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ABSTRACT

Chikungunya virus (CHIKV) is a re-emerging alphavirus transmitted to humans by *Aedes* mosquitoes. Since 2005, CHIKV has been spreading worldwide resulting in epidemics in Africa, the Indian Ocean islands, Asia and more recently in the Americas. CHIKV is thus considered as a global health concern. There is no specific vaccine or drug available for the treatment of this incapacitating viral infection. We previously identified 3-aryl-[1,2,3]triazolo[4,5-*d*]pyrimidin-7(6*H*)-ones as selective inhibitors of CHIKV replication and proposed the viral capping enzyme nsP1 as a target. This work describes the synthesis of novel series of related compounds carrying at the aryl moiety a methylketone and related oximes combined with an ethyl or an ethyl-mimic at 5-position of the triazolopyrimidinone. These compounds have shown antiviral activity against different CHIKV isolates in the very low μM range based on both virus yield reduction and virus-induced cell-killing inhibition assays. Moreover, these antivirals inhibit the *in vitro* guanylylation of alphavirus nsP1, as determined by Western blot using an anti-cap antibody. Thus, the data obtained seem to indicate that the anti-CHIKV activity might be related to the inhibition of this crucial step in the viral RNA capping machinery.

1. Introduction

Chikungunya virus (CHIKV) is an alphavirus that is transmitted to humans by *Aedes* mosquitoes, traditionally *Aedes aegypti* and more recently also *Aedes albopictus*. CHIKV causes chikungunya fever, a disease that is characterized by fever, nausea, headaches, rash and a persistent arthralgia (Burt et al., 2012; Thiberville et al., 2013). Although the clinical course is rarely associated with a fatal outcome, the symptoms can be severe and disabling, and may last for a long period of time (Couderc and Lecuit, 2015). In neonates, elderly people or patients with underlying medical conditions such as diabetes or heart disease, chikungunya fever can be associated with severe complications, including death (Couderc and Lecuit, 2015; Thiberville et al., 2013). Of particular concern are patients with a pre-existing arthritic disease that become infected by CHIKV (Burt et al., 2014).

Since first reported in 1952, CHIKV has been the cause of sporadic and infrequent outbreaks in Africa and Asia. In the last 10 years, the situation has dramatically changed and CHIKV is now considered a re-emerging virus that is a global health threat (Powers, 2015; Rougeron et al., 2015; Thiboutot et al., 2010). From 2004 onwards, CHIKV outbreaks have resulted in millions of cases reported on the five continents. Phylogenetic tools are being used to reconstruct the geographic spread of CHIKV outbreaks and to characterize the circulating virus, a crucial issue for the prediction and control of the CHIKV outbreak (Lo Presti et al., 2016; Weaver and Forrester, 2015). As an example, since December 2013, with the first reported case in the Caribbean island of Saint Martin, the

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virus has expanded to more than 20 countries in the Americas. Mediterranean Europe is also considered a potential area for CHIKV expansion, and autochthonous cases have already been reported in Italy and France (Delisle et al., 2015). Moreover, CHIKV infections are considered to be underestimated since the clinical symptoms and geographic distribution partially overlaps with dengue virus infection (Lo Presti et al., 2016).

There are no specific drugs to prevent or cure CHIKV infections (Abdelnabi et al., 2015; Ahola et al., 2015; Kaur and Chu, 2013; Rashad et al., 2013). Treatment is primarily directed at relieving the symptoms with over-the-counter drugs (Thiberville et al., 2013). Taking the severe impact of this infection into account, there is yet an unmet need for the discovery of compounds that are able to effectively and selectively interfere with the replication of CHIKV (Abdelnabi et al., 2015; Kaur and Chu, 2013). As the likelihood to develop chronic CHIKV disease seems to be correlated with the severity of symptoms during the acute phase of infection, a potent antiviral administered during the acute infection may diminish the chances to develop chronic disease (Abdelnabi et al., 2017). However, it is not clear yet whether anti-CHIKV therapy may be beneficial on the development of chronic CHIKV-induced arthritis. Compounds from natural origin such as flavonoids (Lani et al., 2016), known drugs such as niclosamide (Wang et al., 2016) and suramine (Albulescu et al., 2015; Kuo et al., 2016) or synthetic compounds (Ching et al., 2015; Mishra et al., 2016) have been recently reported to inhibit CHIKV infection in cell-based assays. Moreover, the alkaloid berberine has been shown to inhibit MAPK signaling activated by CHIKV infection, leading to antiviral effects in an animal model (Varghese et al., 2016).

In 2014, we discovered a series of small molecules that selectively block CHIKV replication. We identified compound **1** (Fig. 1) as an initial hit (Gigante et al., 2014). Structure-activity relationship studies revealed that the [1,2,3]triazolo[4,5-*d*]pyrimidin-7(6*H*)-one structure and the *meta*-substituted aryl ring linked at position 3 of the triazole were critical for anti-CHIKV activity. Accordingly, a significant improvement in terms of antiviral activity was observed for compound **2**, which has an ethyl substituent at position 5 of the heterocyclic base (Fig. 1). In addition, we recently showed that under the antiviral pressure of compound **1** drug resistant CHIKV strains were selected that carried a P34S substitution in the non-structural protein (nsP1). The importance of this mutation for the resistance phenotype was confirmed by reverse genetics (Delang et al., 2016), demonstrating that the nonstructural protein 1 (nsP1) carrying the function for the mRNA capping is targeted by our hit compound **1**. Indeed, compound **1** inhibited the guanylyltransfer (GT) activity of the nsP1 of Venezuelan equine encephalitis virus (VEEV) that was used as the enzymatic model for the study (Delang et al., 2016). In this study, we have now synthesized new derivatives analogous to **1** carrying at the aryl moiety a methylketone and related oximes combined with an ethyl or an ethylmimic at 5-position of the triazolopyrimidinone (Fig. 1). We have determined their antiviral activity against CHIKV in virus-cell-based assays, and the inhibition of the GT transfer by VEEV nsP1 for the most potent compounds.

2. Materials and methods

2.1. Chemistry procedures

Melting points were obtained on a Mettler Toledo M170 apparatus and are uncorrected. The elemental analysis was performed with a Heraus CHN-O-RAPID instrument. The elemental compositions of the compounds agreed to within ±0.4 of the calculated values. For all the tested compounds, satisfactory elemental analysis was obtained supporting greater than 95% purity. Electrospray

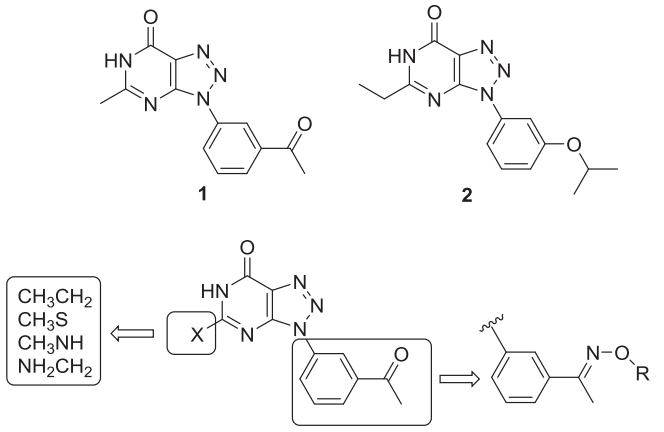


Fig. 1. Previously identified inhibitors of CHIKV replication based on [1,2,3]triazolo[4,5-*d*]pyrimidin-7(6*H*)-ones and general structures of the compounds addressed herein.

mass spectra were measured on a quadrupole mass spectrometer equipped with an electrospray source (Hewlett-Packard, LC/MS HP 1100). ^{1}H and ^{13}C NMR spectra were recorded on a Varian INNOVA-300 operating at 300 MHz (^{1}H) and 75 MHz (^{13}C), respectively, and a Varian INNOVA-400 operating at 400 MHz (^{1}H) and 101 MHz (^{13}C), respectively.

Analytical TLC was performed on silica gel 60 F_{254} (Merck) precoated plates (0.2 mm). Spots were detected under UV light (254 nm) and/or charring with ninhydrin. Separations on silica gel were performed by preparative centrifugal circular thin-layer chromatography (CCTLC) on a Chromatotron^{R} (Kiesegel 60 F_{254} gipshaltig (MercK)), with layer thicknesses of 1 and 2 mm and flow rates of 4 or 8 mL/min, respectively. Flash column chromatography was performed with silica gel 60 (230–400 mesh) (Merck). Microwave reactions were performed using the Biotage Initiator 2.0 single-mode cavity instrument from Biotage (Uppsala). Experiments were carried out in sealed microwave process vials utilizing the standard absorbance level (400 W maximum power).

Detailed synthetic procedures and characterization of all the compounds can be found in the Supplementary data.

2.2. Virus strains and cells

Chikungunya virus (CHIKV) Indian Ocean strain 899 (Genbank FJ959103.1) was generously provided by Prof. C. Drosten (University of Bonn, Germany). Venezuelan equine encephalitis virus (VEEV) vaccine strain TC83, CHIKV strain LR2006_OPY1 (Genbank DQ443544.2) and the clinical isolates Venturini (Italy 2008), Congo 95 (2011) and St Martin (2013) were used in EPV in Marseille and are freely disposable from the European Virus Archive (https://www.european-virus-archive.com/). Sindbis virus (SINV, strain HRsp, GenBank J02363.1) and the Semliki Forest virus (SFV, Vietnam strain, GenBank EU350586.1) belong to the collection of viruses at the Rega Institute of Medical Research, Belgium. All viruses were propagated in African green monkey kidney cells [Vero cells (ATCC CCL-81) or Vero E6 (ATCC CRL-1586)].

Vero cells were maintained in cell growth medium composed of minimum essential medium (MEM Rega-3, Gibco, Belgium) supplemented with 10% Foetal Bovine Serum (FBS, Integro, The Netherlands), 1% L-glutamine (Gibco), and 1% sodium bicarbonate (Gibco). The antiviral assays were performed in the same medium but supplemented with 2% (instead of 10%) FBS. The E6 sub-clone of Vero cells were maintained in Eagles MEM (Gibco) supplemented with antibiotics, 1% glutamine, 1% non essential amino-acids

(Gibco) and 7.5% FCS. Plating prior to infection and antiviral assays were done in the same medium but with reduced serum concentration (2.5%). All cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and 95–99% humidity.

2.3. Antiviral assays

2.3.1. CPE-inhibition assay

Vero cells were seeded in 96-well tissue culture plates (Becton Dickinson, Aalst, Belgium) at a density of 2.5×10^4 cells/well in 100 μl 2% FBS medium and were allowed to adhere overnight. Next, a compound dilution series was prepared in the medium on top of the cells (100 μl) after which the cultures were infected with 0.01 MOI of CHIKV 899 inoculum in 100 μl 2% FBS medium. The starting concentration was 100 μg/ml for all compounds tested. A similar setup was used for SINV and SFV. For SINV, a MOI of 0.001 was used and for SFV, a MOI of 0.0001 was used. Each assay was performed in 3-fold in the same test and assays were repeated three times independently to assess for inter-experiment variability. On day 5 post-infection (p.i.), the plates were processed using the MTS/PMS method as described by the manufacturer (Promega, The Netherlands). The 50% effective concentration (EC_{50}), which is defined as the compound concentration that is required to inhibit virus-induced cytopathic effect by 50%, was determined using logarithmic interpolation.

2.3.2. Virus yield assay

The antiviral activity of the best compounds was validated in an assay for CHIKV OPY 1, Venturini, Congo and St. Martin. The amount of each virus in the assay has been calibrated by trial so that the replication is at the beginning of the plateau at day 2 (multiplicity of infection $3\ 10^{-3}$ for CHIK viruses and $3\ 10^{-4}$ for VEEV). One day prior to infection, 5×10^4 Vero E6 cells were seeded in 100 μl of medium (with 2.5% FCS) in each well of a 96-well titer plate. The next day, two-fold serial dilutions of the compounds, in triplicates, starting either at 10 μM, final concentration, for CHIK Congo assay, or 40 μM, for CHIK OPY, Venturini and VEEV, were added to the cells (25 μl/well). Four Virus Control (VC) wells (per virus) were supplemented with 25 μl medium containing 0.1% DMSO and four cell control wells were supplemented with 50 μl of medium. Fifteen minutes later, 25 μl of a virus mix containing the appropriate amount of viral stock diluted in medium was added to the 96-well plates. Cells were cultivated for 2 days after which 100 μl of the supernatant was collected and transferred to 96 square well plates preloaded with 400 μl of RAV-1 Lysis buffer from Macherey Nagel "NucleoSpin 96 virus kit". Viral RNA was extracted on a Qiacube HT automat using Cador Pathogen 96 HT kit (Qiagen), and quantified by real time RT-PCR to quantify the amount of viral RNA, which is representative for the amount of progeny virions that are produced (SuperScript III Platinium one-step RT-PCR with Rox from Invitrogen). The four control wells were replaced by four 2-log dilutions of an appropriate T7-generated RNA standards. IC_{50} were determined by fitting a sigmoïdal curve on values of virus yield inhibition (in %) versus the log values of drug concentration (Kaleidagraph software).

2.4. Cytotoxicity assays

2.4.1. Cytotoxicity assay on vero cells

The cytotoxic and cytostatic effect of the compounds was evaluated in uninfected cells by means of the MTS/PMS method. Vero cells were seeded in 96-well tissue culture plates (Becton Dickinson, Aalst, Belgium) at a density of 2.5×10^4 cells/well in 100 μl 2% FBS medium and were allowed to adhere overnight. Next, a compound dilution series was prepared in the medium on top of the cells. Following 5 days of incubation, the plates were processed using the MTS/PMS method as described by the manufacturer (Promega, The Netherlands). The 50% cytostatic/cytotoxic concentration (CC_{50}; i.e. the concentration of compound that reduces the overall metabolic activity of the cells by 50%) was calculated using logarithmic interpolation. All assay wells were checked microscopically for alterations of the cell or monolayer morphology.

2.4.2. Cytotoxicity on vero E6 cells

Cytotoxicities were assayed by measuring the cell viabilities in the same culture settings along antiviral activities in parallel P96 wells plates. Two fold serial dilutions, starting 100 μM, in duplicates or triplicates, were added in 25 μl–100 μl of Vero E6 cells that have been plated the day before. At day two, the medium was removed and replaced by 70 μl of fresh medium containing 10 μl of CellTiter blue reagent (Promega) and incubated for 90 min at 37 °C. Cell viabilities were measured as resorufin fluorescence readings on a plate reader (Tecan Infinite M200 Pro).

2.5. Inhibition assays of nsP1 guanylylation

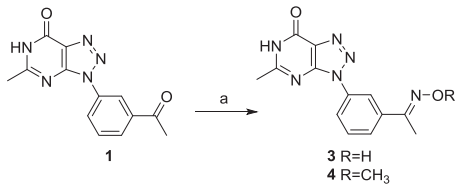
VEEV nsP1 (strain P676, (GenBank accession number L04653.1) amino acid 1 to 535) with a C-terminal 6-His tag was produced and purified following a previously described protocol (Li et al., 2015). The protein was quantified using UV absorbance at 280 nm, and purity was checked on Coomassie blue stained SDS PAGE gel prior the enzymatic assay. The VEEV nsP1 guanylylation reactions were performed in 20 μL buffer containing 50 mM Tris (pH 7.0), 2 mM $MgCl_2$, 2 mM DTT, 10 μM m^7GTP, 100 μM AdoHcy and 2 μM nsP1, with an increasing concentrations of compounds (from 0 to 1 mM) and incubated 1 h at 30 °C. The guanylylation was detected by Western Blot using an anti-m^3G/m^7G-cap monoclonal antibody (Synaptic Systems) and quantified using ImageJ software (Delang et al., 2016).

3. Results and discussion

3.1. Synthesis of new 3-aryl-[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones derivatives

Reaction of the ketone **1** with hydroxylamime hydrochloride or methoxyamine hydrochloride in ethanol afforded the oximes **3** and **4** in 76 and 77% yields, respectively. Compound **4** kept significant anti-CHIKV activity, so the synthesis of the 5-ethyl analogues of the ketone **1** and the methoxyamine **4** was addressed, as shown in Scheme 1. The 3-azidophenylethanone **5** (Chakraborty et al., 2010) reacted with methoxyamine hydrochloride under MW irradiation to afford the methoxime **6** in 88% yield. Reaction of this azide with cyanoacetamide in the presence of NaH in DMF afforded the 5-aminotriazol **7** in 79% yield. Further reaction of **7** with ethyl propionate in dioxane in the presence of ^{t}BuOK under MW irradiation afforded the triazolopyrimidine **8** in 72% yield. It should be mentioned that, when the reaction between the aminotriazol **7** and ethylpropionate was performed in the presence of EtONa in EtOH, as described for the reaction of other aminocarboxamides with esters (Miyashita et al., 1996), besides the cyclized product **8**, a significant proportion of the Dimroth isomer of the staring material was detected (Gigante et al., 2014). Hydrolysis of **8** under strong acidic conditions provided the ketone derivative **9** with an ethyl at position 5 of the base.

The results obtained in our previous series (Gigante et al., 2014) have shown that substituents larger than an ethyl at position 5 of the triazolopyrimidine resulted in a drop of the anti-CHIKV activity. Here we have explored a subset of substitutions for which the steric properties are similar to those of the ethyl group (Scheme 2). Thus,



[a] Reagents and conditions: (a) NH_2OR, EtOH, MW, 80 ºC, 1 h (**3**, 76% yield and **4**,77% yield).

Scheme 1. Synthesis of oxime derivatives on the methylketone **1**.[a] Reagents and conditions: (a) NH_2OR, EtOH, MW, 80 °C, 1 h (**3**, 76% yield and **4**,77% yield).

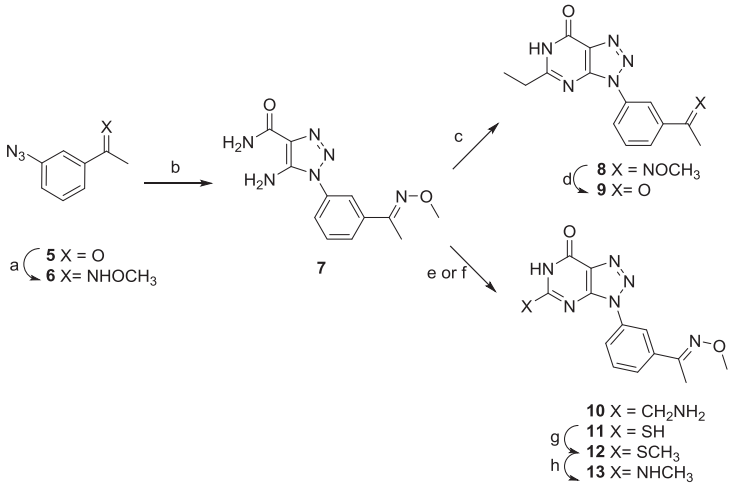
reaction of the 5-amino-4-carboxamide **7** with *N*-Boc-Gly-OMe in the presence of ^{t}BuOK followed by Boc removal by treatment with TFA afforded the aminomethylen derivative **10** in 46% yield for the two steps. On the other hand, reaction of **7** with CS_2 in the presence of ^{t}BuOK afforded the 5-mercapto derivative **11** that was further treated with methyl iodide at rt to provide the 5-methylthio derivative **12** in 60% yield. Additionally, reaction of **12** with $MeNH_2$ in MeOH under MW irradiation at 150 °C afforded the 5-methylamino derivative **13** in 38% yield.

Despite the interesting anti-CHIKV activity of the methoxime **8** (see Antiviral activity section), this compound exhibited poor water solubility. In an effort to overcome this drawback, the incorporation of different amines tethered to the oxygen of the oxime was envisioned. This strategy has been described as a valuable approach to improve water solubility in other oximes (Chen et al., 2011; Pergola et al., 2014; Vougogiannopoulou et al., 2008). In our case, an ethylene linker was employed for the incorporation of a variety of amines onto the oxime. Reaction of the methylketone **5** (Chakraborty et al., 2010) (Scheme 3) with hydroxylamine hydrochloride in ethanol under MW irradiation afforded the hydroximino derivative **14** in 90% yield, that further reacted with 1,2-dibromoethane in 10% NaOH/THF (1:1) in the presence of TBABr to afford the bromo derivative **15** in an excellent yield. Reaction of **15** with a variety of amines (methylpiperazine (**a**); morpholine (**b**); thiomorpholine (**c**); diethylamine (**d**); cyclohexylamine (**e**); piperidine (**f**); pirrolidine (**g**) or 4-dimethylaminopiperidine (**h**), Scheme 3) in DMF and in the presence of NaI afforded compounds **16a-h** in good to excellent yields. The oximes were obtained as pure or almost pure *E* isomers. Reaction of **16a-h** with cyanoacetamide in the presence of NaH in DMF led to the carboxamides **17a-h**, that were further treated with ethyl propionate in the presence of tBuOK to yield the final triazolopyrimidines **18a-h**.

3.2. Biological results

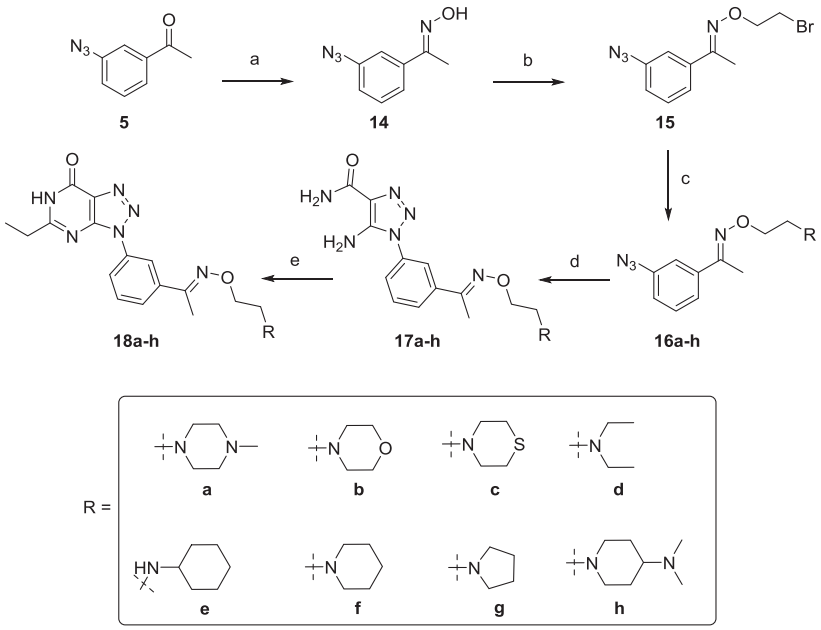
3.2.1. Evaluation of anti-CHIKV activity and cytotoxicity

The synthesized compounds were evaluated for their potential to inhibit the CHIKV-induced cytopathogenic effect (CPE) in Vero cells (Table 1). Dose-response curves for the most potent compounds are provided as Supplementary Fig. S1. Chloroquine and our previously reported hits (compounds **1** and **2**) were included as reference compounds. The antiviral activity is expressed as the 50% effective concentration (EC_{50}) and the 90% effective concentration (EC_{90}), indicating the concentration of compound required to



[a] Reagents and conditions: (a) $NH_2OCH_3 \cdot HCl$, EtOH, MW, 80 ºC, 80 min (**6**, 88% yield); (b) cyanoacetamide, NaH 60%, DMF, 0 ºC to rt, 1 h (**7**, 79% yield; (c) $EtCO_2Et$, ^{t}BuOK, dioxane, MW, 100 ºC, 1 h (**8**, 72% yield); (d) HCl 6N, dioxane, MW, 120 ºC, 1 h (**9**, 33% yield); (e) i) *N*-Boc-Gly-OMe, ^{t}BuOK, THF, MW, 60 ºC, 2 h; ii) TFA, DCM, rt, 15 min, (**10**, 46% yield (two steps)); (f) CS_2, ^{t}BuOK, THF, MW, 120 ºC, 2 h, (**11**, 86% yield); (g) CH_3I, DMF, rt, 30 min, (**12**, 60% yield); (h) CH_3NH_2 2N, MeOH, DMF, MW, 150 ºC, 1 h, (**13**, 38% yield).

Scheme 2. Synthesis of the 5-Ethyl Triazolopyrimidin-7-ones **8**–**13**.[a] Reagents and conditions: (a) $NH_2OCH_3 \cdot HCl$, EtOH, MW, 80 °C, 80 min (**6**, 88% yield); (b) cyanoacetamide, NaH 60%, DMF, 0 °C to rt, 1 h (**7**, 79% yield; (c) $EtCO_2Et$, ^{t}BuOK, dioxane, MW, 100 °C, 1 h (**8**, 72% yield); (d) HCl 6N, dioxane, MW, 120 °C, 1 h (**9**, 33% yield); (e) i) *N*-Boc-Gly-OMe, ^{t}BuOK, THF, MW, 60 °C, 2 h; ii) TFA, DCM, rt, 15 min, (**10**, 46% yield (two steps)); (f) CS_2, ^{t}BuOK, THF, MW, 120 °C, 2 h, (**11**, 86% yield); (g) CH_3I, DMF, rt, 30 min, (**12**, 60% yield); (h) CH_3NH_2 2N, MeOH, DMF, MW, 150 °C, 1 h, (**13**, 38% yield).



[a] Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, EtOH, MW, 80 ºC, 80 min (**14**, 90% yield); (b) $Br(CH_2)_2Br$, TBABr, NaOH 10% aq, THF/H_2O, rt, 3 h (**15**, 90% yield); (c) Amine, NaI, DMF, 80 ºC, 3-16 h (71-98% yield); (d) Cyanoacetamide, NaH 60%, DMF, 0 ºC to rt, 1 h (66-90% yield); (e) $EtCO_2Et$, ^{t}BuOK, dioxane, MW, 100 ºC, 1 h, (52-87% yield).

Scheme 3. Synthesis of the aminoalkyloximes **18a-h**.[a] Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, EtOH, MW, 80 °C, 80 min (**14**, 90% yield); (b) $Br(CH_2)_2Br$, TBABr, NaOH 10% aq, THF/H_2O, rt, 3 h (**15**, 90% yield); (c) Amine, NaI, DMF, 80 °C, 3–16 h (71–98% yield); (d) Cyanoacetamide, NaH 60%, DMF, 0 °C to rt, 1 h (66–90% yield); (e) $EtCO_2Et$, ^{t}BuOK, dioxane, MW, 100 °C, 1 h, (52–87% yield).

Table 1
Antiviral evaluation of the triazolopyrimidines against CHIKV strain 899 in Vero cells.

Compound	EC_{50} (μM) ± SD [a]	EC_{90} (μM) ± SD [b]	CC_{50} (μM) ± SD[c]
1[d]	19 ± 2	38 ± 16	>743
2[d]	2.6 ± 1.0	8.8 ± 6.0	>668
3	69 ± 23	96 ± 36	>704
4	21 ± 9	92 ± 54	>670
8	3.6 ± 1.3	5.3 ± 0.9	101 ± 73
9	27 ± 13	>350	>1000
10	>549	>549	>549
11	>126	>126	>126
12	>24	>24	>24
13	26 ± 3	>160	>160
18a	58 ± 2	151 ± 77	380 ± 29
18b	12 ± 1	20 ± 1	>729
18c	5.2 ± 0.5	9.2 ± 2.7	162 ± 1
18d	16 ± 6	24 ± 9	165 ± 8
18e	135 ± 46	>236	383 ± 269
18f	6.9 ± 2	11 ± 4	95 ± 40
18g	16 ± 1.8	21 ± 1.8	166 ± 26
18h	>424	>424	424 ± 32
Chloroquine	11 ± 7	21 ± 18	89 ± 28

All data are mean values ± standard deviation for at least three independent experiments.
[a] 50% effective concentration or concentration required to protect 50% of the cells against the cytopathic effect of the virus.
[b] 90% effective concentration or concentration required to protect 90% of the cells against the cytopathic effect of the virus.
[c] 50% cytotoxic concentration.
[d] Data as reported in (Gigante et al., 2014).

inhibit the virus-induced CPE by 50 and 90%, respectively. The effect of the compounds on non-infected cells is expressed as CC_{50}, which corresponds to the calculated concentration of compound that reduces the metabolic activity of compound-treated cells by 50%.

Compounds **3, 4, 8** and **9** resulted in significant anti-CHIKV activity at non-toxic concentrations. The best results were obtained with the methoxyimino derivative **8** that showed an EC_{50} value of 3.6 ± 1.3 μM and an EC_{90} value of 5.3 ± 0.9 μM. However, when the ethyl group at position 5 of the triazolopyrimidine in **8** was replaced by a CH_2NH_2 (**10**), SH (**11**), or a SCH_3 (**12**), the antiviral activity was completely lost. The only exception in this subgroup was compound **13** (X = $NHCH_3$) although it was at least 6-fold less

Table 2
Antiviral evaluation of selected triazolopyrimidines against different laboratory strains and clinical isolates of CHIKV, VEEV, SFV and SINV in Vero and Vero E6 cells.

Species	Virus (strain)	EC_{50} (μM) ± SD		
		8	18b	18f
CHIKV	*899 (lab)*[a]	3.6 ± 1.3	12 ± 1	6.9 ± 1.5
	Venturini (Italy 2008)[b]	5.2 ± 0.2	6.7	9.6 ± 1.2
	Venturini (Italy 2008)[a]		4.4	9.8
	Congo 95 (2011)[b]	1.1 ± 0.1	2.5	3 ± 0.5
	St. Martin 20235 (2013)[b]	4.2 ± 0.8		14 ± 3.5
VEEV	*TC83*[b]	ND	~22	>20
SINV	*HRsp (lab)*[a]	>101	>243	22 ± 3
SFV	*Vietnam (lab)*[a]	>101	>243	95 ± 40

VEEV: Venezuelan equine encephalitis virus; SINV: Sindbis virus; SFV: Semliki forest virus; data shown are average values ± SD of at least two independent experiments. CC_{50} values for **8**, **18b** and **18f** on Vero cells are >300 μM, >100 μM and >100 μM, respectively.
[a] EC_{50} value determined by CPE-inhibition assay.
[b] EC_{50} value determined by qRT-PCR.

Table 3
Inhibition of VEEV nsP1 guanylylation as detected by Western Blot using an anti-cap antibody.

Compound	IC_{50} (μM)[a]	Compound	IC_{50} (μM)
1	21	**18d**	16
2	10	**18f**	<2
8	5.8	**18g**	3.2
18b	10		

[a] The intensity of the signal was quantified using ImageJ software and the IC_{50} was defined as the concentration of compound for which the intensity of the signal is decreased by 50%. Gels of these experiments are shown in Supplementary Fig. S3.

active than **8**. This pointed to an important role for the ethyl group at position 5 of the triazolopyrimidine for the anti-CHIKV activity not only in terms of steric properties, as previously reported (Gigante et al., 2014), but also in terms of electronic properties according to these data.

Most of the aminoalkyloximes **18a-h** exerted significant anti-CHIKV activity with EC_{50} values in the range of 5–20 μM. Compound **18c**, with a thiomorpholine at the ethyl chain, and compound **18f**, with a piperidine substituent, had the best EC_{50} and EC_{90} values in this series. Moreover, compound **18b** with a distal morpholine showed the best selectivity index (>60).

3.2.2. Antiviral activity against different CHIKV clinical isolates and other alphaviruses

Compounds **8**, **18b** and **18f** were evaluated for antiviral activity against several CHIKV clinical isolates (Table 2) in Vero E6 cells using virus yield reduction assays quantified by real-time RT-PCR or virus-induced cell-killing measured by a cell viability assay (dose-response curves are provided as Supplementary Fig. S2). The three compounds showed antiviral activity against isolates obtained from outbreaks in Italy (Venturini 2008), Africa (Congo 2011) and the Caribbean Americas (St Martin 2013) with EC_{50} values in the low μM range. It should be highlighted that they were particularly active against the African Congo strain (i.e. EC_{50}= 1.1 ± 0.1 μM for **8**). In addition, no cytotoxicity up to 100 μM was observed in Vero E6 cells using the same experimental settings as the antiviral assay. Because the antiviral assays were based on two different methods in two laboratories, we therefore concluded that these compounds were specific inhibitors of Chikungunya virus replication.

These compounds did not provide significant protection against the replication of other alphaviruses with the only exception of compound **18f** that showed some activity against SINV and compound **18b** that resulted in modest antiviral activity against VEEV (Table 2).

3.2.3. nsP1 evaluation

As mentioned in the introduction, we have recently reported that compound **1** is able to inhibit the guanylation step catalyzed by VEEV nsP1 (Delang et al., 2016), strongly suggesting that this capping enzyme could be a target for this class of compounds. Therefore it was important to determine the characteristics of the new series of compounds in the guanylylation step catalyzed by VEEV nsP1, as the recombinant CHIKV nsP1 does not display any guanylylation activity in our assay (Li et al., 2015). The guanylyltransfer of mGTP on VEEV nsP1 was assessed in presence of increasing concentrations of selected compounds and determined by Western blot (see Supplementary Fig. S3). As shown in Table 3, the oximes **8**, **18b**, **18d**, **18f** and **18g** were able to inhibit the guanylylation of VEEV nsP1 with IC_{50} values in the μM range. . Particularly relevant in this assay were compounds **8**, **18f** and **18g**, with IC_{50} values significantly lower than that of the hit compound **1**. However, it was quite surprising that despite inhibiting VEEV nsP1, these compounds did not or only modestly show antiviral activity against VEEV replication in cell culture.

Moreover, we have already shown (Delang et al., 2016) that the amino acid at position 34 in VEEV nsP1 is relevant for the inhibitory activity of these triazolopyrimidines. Recombinant D34S VEEV nsP1 maintains the GT activity but the inhibitory activity of the triazolopyrimidines, exemplified by compound **2**, was significantly alleviated. The N-terminal region of nsP1 where the resistance mutation occurred is highly conserved and although no structural model can be proposed, secondary structures and catalytic residues have been mapped and correspond to common features to both VEEV and CHIKV nsP1 (Ahola and Karlin, 2015). Thus, the folding of both nsP1 should be comparable but to date there is no mean to assess local structural differences. Therefore we cannot directly correlate the relative differences between inhibition on VEEV nsP1 and CHIKV antiviral effects. Still, the data here presented with the new series of compounds are in agreement with those obtained for our original hit **1**, for which we presented evidences showing that nsP1 could be the target.

4. Conclusions

We previously reported on the identification of 3-aryl-[1,2,3]triazolo[4,5-*d*]pyrimidin-7(6*H*)-ones as selective anti-CHIKV agents, exemplified by compound **1**, and further studied the mechanism of action that involves the viral mRNA capping machinery through nsP1. Here we have addressed the synthesis and evaluation of novel analogues within this series of compounds thereby further dissecting the structural requirements for inhibition of CHIKV replication. The data obtained stress the exigent structural requirements at position 5 of the triazolopyrimdine portion so that both steric and electronic properties are very important. On the other hand, the new synthesized oximes (exemplified by compounds **8**, **18b** or **18f**) clearly show that the functionalization of the methyl ketone at position 3 of the aryl ring through oxime derivatization and incorporation of distal amines improves the antiviral activity against CHIKV. The data obtained in the nsP1 guanylylation assay provide also evidence that these oximes are more potent inhibitors of the guanylylation step than the initial hit **1**. Further work is required to understand why the inhibition of VEEV nsP1 is accompanied with no or only modest antiviral activity against VEEV in cell culture. Still, these triazolopyrimidines represent, to the best of our knowledge, the first compounds able to inhibit the guanylylation reaction in alphavirus mRNA capping.

Author contributions

Alba Gigante and Asier Gómez-SanJuan contributed equally to this work.

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Abbreviations

CC_{50}	50% cytotoxic concentration or concentration that reduces the overall metabolic activity of uninfected cells to 50%
CHIKV	chikungunya virus
CPE	cytopathic effect
EC_{50}	50% effective concentration
EC_{90}	90% effective concentration
GT	guanylyl-transfer
IC_{50}	50% inhibition concentration
nsP1	non-structural protein 1
SFV	Semliki Forest virus
SINV	Sindbis virus
VEEV	Venezuelan equine encephalitis virus

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2017.06.003.

The following is the supplementary data related to this article:

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