table 2). Nine compounds inhibited the luciferase activity (furaltadone, amodiaquine, promazine, fluphenazine, quinacrine, 4-hydroxyderricin, xanthoangelol, (2E)-1-[3-(2-hydroperoxy-3-methyl-3-buten-1-yl)-2-hydroxy-4-methoxyphenyl]-3-(4-hydroxyphenyl)-2-propen-1-one and 3-methyltoxoflavin) $\geq$ 80% and were further assayed for the determination of their EC_{50} and CC_{50} values. These compounds were tested in a dose-dependent manner, and only xanthoangelol, quinacrine and 3-methyltoxoflavin showed activity. Xanthoangelol and quinacrine decreased the GLuc signals in a dose-dependent manner, with EC_{50} values in the low micromolar range (Figure 1 A,B). These compounds exhibited high cytotoxicity to the replicon cells, resulting in poor selectivity indexes (CC_{50}/EC_{50}, SI)) of 1.66 and 1.64, respectively (Figure 1A,B). In contrast, 3-methyltoxoflavin had an EC_{50} of 0.2 ± 0.04 μM and a low cytotoxicity to the same cells with an CC_{50} of 6.2 ± 5.5 μM and SI = 30.8 (Figure 1C). We also evaluated 3-methyltoxoflavin cytotoxicity in an additional commonly used cell line available to us, namely HepG2 cells, which resulted in a CC_{50} of 11.0 ± 1.73 μM (Figure 1D).

2.2. Antiviral activity against CHIKV and other viruses

3-methyltoxoflavin was tested in Vero 76 and Huh-7 cells infected with CHIKV (S27 strain) and the activity was investigated using a plaque assay. 3-methyltoxoflavin had an $EC_{50\ of}$ 0.19 μM and an SI = 17 in Huh-7 cells, however when tested in Vero 76 this compound was toxic with SI 0 (Table 3). 3-methyltoxoflavin was also tested against a diverse panel of 17 additional viruses available through the NIAID screening resource (Table S1) and only showed activity against yellow fever (YFV 17D strain) with an EC_{50} 0.37 μM and SI 3.2 in the visual cytopathic toxicity assay when tested in Huh-7 cells (Table 3).

2.3. Predictive and experimental Absorption, Distribution, metabolism and excretion (ADME) properties

We first used a web based software SwissADME[31] to predict 3-methyltoxoflavin physicochemical and pharmacokinetic properties, ADME parameters, drug likeness and medicinal chemistry properties, which suggested a low predicted logP, good solubility, permeability and no alerts for PAINS (Table S3). We also used our proprietary software Assay Central[32–33] and models from our toxicology collection (MegaTox)[34] to predict solubility, permeability and human microsomal clearance (Table S4).

We then assessed several ADME properties *in vitro* to verify if 3-methyltoxoflavin could be suitable for future *in vivo* pharmacokinetic (PK) studies. 3-methytoxoflavin showed good solubility > 200 μM in all buffers tested (pH 1.2, 6.8 and 7.4), excellent metabolic stability with $t_{1/2}$ > 186.4 min in human and mouse liver microsomes, and a low hepatic intrinsic clearance Cl_{int} < 7.4 μL/min/mg protein in both species (Table 4). As 3-methyltoxoflavin has good Caco-2 permeability, it indicates that it can potentially have good absorption and oral bioavailability while it is unlikely to be a P-glycoprotein (P-gp) substrate (Table 4).

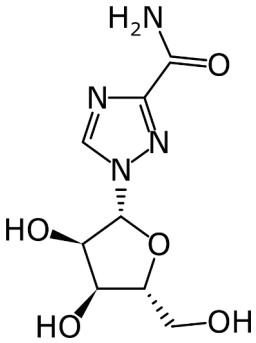
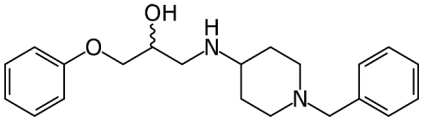
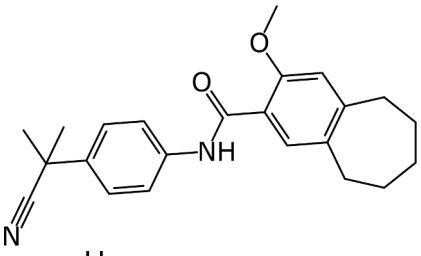
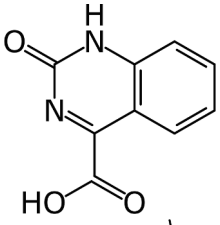
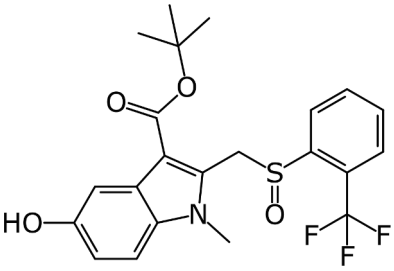
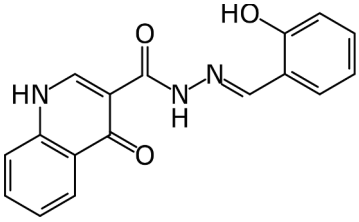
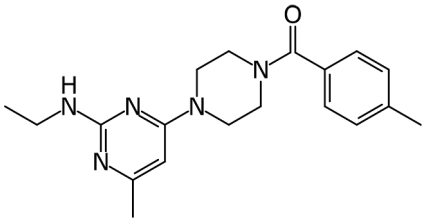
2.4. Discussion

CHIKV is a (re)emerging arbovirus and about 3–5 million cases of CHIKV infections are reported globally every year.[35] After an acute phase with fever and joint arthralgia, viral infection can lead to chronic debilitating arthritis, which has important social and economic consequences. Although mortality is low, in neonates, elderly people or patients suffering from diabetes or heart disease, infection with CHIKV can lead to severe complications including death[36–37]. In contrast, morbidity is high, with important social and economic consequences. Moreover, coinfections with dengue virus, transmitted by the same vector, also raises serious health concerns.[38].

Alphaviruses require conserved cysteine residues for proper folding and assembly of the E1 and E2 envelope glycoproteins,[39] and this likely depends on the host protein disulfide isomerase (PDI) to aid in facilitating disulfide bond formation and isomerization in these proteins. 3-methyltoxoflavin is a potent PDI inhibitor, with a previously reported IC_{50} of 170 nM[40]. We hypothesize that the mechanism for the activity of 3-methyltoxoflavin in CHIKV may be due in part to inhibition of PDI[40]. PDI is also a promising target for cancer therapy[40–41] and many viruses, such as HIV[42], CHIKV[43], Dengue[44–45], Zika[45], Hepatitis C[46], influenza[47], as well as others.[48–49] 3-Methyltoxoflavin also induces the Nrf2 antioxidant response, ER stress response, and autophagy[40] which suggests a broad range of diseases for which it could be applied. 3-Methyltoxoflavin bears some 2D structural resemblance to nucleotide and nucleoside type antiviral inhibitors so this may also represent another mechanism by interfering directly with viral targets which could be worthy of future study. We therefore determined the Tanimoto similarity using (Molecular ACCess System) MACCS keys fingerprints[50] comparing 3-methyltoxoflavin with the most active compounds (EC_{50} < 2 μM) against CHIKV deposited in CHEMBL (CHEMBL4296563) and compounds listed in Table 1. 3-methyltoxoflavin shows structural similarity with ribavirin, 6-azauridine, 2-oxo-quinazoline- 4-carboxylic acid a recently described inhibitor of the macrodomain of nsP3 (important for replication),[20] and 6—H—N'-(2-hydroxybenzylidene)- 4-oxo-1,4-dihydroquinoline-3-carbohydrazide (an inhibitor of replication)[22] (Table 1 and Table S4).

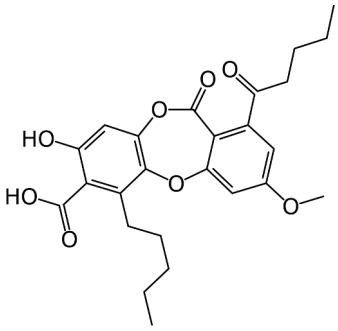
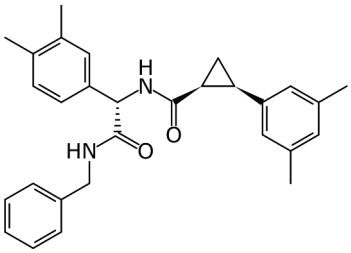
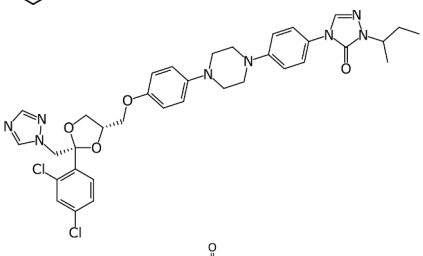
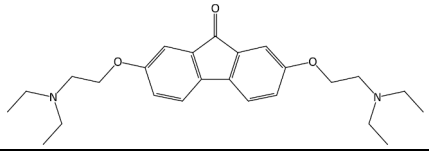
So far there have been relatively few compounds described with promising *in vitro* activity for CHIKV[25–26,18–22] (Table 1 and S4). Notable examples include previous work using a CHIKV replicon cell line which screened ~ 3000 bioactive molecules including FDA approved drugs and

Table 1
Compounds with reported activity against CHIKV.

Molecule	Structure	Comment	Reference
Ribavirin		Broad spectrum antiviral	16
1-[(1-Benzylpiperidin-4-yl)amino]-3-phenoxypropan-2-ol		Inhibitor of the E2-E1 envelope glycoprotein	18
benzo[6]annulene, 4-(*tert*-butyl)-N-(3-methoxy-5,6,7,8-tetrahydronaphthalen-2-yl) benzamide		Inhibitor of human dihydroorotate dehydrogenase in addition to CHIKV nsP3 macrodomain	19
2-oxo-5,6-benzopyrimidine-4-carboxylic acid		CHIKV nsP3 macrodomain	20
tert-butyl 5-hydroxy-1-methyl-2-((2-trifluoromethylphenyl)sulfinylmethyl)-indole-3-carboxylate			21
6—H—*N*'-(2-hydxoxybenzylidene)- 4-oxo-1,4-dihydroquinoline-3-carbohydrazide		Inhibition of replication	22
{4-[2-(Ethylamino)-6-methyl-4-pyrimidinyl]-1-piperazinyl}(p-tolyl)methanone		Viral capping machinery	25
Lobaric acid		nsP1 inhibitor	26

(*continued on next page*)

Table 1 (*continued*)

Molecule	Structure	Comment	Reference
			
(1S,2S)-N-((S)-2-(Benzylamino)-1-(3,4- dimethylphenyl)-2-oxoethyl)-2-(3,5- dimethylphenyl)cyclopropane-1-carboxamide		nsP2 inhibitor	[27]
Itraconazole		Inhibitor of replication	[23]
Tilorone		Broad spectrum antiviral	[24]

identified the isoquinoline alkaloid berberine as a potent inhibitor of CHIKV replication[51]. The efficacy of this compound was tested in a CHIKV-induced arthritis mouse model and showed the alleviation of the inflammatory symptoms.[52] Another approach has been described to identify effective antiviral drugs against CHIKV based on a human genome-wide loss-of-function screen[53] which resulted in the identification of important targets and the combination of two existing drugs, 5-(tetradecyloxy)-2-furoic acid (TOFA, and pimozide, targeting fatty acid synthesis and calmodulin signaling respectively, showing anti-CHIKV activity *in vitro* and *in vivo*.[53] The broad-spectrum antiviral ribavirin was also reported to have an EC_{50} of 2.42 μM in Vero cells.[22] We now report that 3-methyltoxoflavin shows activity in human Huh-7 cells but not Vero cells. Another PDI inhibitor PAC31 and auranofin (an inhibitor of thioredoxin reductase) have showed activity against CHIKV as well as toxicity in cell lines, however, they were evaluated for their in vivo efficacy due to their previous use in animals and humans, which provided a precedent for safe dosing regimens.[54–55] This cell type-dependent effect was previously noted in our work on SARS-CoV-2 with several different compounds[56] and points to the importance of assessing antiviral activity in human cell lines. For example, pyronaridine is a safe drug approved in Europe as a combination therapy for malaria, however it shows cytotoxicity in several cell lines.[56].

3-methyltoxoflavin was previously demonstrated to have activity against glioblastoma[40]. Redox cycling toxoflavins, such 3-methyltoxoflavin are known to interfere with enzymatic assays broadly[57], especially in the case of cysteine/GSH dependent enzymes, such as PDI. However, the activity of this compound against PDI was further validated using thermal shift and CETSA assays.[40] Toxoflavins have been described as inhibitors of the tyrosyl-DNA phosphodiesterase II (TDP2) and some analogs were described as potent CYP inhibitors ($IC_{50} < 1$ μM) while also undergoing redox cycling (leading to assay interference)[58], presenting challenges to further development and leading ultimately to the deazaflavin class of TDP2 inhibitors without these liabilities.[59] In this study we have assessed antiviral activity in cells and this assay is unlikely to be affected by the redox cycling. However, if we were to identify a target it is likely we would have issues optimizing this molecule.

In summary, we initially tested 36 compounds (previously tested against the Zika virus[30]and discovered that quinacrine, xanthoangelol and 3-methyltoxoflavin were active using a CHIKV reporter replicon cell line BHK-21-Gluc-nSP-CHIKV-99659, with 3-methyltoxoflavin showing an EC_{50} 0.2 ± 0.04 μM and a low cytotoxicity to the same cells with an CC_{50} of 6.2 ± 5.5 μM and SI = 30.8. We then screened this compound against CHIKV and also against a panel of 17 different viruses (Table S1). 3-Methyltoxoflavin was active against CHIKV with an $EC_{50\ of}$ 0.19 μM and an SI = 17 in Huh-7 cells and only showed additional activity against yellow fever (YFV 17D strain) with an EC_{50} 0.37 μM (comparable to the PDI activity IC_{50} of 170 nM[40] and SI 3.2 in the visual cytopathic toxicity assay (Table 3). As no other viruses tested showed activity this suggests that the compound is not a promiscuous inhibitor. The compounds listed in Table 1 have a wide range of activity against CHIKV. We previously reported that tilorone has activity against CHIKV in Vero cells with an EC_{50} of 4.2 μM.[24] Compound 1-[(1-Benzylpiperidin-4-yl) amino]-3-phenoxypropan-2-ol showed an EC_{50} of 1.6 μM in Vero

Table 2
Antiviral screening of selections from the CPI compound library at 20 μM using the CHIKV reporter replicon cell line BHK-21-Gluc-nSP-CHIKV-99659. Average results and standard deviations of two independent assays performed in duplicates.

Compound	Name	Luciferase activity (%)
1	Atropine	149.2 ± 2.6
2	Furaltadone	20.4 ± 5.2
3	Naloxone	121.5 ± 19.7
4	Acetohexamide	100 ± 60.9
5	Ziprazidone	101
6	Amodiaquine	19 ± 5.9
7	Bergenin	100 ± 13.8
8	Pipamperone	102.9 ± 32.8
9	Risedronate	86.7 ± 21.6
10	Raltegravir	58.9 ± 3.2
11	Estragole	100 ± 17.7
12	D(-)salicin	100 ± 13.8
13	Eugenol	100 ± 36
14	Promazine	18 ± 4.6
15	Phenothiazine	73.7 ± 16.4
16	Fluphenazine	11.6 ± 8.5
17	Lumefantrine	100 ± 45.2
18	Quinacrine	12.5 ± 1.9
19	Tilorone	100 ± 30.1
20	Piperaquine	138.1 ± 7.9
21	MolPort-005–044-056	100 ± 29.5
22	MolPort-004–275-784	100 ± 66.9
23	2-(4-carbamoylpiperidin-1-yl)-N-(2-chlorophenyl)-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido[2-3d]pyrimidine-5-carboxamide	140 ± 5.2
24	4-{[4-(pyridin-2-ylsulfamoyl)phenyl]sulfamoyl} benzoic acid	100 ± 57
25	N-(4-fluorophenyl)-2-({[4-(5-oxopyrrolidin-3-yl) phenyl]sulfonyl}amino)benzamide	100 ± 47.9
26	4-{5-[6-hydroxy-1-(propan-2-yl)-4,5-dihydro-1H-pyrazolo[3,4-b]pyridin-4-yl]furan-2-yl} benzoic acid	100 ± 44
27	3-methyl-2,4-dioxo-N-[3-(tricyclo[3.3.1.1 ~ 3,7 ~]dec-1-yloxy)propyl]-1,2,3,4-tetrahydroquinazoline-6-sulfonamide	100 ± 26.3
28	7-methyl-2-[5-(2-methyl-4-nitrophenyl)furan-2-yl]-1,2,5,6,7,8-hexahydropyrido[4',3':4,5]thien [2,3-d]opyrimidin-4-ol	100
29	4-hydroxyderricin	13.5 ± 1.9
30	Xanthoangelol	8.4
31	(2E)-1-[3-(2-Hydroperoxy-3-methyl-3-buten-1-yl)-2-hydroxy-4-methoxyphenyl]-3-(4-hydroxyphenyl)-2-propen-1-one	
32	LOC14	14 ± 2.6
33	3-Methyltoxoflavin	2.0 ± 6.6
34	Orlistat	48.7 ± 6.6
35	Dexamethasone	70.9 ± 24.8
36	Haloperidol	40.5 ± 5.0

cells,[18] benzo[6]annulene, 4-(*tert*-butyl)-N-(3-methoxy-5,6,7,8-tetrahydronaphthalen-2-yl) benzamide showed an EC_{90} of 0.27 μM in normal human dermal fibroblasts (NHDF),[19] 2-oxo-5,6-benzopyrimidine-4-carboxylic acid showed anti-CHIKV activity with an IC_{50} of 23 μM[20] in normal human foreskin fibroblasts; *tert*-butyl 5-hydroxy-1-methyl-2-((2-trifluoromethylphenyl)sulfinylmethyl)-indole-3-carboxylate showed an EC_{50} of 6.5 μM in Vero cells.[21] Recently, itraconazole has been reported to be active against CHIKV with an EC_{50} of 320 nM in BHK-21 cells infected with CHIKV-*nanoluc*[23] and lobaric acid showed an EC_{50} of 5.3 μM in Huh7 cells.[26] Therefore, 3-methyltoxoflavin is amongst these compounds with the best $EC_{50's}$ reported against CHIKV tested in different cell types.

Importantly, CHIKV and YFV are both arboviruses transmitted by the *Aedes aegypti* mosquito, however CHIKV belongs to the family *Togaviridae* and YFV belongs to the *Flaviviridae*. While the mechanism of action of 3-methyltoxoflavin remains to be fully elucidated our data suggests that it could be used as a chemical probe even when the known redox cycling liabilities are considered. It may be worth also evaluating deazaflavin analogs which do not have the redox cycling issue.[59] The ADME properties for 3-methyltoxoflavin were previously predicted and showed a high likelihood of blood–brain-barrier permeation.[40] We have also now shown for the first time that 3-methyltoxoflavin has good *in vitro* ADME properties, such as high solubility (399.5 μM in pH 7.4), Caco-2 permeability, with an efflux ratio of 1, showing that there is no drug efflux, and metabolic stability ($T_{1/2} > 186.4$ min, *in vitro* $CL_{int} < 7.4$ μL/min/mg protein and $CL_{hep} < 22.1$ mL/min/Kg tested in human liver microsomes as well as similar data in mouse liver microsomes). These favorable *in vitro* ADME properties suggest that this molecule is likely suitable for further *in vivo* pharmacokinetic (PK) and efficacy studies (outside the scope of the current study). We have not determined the potential for CYP inhibition, and this would be important to consider clinically.

In conclusion, while several molecules have previously been reported to have antiviral activity against CHIKV, there is currently no approved drug for this disease and hence there is still a need for new chemical matter and leads to advance drug discovery. As there is a significant unmet global health need, 3-methyltoxoflavin therefore represents a promising starting point for probing potential targets with activity in CHIKV and understanding antiviral activity of this compound.

3. Experimental section

3.1. Cells and compounds

BHK-21 [C-13] and HepG2 cell lines were purchased from Banco de Células do Rio de Janeiro (BCRJ) and maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) containing 10% heat-inactivated fetal bovine serum (FBS, GIBCO), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2-humidified incubator. BHK-21-GLuc-nSP-CHIKV-99659 cell line, harboring a CHIKV replicon expressing Gaussia luciferase (Gluc) and neomycin phosphotransferase (Neo) genes, were maintained in DMEM 10% FBS with 500 μg/ml G418 (Sigma-Aldrich). The development and characterization of this CHIKV replicon cell line will be described elsewhere. CPI compounds (>90% purity) were solubilized in 100% DMSO (v/v) and further diluted with assay media to a final DMSO concentration of 1% (v/v) for the antiviral assays.

3.2. Replicon-based antiviral assays

We screened a small set of chemical diverse structures from our library ((36 compounds (Table 1)) that were evaluated as potential inhibitors of the viral replication using the BHK-21-T7-Gluc-nSP-CHIKV-99659 cell line. Primary screening of each compound was performed at 20 μM with 1% DMSO final concentration in a 96-well format, as described previously.[60] Approximately 2×10^4 replicon cells/well in DMEM 10% FBS were seeded in a 96-well plate. After 16 h of incubation at 37 °C 5% CO_2, medium was replaced with fresh DMEM supplemented with 2% FBS and compounds were added to the cells. After a 48 h-incubation, 40 μL of the cells' supernatant containing secreted GLuc were mixed with *Renilla* luciferase Assay Reagent (100 μL) (Promega). GLuc activity was measured using SpectraMax i3 Multi-mode Detection Platform (Molecular Devices).

Compounds that inhibited GLuc activity in ≥ 80% were assayed for the determination of their effective (EC_{50}) and cytotoxicity (CC_{50}) concentrations. As described previously,[60] replicon cells were seeded in 96-well plates and after 16 h of incubation at 37 °C 5% CO_2, medium was replaced with fresh DMEM with 2% FBS and compounds at 2-fold serial dilutions were added to the cells. After a 48 h-incubation, 40 μL of the cells' supernatant were mixed with *Renilla* luciferase Assay Reagent (100 μL) (Promega) and Gluc activity were measured using SpectraMax i3 Multi-mode Detection Platform (Molecular Devices). The compound concentrations required to inhibit 50% of the Gluc activity (EC_{50}) were

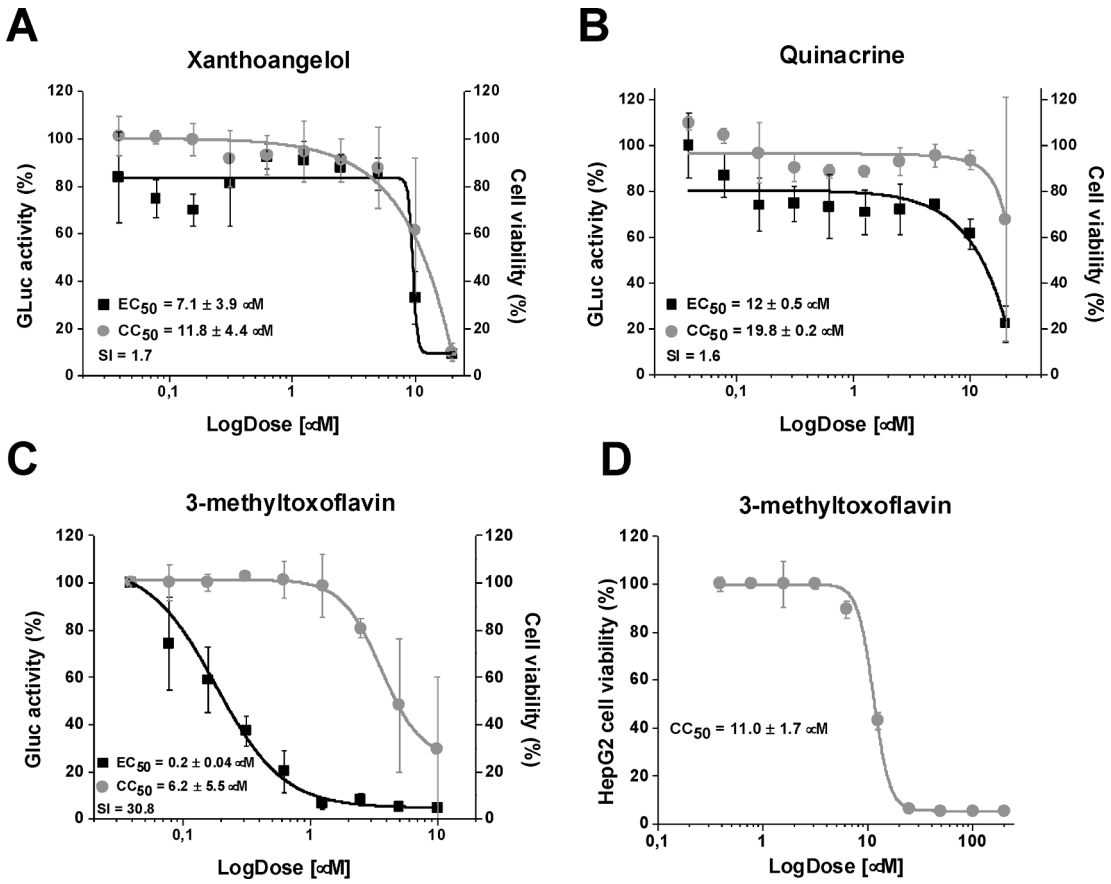


Figure 1. The dose–response curves. A) Xanthoangelol, B**)** Quinacrine and **3)** 3-methyltoxoflavin tested in replicon cell lines. CHIKV replicon cells were incubated with compounds at 2-fold serial dilutions (from 20 μM to 0.03 μM) for 48 h and Gluc activity was measured from cells' supernatant. **D)** Cytotoxicity of 3-methyltoxoflavin was tested in HepG2 cells. The cells were incubated with compound at 2-fold serial dilutions (from 200 μM to 0.39 μM) for 48 h and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/mL in PBS) were added to the wells. After 3–4 h of incubation, the formazan crystals were solubilized in DMSO, and absorbance was read at 570 nm. Average results of two independent experiments. Error bars represent the standard deviations.

Table 3
3-Methyltoxoflavin was tested against CHIKV and Yellow Fever.

Virus/strain	Cell type	Drug Assay Name	EC_{50} (μM)	EC_{90} (μM)	CC_{50} (μM)	SI (μM)
Chikungunya virus/ S27	Huh-7	Visual (cytopathic effect/toxicity)	0.19		3.2	17
Chikungunya virus/ S27	Huh-7	Neutral Red (cytopathic effect/toxicity)	<0.1		1.1	>11
Chikungunya virus/ S27	Vero 76	Visual (Virus yield reduction)/ Neutral Red (Toxicity)		>1.7	1.7	0
Chikungunya virus/ S27	Vero 76	Neutral Red (Cytopathic effect/Toxicity)	>1.7		1.7	0
Yellow Fever Virus/ YFV 17D	Huh-7	Visual (cytopathic effect/toxicity)	0.37		1.2	3.2
Yellow Fever Virus/ YFV 17D	Huh-7	Neutral Red (cytopathic effect/toxicity)	0.42		1.2	2.9

Table 4
In vitro ADME properties for 3-methyltoxoflavin.

ADME property	Value
Solubility (μM)	
Buffer (pH 7.4)	399.5
FaSSIF (pH 6.8)	227.5
FaSSGF (pH 1.2)	423.5
Mouse liver microsomes	
$T_{1/2}$ (min)	>186.4
In vitro CL_{int} (μL/min/mg protein)	<7.4
CL_{hep} (mL/min/Kg)	<22.1
Human liver microsomes	
$T_{1/2}$ (min)	>186.4
In vitro CL_{int} (μL/min/mg protein)	<7.4
CL_{hep} (mL/min/Kg)	<6.1
Caco-2	
P_{app} (10^{-6} cm/s) A-B	17.8
Recovery (%) A-B	103.0
P_{app} (10^{-6} cm/s) B-A	17.2
Recovery (%) B-A	93.5
Efflux ratio	1.0

calculated using the OriginPro 9.0 software. BHK-21 cells in 1% DMSO were used as negative control.

The cytotoxicity of compounds was evaluated by a cell proliferation-based MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[61] Replicon cells were seeded in 96-well plates as described above. After 48 h of incubation with the compounds at 2-fold serial dilutions, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/mL in PBS) was added to the wells at one tenth of the well volume and the plates were incubated at 37 °C 5% CO_2 for 3–4 h. Next, the medium was removed, and formazan crystals were solubilized in DMSO. Absorbance was measured at 570 nm wavelength in SpectraMax 384 plate reader (Molecular Devices).[60] The compounds

concentrations required to cause 50% cytotoxicity (CC_{50}) were estimated using the OriginPro 9.0 software. All the antiviral assays were performed twice in duplicates.

3.3. Cytotoxicity of 3-methyltoxoflavin in HepG2 cells

The cell proliferation-based MTT assay was also used to evaluate the potential toxicity of 3-methyltoxoflavin using hepatocellular carcinoma cells (HepG2). Approximately 2×10^4 HepG2 cells per well were seeded in 96-well plates and the assay was performed as described above. The compound concentration required to cause 50% cytotoxicity (CC_{50}) was estimated using the OriginPro 9.0 software. The assay was performed twice in duplicate.

3.4. Primary CPE and secondary VYR assays for viruses

Using the non-clinical and pre-clinical services program offered by the National Institute of Allergy and Infectious Diseases we screened the following viruses: Dengue virus 2, Eastern equine encephalitis virus, Enterovirus-71, MERS-Coronavirus, Influenza A (H1N1), Japanese encephalitis virus, Mayaro virus, Measles, Respiratory syncytial virus, Rift Valley fever virus, Tacaribe virus, Usutu Virus, Venezuelan equine encephalitis virus, West Nile virus, Western Equine Encephalitis and Zika Virus (Table S1). The assays were conducted as described.[56] Two sets of data are provided: (1) EC_{50}, CC_{50}, and SI_{50} obtained from the neutral red assay, and (2) EC_{90} (obtained from the VYR assay), CC_{50} (same value as for item 1), and SI_{90}. A more detailed protocol is described in the Supplemental Methods.

3.5. Data analysis

For the replicon-based assays, statistical calculations of Z′-values were made as follows: Z′ =1– ((3SD of sample + 3SD of control)/|Mean of sample – Mean of control|). Here, SD is the standard deviation of the luminescent signals from cell control (BHK-21) or sample. Z′ values between 0.5 and 1 are considered good quality.[61–63]

3.6. In vitro ADME assays

In vitro ADME studies were performed by BioDuro (San Diego, CA) and the assay protocols for kinetic solubility, Caco-2 permeability, and mouse liver microsomes were performed as described previously [64]. The human liver microsome studies were performed as described for the mouse procedure. Besides solubility at pH 7.4, we also evaluated FaSSIF (Fasted State Simulated Intestinal Fluid) pH 6.8 and FaSSGF (Fasted State Simulated Gastric Fluid) pH 1.2 for kinetic solubility studies.

Declaration of Competing Interest

SE is owner and ACP is an employee of Collaborations Pharmaceuticals, Inc. All other co-authors have no competing interests.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2023.117239.

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