Approved drugs screening against the nsP1 capping enzyme of Venezuelan equine encephalitis virus using an immuno-based assay

Ana S. Ferreira-Ramos[a], Changqing Li[a], Cécilia Eydoux[a], Jean Marie Contreras[b], Christophe Morice[b], Gilles Quérat[c], Alba Gigante[d], María-Jesús Pérez Pérez[d], Marie-Louise Jung[b], Bruno Canard[a], Jean-Claude Guillemot[a], Etienne Decroly[a,1], Bruno Coutard[a,c,*,1]

[a] Aix Marseille Université, CNRS, AFMB UMR 7257, Marseille, France
[b] Prestwick Chemical, ILLKIRCH, Strasbourg, France
[c] Unité des Virus Emergents (UVE: Aix-Marseille Univ-IRD 190-Inserm 1207-IHU Méditerranée Infection), Marseille, France
[d] Instituto de Química Médica (IQM, CSIC), Madrid, Spain

ARTICLE INFO

ABSTRACT

Alphaviruses such as the Venezuelan equine encephalitis virus (VEEV) are important human emerging pathogens transmitted by mosquitoes. They possess a unique viral mRNA capping mechanism catalyzed by the viral non-structural protein nsP1, which is essential for virus replication. The alphaviruses capping starts by the methylation of a GTP molecule by the N7-guanine methyltransferase (MTase) activity; nsP1 then forms a covalent link with m^7GMP releasing pyrophosphate (GT reaction) and the m^7GMP is next transferred onto the 5′-diphosphate end of the viral mRNA to form a cap-0 structure. The cap-0 structure decreases the detection of foreign viral RNAs, prevents RNA degradation by cellular exonucleases, and promotes viral RNA translation into proteins. Additionally, reverse-genetic studies have demonstrated that viruses mutated in nsP1 catalytic residues are both impaired towards replication and attenuated. The nsP1 protein is thus considered an attractive antiviral target for drug discovery. We have previously demonstrated that the guanylylation of VEEV nsP1 can be monitored by Western blot analysis using an antibody recognizing the cap structure. In this study, we developed a high throughput ELISA screening assay to monitor the GT reaction through m^7GMP-nsP1 adduct quantitation. This assay was validated using known nsP1 inhibitors before screening 1220 approved compounds. 18 compounds inhibiting the nsP1 guanylylation were identified, and their IC_{50} determined. Compounds from two series were further characterized and shown to inhibit the nsP1 MTase activity. Conversely, these compounds barely inhibited a cellular MTase demonstrating their specificity towards nsP1. Analogues search and SAR were also initiated to identify the active pharmacophore features. Altogether the results show that this HT enzyme-based assay is a convenient way to select potent and specific hit compounds targeting the viral mRNA capping of Alphaviruses.

1. Introduction

Emergence or re-emergence of alphaviruses represents a serious health concern, as exemplified by the worldwide epidemics of Chikungunya virus in recent years (Charrel et al., 2014). *Alphavirus* genus comprises 31 species among which at least ten of the arthropod-borne alphaviruses are important in terms of public health (Gould et al., 2010). Alphaviruses can be organised into two major groups based on their geographical distribution: Alphaviruses from the Old World (OW), most commonly causing febrile illness and painful arthralgia or polyarthralgia and New World (NW) alphaviruses mostly leading to neurological diseases. Among NW alphaviruses, Venezuelan equine encephalitis virus (VEEV) is an important pathogen present in the Americas from Texas to Argentina (Adams et al., 2012; Pisano et al., 2013). In 1995, an outbreak in Venezuela and Colombia resulted in roughly 100,000 human cases including more than 300 fatal encephalitis cases (Rivas et al., 1997). Other epidemics were also reported indicating that VEEV represents a real public health concern. Moreover, VEEV infection symptoms resemble those of Dengue fever, likely leading to an underestimation of the number of cases related to this

virus infection in regions where both Dengue virus and VEEV are endemic (Aguilar et al., 2011). In humans, whilst the overall mortality rate is low (< 1%), neurological disease, including disorientation, ataxia, mental depression, and convulsions can be detected in up to 14% of infected individuals, especially in children (Johnson and Martin, 1974).

Alphaviruses belong to the positive stranded RNA viruses replicating their genome in the cytoplasm of host cells. The genome contains two open reading frames (ORFs), encoding for the non-structural proteins (nsPs) and the structural proteins, which are expressed later from the sub-genomic RNA. Following viral entry, the genomic RNA is translated into the non-structural polyprotein P123 and P1234 which are processed into non-structural proteins (nsP1 to nsP4). The non-structural polyproteins and proteins form a complex associated to the plasma membranes, and this complex is embedding the enzymatic activities necessary for the viral replication and transcription. Namely, the nsP4 carries the RNA dependent RNA polymerase activity and drives antigenome synthesis and its subsequent transcription into genomic and sub-genomic mRNAs. The 5′ ends of the genomic and subgenomic RNAs are decorated by a cap-0 structure (m^{7}G-pppN-RNA) and RNA are polyadenylated at their 3′ extremity. The capping of viral mRNA is a key step for virus replication as cap structures protect viral RNA from cellular 5′-3′ exonucleases and promote the initiation of RNA translation into viral proteins. In addition, the cap structure, together with conserved hairpin structure present in the 5′UTR, also hides viral RNA from detection by sensors of the innate immunity, such as RIG-I and/or MDA5 or by antiviral restriction factors (Hyde et al., 2014). The cap structure of alphavirus mRNA is thought to be synthetized by an unconventional capping mechanism involving the methyltransferase/guanylyltransferase activities of nsP1 and the 5′ RNA triphosphatase activity of nsP2 (Li et al., 2015; Vasiljeva et al., 2000). Reverse genetic experiments have confirmed that these activities are essential for the viral life cycle. Indeed, the mutation of either MTase or nsP1 guanylylation catalytic residues strongly impairs viral replication (Wang et al., 1996). Capping enzymes are thus considered as promising antiviral drug targets.

Despite important efforts, antiviral drugs against alphaviruses such as VEEV are still lacking and to date, the treatment of VEEV infections is mainly supportive. For instance, the broad spectrum antiviral ribavirin, that was found to be efficient against Chikungunya virus (CHIKV) in resolving joint and soft tissue swelling (Ravichandran and Manian, 2008), has almost no significant activity against VEEV in infected cell cultures (Markland et al., 2000). Therefore, efforts to discover molecules inhibiting alphaviruses such as VEEV have increased in the last years. Small molecule library screenings were recently developed on cell based assays to discover compounds active against VEEV or other alphaviruses (Chung et al., 2013) (Pohjala et al., 2011; Spurgers et al., 2013) (Delekta et al., 2015; Lucas-Hourani et al., 2014; Seyedi et al., 2016; Varghese et al., 2016). Several compounds were found to be associated to the inhibition of functions carried out by the proteins constituting the viral replication complex. Among the selected compounds, the broad-spectrum antiviral candidate Favipiravir (T-705), initially developed to treat human influenza, shows a potent antiviral effect in small animal models (Furuta et al., 2013). This nucleobase mimetic was proposed to target the polymerase activity of nsP4 after intracellular activation into its 5′-triphosphate ribonucleotide form (Delang et al., 2014). In addition, nucleoside or non-nucleoside analogues have revealed antiviral effects supposedly by targeting nsP2 or nsP4 (Chung et al., 2013, 2014; Urakova et al., 2017; Wada et al., 2017). nsP1 is also considered as an antiviral target: the recent identification of compounds inhibiting the mRNA nsP1 mediated-capping (Delang et al., 2016; Feibelman et al., 2018; Gigante et al., 2014; Gomez-SanJuan et al., 2018) sheds light on this viral enzyme. Screening strategies based on the inhibition of alphavirus nsP1 enzymatic activities can thus be considered for drug discovery.

The enzymatic activities of VEEV nsP1 involved in the formation of the viral mRNA cap-0 structure were previously characterized (Li et al., 2015). NsP1 first methylates a GTP molecule into its guanine N7 position (MTase activity). The methylation reaction requires the S-adenosylmethionine (SAM) as methyl donor and generates S-adenosylhomocysteine (SAH) as by-product. The m^{7}GTP product is next used for nsP1 guanylylation (GT reaction) leading to the formation of an m^{7}GMP-nsP1 covalent adduct. m^{7}GMP forms a covalent link with a catalytic histidine of VEEV nsP1. The formation of guanylylated-nsP1 adduct is regulated by the SAM/SAH balance, and the release of SAH by the MTase reaction stimulates the GT reaction. Finally, the m^{7}GMP molecule is transferred onto the 5′-diphosphate RNA end to yield the capped RNA (GTase reaction). In order to characterize nsP1 functions as well as compounds inhibiting nsP1 we developed a Western blot assay to detect and quantify the guanylylated nsP1. However, this labor intensive assay is not suitable for high throughput screening of guanylylation inhibitors.

In this work, we developed an assay in order to uncouple the capping reactions carried by VEEV nsP1 and to specifically quantify the synthesis of m^{7}GMP-nsP1 adduct using ELISA. After validation of the assay using known nsP1 inhibitors, we screened a library containing 1220 approved drugs. The best compounds were selected at 50 μM and further characterized. We determined their IC$_{50}$ on both the nsP1-mediated MTase and GT reactions. In addition, the specificity of the inhibitors was assessed comparatively using a cellular MTase and viral MTase involved in capping process. Compounds analogues and/or fragments available from commercial libraries were also tested in order to better understand the structure-activity relationships of molecules inhibiting nsP1 activities.

2. Material and methods

Chemicals. Chemicals such as GTP, m^{7}GTP, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) were purchased from Sigma Aldrich. The Prestwick Chemical Library (PCL) is a library of 1280 small molecules, mostly approved drugs (FDA, EMA and other agencies) from which 1220 compounds were picked for the screening (Strasbourg, France). Other compounds used in this study are commercially available and were purchased from Toronto Research Chemicals (TRC), Key Organics, Enamine, Chembridge, Combi Blocks, Enzo Life Science, Sigma Aldrich, Tocris and Alfa Aesar. The synthesis of compounds MADTP-314 (3-(3-acetylphenyl)-5-methyl-3,6-dihydro-7H-[1,2,3]triazolo[4,5-d]pyrimidin-7-one) and MADTP-393 (3-(3-acetylphenyl)-5-ethyl-3,6-dihydro-7H-[1,2,3]triazolo[4,5-d]pyrimidin-7-one) has already been described (Gigante et al., 2014, 2017). The FDA-approved compounds and the compounds purchased from other suppliers were dissolved in DMSO at a stock concentration of 20 mM.

2.1. Expression and purification of VEEV nsP1 wild-type (wt) and mutant D34S

The codon optimized DNA encoding nsP1 of VEEV (strain P676, amino acid 1 to 535) was cloned into the expression vector pET28b (Novagen) to enable the fusion of a 6xHis tag at the C-terminus of nsP1. The protein was produced in *E. coli* Rosetta pLysS (DE3) (Novagen) cells and purified by IMAC chromatography on a 5 ml His TrapFF crude column (GE Healthcare). The proteins eluted 250 mM imidazole, 20 mM tris pH 7.5, 100 mM NaCl and 5% glycerol were dialysed in a dialysis buffer with the following composition; 20 mM Tris pH 7.5, 100 mM NaCl and 10% Glycerol. The protein purity was assessed on Coomassie blue stained SDS-PAGE gel and protein concentration was determined by Bradford protein assay (Bio Rad). The same procedure was performed for D34S nsP1.

2.2. Methyltransferase filter binding assay

The methyltransferase assays were carried out in a 20 μL reaction

mixture, containing 50 mM Tris (pH 7.0), 2 mM DTT, 10 mM KCl, 2 mM GIDP, 330 nM S-adenosyl [methyl-^{3}H] Methionine (83.1 Ci/mmol, PerkinElmer), 10 μM S-adenosylmethionine, 2 μM VEEV nsP1 and increasing concentrations of inhibitors with 5% final concentration of DMSO. After incubation at 30 °C for 1 h, the reaction samples were loaded on DEAE-cellulose filter (PerkinElmer), and the filter was washed twice with 20 mM ammonium formate, once with H$_2$O, and once with absolute ethanol. The filter was dried, and the radioactivity was measured by scintillation counting with SCINT BETAPLATE solution in a Wallac MicroBeta Trilux 1450 counter (PerkinElmer). Compounds were tested in triplicate at a final concentration of 50 μM for single point analysis.

2.3. Guanylylation assay and detection of the m^{7}GMP-nsP1 adduct by ELISA

The guanylylation reaction (GT) of nsP1 VEEV was performed in 20 μL as previously described (Li et al., 2015). Briefly, 5 μM of nsP1 was incubated at 30 °C in a buffer containing 20 mM HEPES (pH 7.0), 2 mM MgCl$_2$, 1 mM DTT, 100 μM m^{7}GMP, 100 μM S-Adenosylhomocysteine (SAH). Time course experiments using 1 μM, 2.5 μM and 5 μM of nsP1 were performed to optimize the incubation time and enzyme concentration for the ELISA assay. The screening of GT inhibitors was performed in a buffer containing 100 μM m^{7}GTP, 100 μM SAH, 5 μM nsP1, and the reaction were incubated during 30 min at 30 °C. Reactions with DMSO but without compound were used as negative control (NG) of inhibition and, as background control (BG) were used reactions without compound and m^{7}GTP. For the validation of the assay, 5 μM of enzyme and 1 h incubation of the reaction at 30 °C were selected, speculating that a high signal at OD$_{430nm}$ and a complete reaction would correspond to discriminating and stringent conditions suitable for the selection of the most potent inhibitors.

The ELISA detection of nsP-1 m^{7}GMP adducts was performed as follow: 8 μL of the GT reaction were transferred into 92 μL of PBS (Phosphate buffered saline, Sigma #P3813) in Nunc MaxiSorp flat-bottom 96 well plate (eBioscience). The plates were incubated overnight at 4 °C to allow nsP1 capture before plates washing (twice with PBS), and blocking with 2% (w/v) of non-fat milk in PBS for 2 h at 37 °C. The plate was then washed once with PBS, before incubation (1 h at RT) with 100 μL of primary antibody anti-m$_3$G/m^{7}G-cap monoclonal antibody (Synaptic Systems, Götingen, Germany) at a dilution of 1:1000 in PBS with 1% (w/v) of non-fat milk. The wells were washed three times with PBS-Tween, and three times with PBS solution before incubation with the peroxidase-conjugated rabbit anti-mouse antibody (Sigma, #A9044) diluted at 1: 2000 in PBS with 1% (w/v) of non-fat milk at RT for 1 h. The plate was then washed sequentially three times with PBS-Tween and three times with PBS. Finally, 3,3′,5,5′-Tetramethylbenzidine (TMB) solution was prepared by dissolving one 3,3′,5,5′-Tetramethylbenzidine tablet (Sigma T5525) in 100 μL of DMSO, which was then added into 9.9 mL of 0.1 M Sodium-acetate buffer, pH 6.0 with 2 μL of fresh 30% hydrogen peroxide. For each well, 100 μL TMB solution were added followed by incubate at 20 min at RT, then 50 μL of 2 M H$_2$SO$_4$ were added to stop the reaction, and then the absorbance of each well was read at 430 nm. Each 96-wells reaction and ELISA plate was used to test 80 compounds. The plates also contained two negative controls of the inhibition (reactions with no compound) and two controls for the background of the signal (reactions with no compound and no m^{7}GTP). For each plate, the controls were used to define the following values: **OD_{NG}** represents the mean value of the OD$_{430nm}$ measured for the duplicates for the negative control which is representative of 100% GT activity (reaction without compounds); **OD_{BG}** represents the mean value of the OD$_{430nm}$ measured for the duplicates for the background control which is representative of 0% GT activity (reaction without compounds and without m^{7}GTP).

The percentage of inhibition was calculated according to the following equation (1).

$$Y\ (\%) = \frac{OD_{NG} - OD_{[sample]}}{OD_{NG} - OD_{BG}} \times 100 \tag{1}$$

Where, **Y (%)** represents the inhibition percentage of GT activity mediated by nsP1, which is dose dependent of inhibitor concentration used; **OD_{sample}** represents the OD$_{430nm}$ measured from the sample.

2.4. Hit validation

The hit compounds, showing more than 80% of inhibition at 50 μM were selected for hit validation. The hit validation consisted in the re-determination of the percentage of inhibition in triplicates at 50 μM using fresh powders of compounds and in the determination of the IC$_{50}$ value for the compounds showing more than 80% of inhibition by Western blot (WB).

The IC$_{50}$ of the inhibitors was assessed by WB, starting from 500 μM or 100 μM concentration followed by nine 1:2 serial dilutions. For each inhibitor, a negative control for GT (without both m^{7}GTP and inhibitor) and positive control for GT (without inhibitor) were included. Reactions were prepared in a final volume of 20 μL, containing 20 mM HEPES pH 7.0, 2 mM MgCl$_2$, 1 mM DTT, 100 μM SAH, 100 μM m^{7}GTP, 2% DMSO and 5 μM of nsP1 enzyme and were incubated at 30 °C for 45 min. The reactions were stopped by adding 2.5 μL of 4x Laemmli Buffer followed by heat at 95 °C for 5 min prior to the loading in 8% (SDS-PAGE) acrylamide Tris-Glycine gels. Reactions were performed in independent duplicates. In order to improve the throughput of the detection by WB, the products of titration of four compounds were loaded on a single 8% acrylamide SDS PAGE gel. 7 μL of each reaction from a same titration were loaded on a gel prior 14 min of electrophoresis at 200 V. Negative and positive controls were loaded on the same gel with each titration for an appropriate quantification. The gel was then reloaded with second titration. The procedure was repeated for the third and fourth titrations. After the last loading the electrophoresis was performed for 30 min at 200 V. The Western blotting was done using the already described procedure (Li et al., 2015). After the signal acquisition of the WB, the PVDF membranes were stained during 3 min with Coomassie blue and the distained with 50% Ethanol (EtOH) during 5 min.

For each PVDF membrane, a BIP file was acquired with Kodak system, the file was converted in a TIF format that was used for analysis and quantification of the intensity (I) of the bands using ImageJ. After the quantification, the raw data was used to determine the percentage of activity for each compound, using the negative control for activity (NA) and the positive control for activity (MA) as references. The percentage of activity for each inhibitor concentration was determined using equation (2).

$$Y(\%) = \left[\frac{(I_{sample} - I_{NA})}{I_{MA} - I_{NA}} \right] \times 100 \tag{2}$$

Where, **Y (%)** represents the percentage of remaining guanylylation activity mediated by nsP1, which is dose dependent of inhibitor concentration used; **I_{sample}** is the intensity calculated from the band, of the sample, obtained through the signal acquisition of the WB, **I_{NA}** represents the average of the duplicates of the intensity calculated for the control representative of 0% guanylylation activity (reaction without compounds and without m^{7}GTP), **I_{MA}** represents the average of the duplicates of the intensity calculated for the control representative of 100% guanylylation activity (reaction without compound but with m^{7}GTP).

IC$_{50}$ values of selected inhibitors were determined with GraphPad Prism 6, using a non-linear regression dose response inhibition.

2.5. Analogues search and chemical group validation

The analogues search was first achieved through the internal drug approved library (The Prestwick Chemical Library). The second

analogue search was done using the commercial databases Reaxys and eMolecule. In a similar manner to the analogue search, we used the 4 initial hit compounds and from each compound we selected different chemical groups, which were used to search for close analogues at commercial databases. Some of these chemical groups and close analogues were commercially available and purchased for test against the guanylylation activity of nsP1.

3. Results

3.1. Monitoring the nsP1 guanylylation by ELISA

Alphavirus mRNA are capped by an unconventional mechanism in which nsP1 catalyses first methylation onto the N7 of GTP (MTase) followed by the formation of m^7GMP-nsP1 adduct (GT). Both MTase and GT reactions precede the transfer of m^7GMP onto the 5′-end of the ppRNA. In order to identify small molecules inhibiting specifically the GT activity of nsP1, we developed an assay detecting the synthesis of m^7GMP-nsP1 adduct. The GT reaction assay was performed with purified recombinant VEEV nsP1 expressed in *E. coli*. Since the purified nsP1 protein carries N7 MTase and GT activities, we tried to uncoupled the two reactions in order to specifically monitor the GT activity. We thus used m^7GTP (100 μM) instead of GTP as substrate and the GT reactions were performed in an optimized buffer supplemented with 2 mM $MgCl_2$ and 100 μM of SAH as both $MgCl_2$ and SAH have been shown to stimulate the nsP1 mediated-GT activity (Li et al., 2015).

After the GT reaction, the guanylylated-nsP1 was detected by ELISA assay. For this purpose, nsP1 was captured on an ELISA plate and the m^7GMP linked to nsP1 was next detected by the successive addition of an anti-m_3G/m^7G-cap antibody and peroxidase-conjugated rabbit anti-mouse antibody. Fig. 1A shows that using this assay, the m^7GMP-nsP1 covalent complex resulting from the GT reaction could be detected in presence of m^7GTP, SAH and nsP1. Conversely, no signal is detected in absence of SAH, m7GTP or nsP1, demonstrating the specificity of the ELISA detection system. We next determined the optimal experimental conditions for the screening. The time course experiment presented in Fig. 1B indicates that when using 5 μM of VEEV nsP1 the saturation phase is reached after 30–40 min of reaction whereas at lower nsP1 concentration (*i.e.* 1 and 2.5 μM) the reaction remains in a linear phase during the 30 min incubation period, but the ELISA signal is lower. Altogether these results indicate that the ELISA detection system provides a suitable tool to follow specifically the VEEV nsP1 mediated-GT reaction in a 96-well format.

The robustness of the GT assay was further assessed by testing already described VEEV nsP1 inhibitors for validation. In these control reactions, we used Sinefungin, a SAM analogue, which is known to inhibit viral and cellular MTases, as well as MADTP compounds, previously demonstrated to inhibit VEEV nsP1 mediated GT reaction

(Delang et al., 2016; Gigante et al., 2017). The inhibitory effect of these compounds was determined at 50 μM and the remaining GTase activity was normalized with the activity detected in absence of inhibitors. Sinefungin inhibits 47.9 ± 10.9% of the GT activity whereas MADTP-393 and MADTP-314 showed 99.2 ± 0.6% and 81.4 ± 2.8% of inhibition on the GT activity, respectively. The inhibition induced by MADTP-393, MADTP-314 and Sinefungin were further confirmed by determination of their IC_{50}. The GT reaction of VEEV nsP1 was performed in the presence of increasing concentration of inhibitor as previously described, and the amount of the m^7GMP-nsP1 complex produced was quantified by WB, using the anti m_3G/m^7G-cap antibody for detection. Fig. S1 shows that, after WB quantification and Hill plot curve fitting, Sinefungin, MADTP-393, and MADTP-314, inhibit the GT activity of VEEV nsP1 in a dose-dependent manner with IC_{50} of 96 μM, 69 μM and 177 μM, respectively. Of note, the IC_{50} determined for Sinefungin in the current study and the previous one differs (29 and 96 μM, respectively) likely because the reaction conditions slightly differ from the original assay (Li et al., 2015). Altogether these results indicate a correlation between inhibitions observed using 50 μM of inhibitors by ELISA assay, and IC_{50} determined by WB. The latter method was validated in a previous study (Li et al., 2015), confirming that the developed ELISA assay can be used to screen small molecules inhibiting specifically the VEEV nsP1 GT activity.

3.2. Screening of 1220 approved compounds inhibiting GT by ELISA

We next used the ELISA nsP1 GT assay to screen a library containing 1220 compounds from the Prestwick Chemical Library (PCL), a library containing approved drugs. The compounds were incubated with VEEV nsP1 at a final concentration of 50 μM and the GT reaction was started by addition of m^7GTP. After an incubation period of 30 min, the m^7GTP-nsP1 adduct was quantitated by ELISA assay and the % of inhibition of each compound was determined (Fig. 2A). The percentage of inhibition follows a right skewed bell-shaped distribution close to the normal distribution (Fig. 2B) with a mean inhibition "σ" of 16.5% close to the median (19.0%) ≈ Mode (20%) and a standard deviation (σ) of 26.8%. 229 compounds present a negative value, most of them yielding to a weak increase of GT activity. The compounds showing more than 40% activation were tested twice in order to determine if some compounds could efficiently stimulate nsP1 GT activity, but none of them showed reproducible activation of the enzyme. We selected 18 compounds presenting at least ~80% of inhibition among which 6 were considered as "strong inhibitors" as they showed more than 3σ above the average (> 96.9% of inhibition) (Table 1).

3.3. Analysis of the screen and hits confirmation

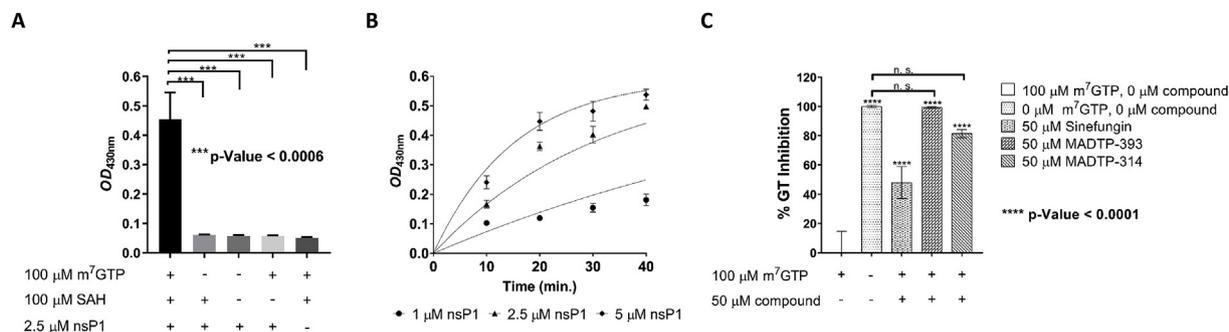We confirmed the initial screen results by determining the IC_{50}

Fig. 1. Setup, optimization and validation of the ELISA monitoring the GT activity of VEEV nsP1. Panel A: Setup of the assay. The m^7GTP-nsP1 complex is formed with 2.5 μM of VEEV nsP1 in the presence of 100 μM m^7GTP, and 100 μM SAH. Panel B: Time course experiment performed with intervals of 10 min and the last time point was collected at 40 min. Panel C: percentage of GT inhibition for selected compounds used as reference and tested at 50 μM, namely Sinefungin, MADTP-393 and MADTP-314.
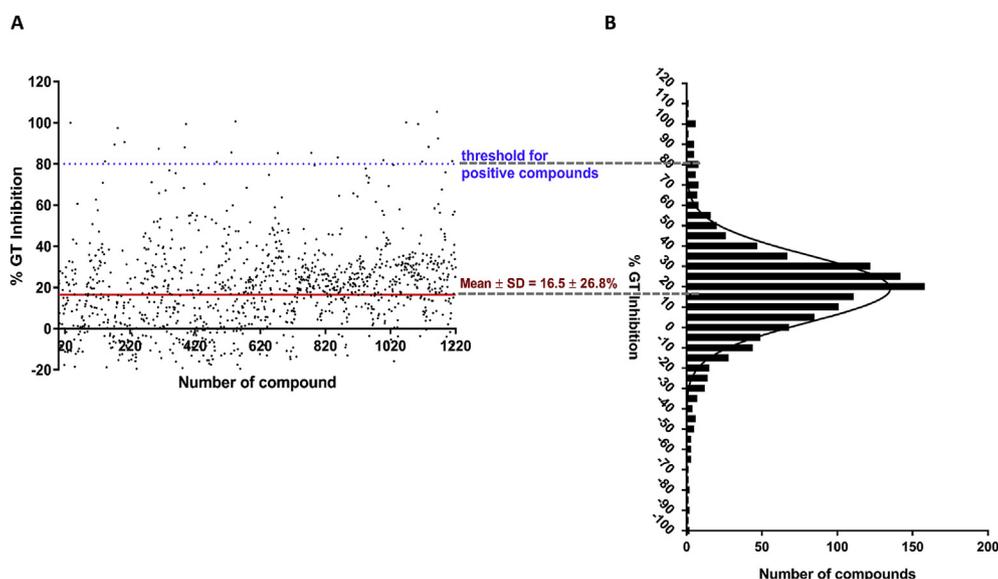
Fig. 2. Screening of approved drugs from Prestwick Chemical Library and hit selection. Panel A: Graph representing the GT inhibition of each tested compound. The average GT inhibition is presented in red and in blue the threshold (80% of GT inhibition) for the hit selection. Panel B: frequency histogram of the GT inhibition with a bin range of 10 and a Gaussian distribution curve fitting performed using GraphPad.

value using the anti-m$_3$G/m^{7}G-cap WB assay on fresh solutions from commercially available powders. The WB assay is more sensitive than the ELISA assay and presents the advantage to detect unambiguously the formation of m^{7}GMP-nsP1 complex with a low background. The limitation of this assay being the throughput, it was improved by serial loading of 4 titrations on a single SDS Page gel allowing the determination of IC$_{50}$ of 4 compounds in a single experiment (Fig. 3). The IC$_{50}$ value was determined for the selected compounds, except Prest-998 that was not available. 7 compounds display IC$_{50}$ values below 50 μM and 2 show IC$_{50}$ value between 50 and 110 μM (Table 1). The best compound corresponds to Prest-37 (pyrimethamine) and shows an IC$_{50}$ of 2.7 ± 0.4 μM. Interestingly, two other compounds, Prest-392 and Prest-531, both containing a 4-F-benzoylpiperidine, show IC$_{50}$ values in the 10 μM range, highlighting the accuracy of the assay to detect potent inhibitors. Based on the IC$_{50}$ and chemical nature of the compounds we arbitrarily decided to further investigate additional compounds based on the scaffold of Prest-37 (series 1) and that of Prest-392/Prest-531 (series 2).

We first searched for analogues already present in the screened library (PCL). Three compounds having structural and chemical similarities with Prest-37 were identified and their IC$_{50}$ for the GT reaction was determined. The 3 compounds (Prest-347, 858, and 947) share with Prest-37 an aminopyrimidine included in a fused bicyclic ring, and were demonstrated to inhibit the GT reaction less efficiently than the initial hit (Table 2), though. Interestingly, one of these compounds (Prest-858) was picked in the initial screen whereas the two others showed an inhibitory effect at 50 μM, but below the 80% threshold. Nine analogues of Prest-392, bearing a piperidine linked to an aromatic moiety through a flexible chain, were selected in the PCL to confirm the hit lead (Table 3). All of them inhibit more than 50% of the nsP1 GT activity at 50 μM, highlighting the robustness of the ELISA screening and suggesting the possible presence of active common pharmacophore (s) in the two series. IC$_{50}$ determined by WB showed that none of the compound has a higher potency than the original hit to inhibit nsP1 GT activity. Table 3 also shows that 3 compounds display a weak inhibition effect on the GT reaction (Prest-1117, 115, 1029), suggesting that the detection of m^{7}GMP-nsP1 adduct by ELISA might yield false positive results.

As the GT and the MTase reaction involve the same protein domain, we next tested if the selected GT inhibitors also inhibit the VEEV nsP1 MTase activity. The selected compounds were thus incubated with VEEV nsP1 in presence of radio-labelled SAM and the [3H] methyl transfer on the non-hydrolysable GTP (GIDP) was measured by DEAE

filter binding assay (FBA) (Tables 2 and 3). In this experimental condition uncoupling the MTase activity from the GT, the two heads of series, Prest-37 and Prest-392, display the best inhibitory effect on the MTase activity with 73.5 ± 2.86% and 79.3 ± 1.7% of inhibition at 50 μM, respectively. This analysis highlights the robustness of the inhibition observed for the two compounds and confirms the interplay between nsP1 mediated GT and MTase activities.

3.4. Hit/series validation beyond the screened library and pharmacophore evaluation of Prest-392

We next looked for analogues of Prest-37 and Prest-392 available in commercial libraries and their IC$_{50}$ for the GT reaction was determined by WB. 5 analogues of Prest-37 were purchased. None of them showed higher or equal inhibitory effect to limit the GT reaction (Table 2). However, the comparative analysis of the inhibitors suggests that the alkyl group (ethyl or methyl) in the 6 position of diaminopyrimidine ring may play a key role in the GT inhibition (Prest 37, E925220 and M338835 vs EN300-183279 and EN300-208764). Indeed, when the alkyl group is missing, the analogues are inactive. The nature of substitution on the phenyl group in the 5 position seems also to modulate the inhibition (Prest 37, E925220 and M338835).

For Prest-392, three commercial compounds were tested, among which Altanserin and Tocris-DV7028 show high chemical similarity (Table 3). The three compounds present pharmacophore similarities to Prest-392: a central piperidine, a bicycle diamide on one side and a phenyl ring on the other side. Altenserin, the direct thio analogue of Prest-392 has a higher to equivalent IC$_{50}$ on the GT activity (IC$_{50}$ = 9.3 ± 2.7) than the one of the head of series confirming the structure related activity of the lead compound. Tocris-DV7028 lacking the quinazoline-2,4-dione motif for the benefit of an unsaturated ring is inactive. This single modification compared to Prest-392 indicates a determinant role of this heteroaromatic bicycle in the inhibitory activity. In Chembridge-9231888 molecular structure, the inversion of flexibility and rigidity on either side of the central piperidine seems detrimental for the activity. This conformational change could be responsible for the complete loss of affinity to the target. To further investigate the role of the structural features in the inhibitory activity, we performed a chemical group assessment on Ketanserin and selected four fragments representative of the main chemical groups present in the molecule. Unfortunately, none of these fragments showed IC$_{50}$ below 100 μM. Although these fragments could have an individual affinity to the target, the level of binding energy was not high enough to result in

Table 1
List of compounds presenting more than 80% inhibition on the GT activity carried out by VEEV nsP1. n.d.: not determined.

Compound information			GT reaction	
			ELISA	WB
Prestw n°	Commercial name	Structure	% Inhi. at 50 μM	IC$_{50}$ (μM) ± SD
37 Head of series 1	Pyrimethamine		100	2.7 ± 0.4
143	Chlorhexidine		81.3	> 200
172	Etofylline		89.4	15.8
182	Levamisole hydrochloride		97.5	> 200
203	Flufenamic acid		90.6	> 200
308	Pimozide		87.4	102.3 ± 40.3
388	Dequalinium dichloride		88.1	15.7 ± 7.5
392 Head of series 2	Ketanserin tartrate hydrate		99.4	14.6 ± 2.9
531 Head of series 2	Pirenperone		85.6	39.6 ± 18.3
544	Tobramycin		101	> 200
1447	Nalidixic acid sodium salt		85.3	59.8 ± 32.2
777	Alexidine dihydrochloride		85.5	> 200
858	Doxazin mesylate		83.2	29.6 ± 23.9
998	Zuclopenthixol dihydrochloride		81.9	n. d.
1029	Risperidone		79.5	> 200
1068	Thiethylperazine dimalate		100	n. d.
1105	Verteporfin		99.5	13.5 ± 3.1
1117	Azaperone		81.1	> 200
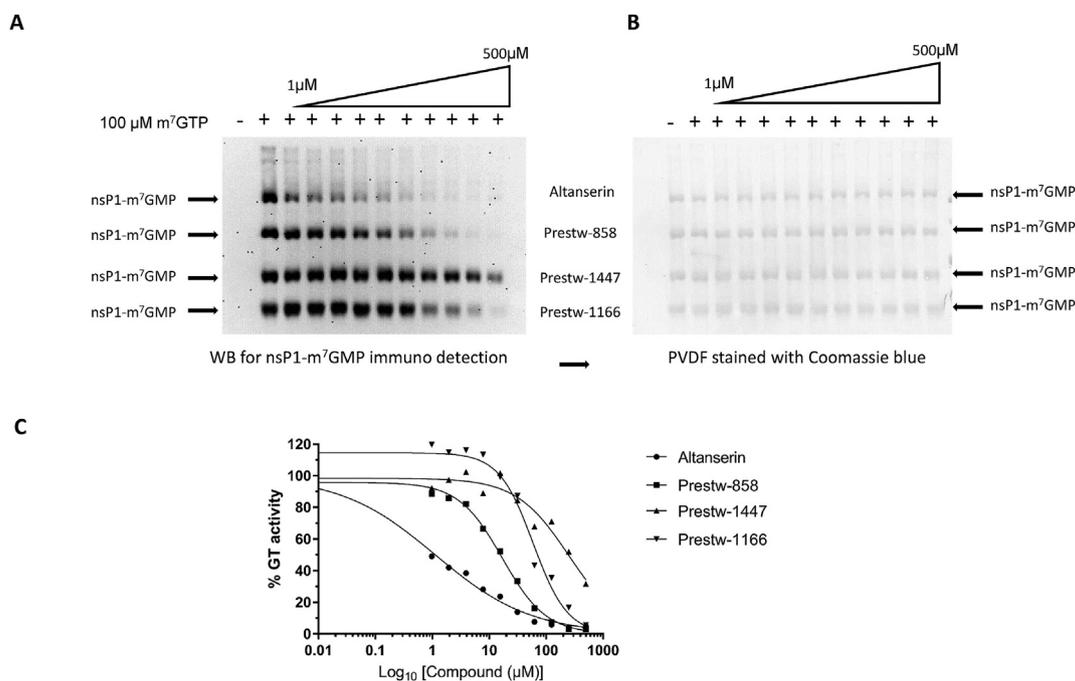
Fig. 3. IC$_{50}$ determination of compounds on the VEEV nsP1 GT activity determined by Western blot. Panel A: Detection of m^{7}GMP-nsP1 complex by Western blot. Panel B: PVDF membrane stained with Coomassie blue. Panel C: Hill plot fitting curve for IC$_{50}$ determination deduced after Western blot quantification.

Table 2
Selected compounds from the analogue search of the head of series 1 (Prest-37) used for the evaluation of the GT activity carried by nsP1 VEEV. The analogue search was performed using the screened library from Prestwick Chemical and commercially available databases. n.d.: not determined.
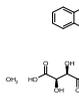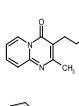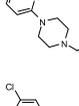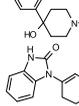
Compound information				GT reaction		MTase reaction
				ELISA	WB	FBA
Supplier	Supplier reference	Commercial name or CAS (nº)	Structure	% Inhi. at 50 μM	IC$_{50}$ (μM)	% Inhi. at 50 μM
Series 1: Head of series						
Prestwick Chemical	37 Head of serie 1	Pyrimethamine		100	2.7 ± 0.4	73.5 ± 2.9
Series 1: Analogue search in screened librarys						
Prestwick Chemical	347	Thioguanosine		47.8	115 ± 69.4	n.d.
Prestwick Chemical	858	Doxazoxin mesylate		83.2	29.6 ± 12.6	38.3 ± 3.3
Prestwick Chemical	947	Prazosin hydrochloride		74.3	29.5 ± 23.9	37.2 ± 3.0
Series 1: Analogue search in commercial databasess						
TRC	E925220	N. A CAS: 71552-34-6		n. d.	39.9 ± 21.3	n.d.
TRC	M338835	Metoprine CAS: 7761-45-7		n. d.	16.1 ± 3.6	n.d.
Key Organics	1B-058	N. A CAS: 320424-61-1		n. d.	> 200	n.d.
Enamine	EN300-183279	N. A CAS: 18588-49-3		n. d.	> 200	n.d.
Enamine	EN300-208764	N. A CAS: 17039-14-4		n. d.	> 200	n.d.
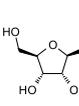
Table 3

Selected compounds from the analogue search of the head of series 2 (Prest-392 and 531) used for the evaluation of the GT activity carried by nsP1 VEEV. The analogue search was performed using the screened library from Prestwick Chemical and commercially available databases.
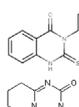
Compound information				GT reaction		MTase reaction
				ELISA	WB	FBA
Supplier	Supplier reference	Commercial name CAS (nº)	Structure	% Inhi. at 50 μM	IC$_{50}$ (μM)	% Inhi. at 50 μM
Serie 2: Head of series						
Prestwick Chemical	392 Head of serie 3	Ketanserin tartrate hydrate		99.4	14.6 ± 2.9	79.3 ± 1.7
Prestwick Chemical	531 Head of serie 3	Pirenperone		85.6	39.6 ± 18.3	44.6 ± 0.5
Serie 2: Analogue search in screened library						
Prestwick Chemical	1117	Azaperone		81.0	> 200	n.d.
Prestwick Chemical	115	Haloperidol		62.7	> 200	n.d.
Prestwick Chemical	360	Droperidol		75.5	22.5	n.d.
Prestwick Chemical	484	Benperidol		51.2	62.6	n.d.
Prestwick Chemical	136	Astemizole		70.8	97.8 ± 59.2	n.d.
Prestwick Chemical	308	Pimozide		87.4	102 ± 40.3	n.d.
Prestwick Chemical	1029	Risperidone		79.5	> 250	n.d.
Prestwick Chemical	1166	Rebamipide		74.0	94.5 ± 49.8	n.d.
Serie 2: Analogue search in Commercial data bases						
ChemBridge	9231888	N. A / N. A		n. d.	> 200	n.d.
Aldrich Sigma	A8106	Altanserin CAS: 76330-71-7		n. d.	9.3 ± 2.7	n.d.
Tocris	DV7028	N. A CAS: 133364-62-2		n. d.	> 200	n.d.
Serie 2: Fragments in commercial databases						
Alfa Aesar	B23142	N. A CAS:25519-78-2		n. d.	> 200	n.d.
Alfa Aesar	AS 142026	Benzoylneurea CAS: 86-96-4		n. d.	> 200	n.d.
Enamine	EN300-23764	N. A CAS: 607-19-2		n. d.	> 200	n.d.
Chembridge	9195974	N. A CAS:144734-42-9?		n. d.	> 200	n.d.
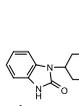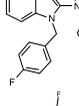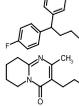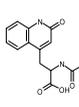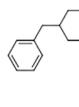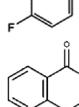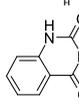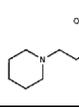
Table 4
Evaluation of the inhibition of the head of series 1 and 2 in both GT and MTase activity carried by VEEV nsP1 and/or Human N7 methyltransferase. Determination of IC_{50} for the GT activity was performed by Western blot and the determination of the %age of inhibition of MTase activity at 50 µM was measured by DEAE filter binding assay (FBA). Sinefungin was included as a reference compound. n.d.: not determined. n.i.: no inhibition.

Heads of series used for analogue search							
Compound information				GT reaction		MTase reaction	
Hit series	Head compound	Commercial name	Structure	WB		FBA	
				IC_{50} (µM)		% Inhi. at 50 µM	
				nsP1(*WT*) VEEV	nsP1(D34S) VEEV	nsP1 VEEV	H7N
1	37	Pyrimethamine		2.7 ± 0.4	2.8 [+]	73.5 ± 2.9	n. i.
2	392	Ketanserin tartrate hydrate		14.6 ± 2.9	n.d.	79.3 ± 1.7	2.8 ± 4.0
	531	Pirenperone		39.6 ± 18.3	65.3 [+]	44.6 ± 0.5	n. i.
Reference compound	–	Sinefungine		[i]29.12 ± 2.6		[i]1.5 ± 0.6	98.2 ± 0.3

i: The value is original from Li, C., Guillen, J., Rabah, N., Blanjoie, A., Debart, F., Vasseur, J. J., Canard, B., Decroly, E. & Coutard, B. (2015). *J Virol* **89**, 8292–8303.
n. i.: no inhibition; n. d.: not determined; +: data from a single experiment.

detectable inhibition. We also mixed fragments representative of the two distinct chemical groups. The groups tested were the B23142 together with AS 142026, the B23142 together with EN300-23764 and the B23142 together with Chembridge 9195974. None of the combination could give IC_{50} in the 100 µM range or below (data not shown).

3.5. Prest-37 and Prest-531 specificity and preliminary characterization of their mode of action

A series of compounds with a chemical structure of 3-aryl-[1,2,3] triazolo[4,5-*d*]pyrimidin-7(6*H*)-ones called MADTPs, targeting alphavirus nsP1 has recently been described (Gigante et al., 2014, 2017) (Delang et al., 2016). A resistance mutation (D34S) to these compounds was found in the N-terminal region of nsP1. The D34S mutation was introduced into the VEEV nsP1 sequence, and allowed the enzyme to keep its GT activity in the presence of MADTP whereas MADTP inhibited the *wt* VEEV nsP1. To evaluate if some of the identified inhibitors could share the same mode of action (MoA) with MADTPs, we tested Prest-37 and Prest-531 on both *wt* and D34S VEEV nsP1. The data in Table 4 show that the D34S mutation did not reduce the inhibitory effect of the compounds on the GT activity, suggesting the MoA is different from that of MADTPs series.

The inhibition of the Human N7-MTase (also known as RNMT) was also tested to evaluate the specificity of the compounds. Prest-37 and Prest-392 were tested at 50 µM, and no significant inhibition effect was observed (Table 4), suggesting that the hit compounds are VEEV nsP1 specific inhibitors.

4. Discussion

Arboviruses, among which alphaviruses, are responsible for many viral emergences causing worldwide health and economic troubles. Many efforts on the development of therapeutics are currently on the way but to date there is no approved treatment dedicated to alphaviruses. One of the first steps toward the development of drugs is the selection and validation of drug targets defined as proteins bearing functions which are essential for the virus replication and which can be modulated by small compounds. Alphavirus nsP1 is the central enzyme of the viral mRNA capping as it is involved in at least three steps of the cap synthesis, namely GTP methylation (MTase), nsP1 guanylylation

(GT) and cap transfer on mRNA (GTase). Mutational abrogation of one of these functions leads to loss of viral replication (Kallio et al., 2016). Since the first reports of nsP1 production and purification suitable for inhibition assays, compounds inhibiting MTase and GT reactions were characterized and showed antiviral effect in cell based assays (Feibelman et al., 2018; Tomar et al., 2011) (Delang et al., 2016), thus validating nsP1 as a *bona fide* antiviral target suitable for enzyme-based drug design. Enzyme-based screening assays have recently been developed for CHIKV nsP1, both based on the detection of the formation of m⁷GMP-nsP1 complex (Bullard-Feibelman et al., 2016; Feibelman et al., 2018; Kaur et al., 2018). Our study presents here the development of an ELISA-based assay in which the GT reaction was uncoupled from the MTase. This assay was validated with known nsP1 inhibitors, and then used for the screening of a library of more than 1200 approved drugs on the VEEV nsP1 GT reaction. We identified 18 compounds efficiently inhibiting the GT reaction at 50 µM, and 7 of them were further validated by a specific WB assay already used to determine their mode of action (Delang et al., 2016; Gigante et al., 2017). Thus, our results validated a high throughput ELISA assay to identify compounds blocking the VEEV nsP1-mediated GT reaction. During this work, Kaur and colleagues also reported a similar ELISA assay to detect the CHIKV m⁷GMP-nsP1 adduct, confirming the robustness of this technique for small molecules screening (Kaur et al., 2018). The main difference between the two reports is that we have found experimental conditions uncoupling the GT from the MTase reaction.

From this screening, 18 compounds (1.48% success rate) showed more than 80% inhibition at 50 µM, among which 9 had IC_{50} values below the 100 µM range, as determined by WB. None of these compounds show structure similarity when compared to compounds selected from a GTP competition assay on CHIKV nsP1 (Feibelman et al., 2018). The best compounds Prest-37 and Prest-392 that were chosen as head of series show a better IC_{50} on the GT activity of VEEV nsP1 than Sinefungin (29.1 ± 2.6 µM (Li et al., 2015),), and IC_{50} in the same range as compounds already described to have an antiviral effect on CHIKV (Gigante et al., 2017). The search of analogues for compounds Prest-37 (series 1) and Prest-392 (series 2) was first done within the PCL screened library, and then extended to commercial databases. With the search of compounds in the PCL screened library, 12 structurally related compounds were selected. All of them show a significant inhibition activity on the GT reaction, suggesting that the screening assay

reproducibly detect inhibitors, even when not highly potent. In particular, Prest-531 which is very close to Prest-392 had been selected as a hit, highlighting a potent scaffold for GT inhibition. The second phase of the analogue search was performed in commercial libraries. None of the selected compounds for series 1 gave inhibition higher than or equivalent to that of Prest-37 but the structure analysis revealed the possible key role of an aliphatic group in position 6 of the diamino-pyrimidine ring, as the three compounds lacking this group showed no GT inhibition. However, the number of tested compounds remains low (including Prest-37, n = 6) and deeper structure activity relationship studies on a larger number of compounds is required to confirm this hypothesis. The number of selected analogues for series 2 was higher (n = 13, including hits Prest-392 and 531). Altanserin, known to be bind 5-HT$_{2A}$ receptors (5-Hydroxytryptamine (serotonin) 2A receptor), is a more potent inhibitor than the hits of series 2. Altanserin differs from Prest-392 by harbouring a thioketone instead of a ketone in position 2 of the quinazoline-2,4-dione group. A preliminary SAR seems to emerge in this chemical series. Two aromatic moieties are mandatory at each extremity of the molecule with a defined spatial orientation. However, at this stage of the study and regarding the low number of analogues tested, we cannot conclude on the best nature of these aromatic moieties. Moreover, we do not know the importance of the central piperidine ring, or the nature and the length of the chains linking the piperidine to both external rings.

In order to gain insight into the mechanism of inhibition, we assessed the inhibition of head of series 1 and 2 on the MTase activity carried by VEEV nsP1. Prest-37 and 531 can significantly affect the MTase reaction (Table 1). This dual activity is not surprising as it has been already demonstrated that both MTase and GT reactions are coordinated (Li et al., 2015). Other developed compounds were also found to affect both GT and MTase activity of VEEV nsP1 among which compounds from MADTP series or SAM/SAH analogues represented by Sinefungin (Delang et al., 2016; Li et al., 2015), prompting us to evaluate a possible common mode of action despite structural differences. To this aim, we assessed the inhibition of GT activity of VEEV D34S nsP1 reluctant to MADTP inhibition. Both Prest-37 and 531 showed comparable inhibitory effect on both VEEV D34S and *wt* nsP1, suggesting that their mechanism of action is likely different from that of the MADTP which targets the N-terminal region of nsP1.

One of the key issue in the development of antivirals targeting capping enzymes is the specificity towards the viral protein, as cellular methyltransferases may share common structural features and have conserved substrate binding pockets with the viral target (Lim et al., 2011). The lack of structural information for nsP1 does not allow to claim that the binding pockets such as the SAM/SAH binding site are conserved with cellular MTases, it is therefore useful to address the specificity issue at the early stage of the study by testing the compounds on a cellular MTase of similar activity spectrum (Aouadi et al., 2017) (Coutard et al., 2017). Unlike Sinefungin, none of the tested compounds are able to inhibit the human N7-MTase used as a reference enzyme to address specificity. The development of an initial screening on the GT activity instead of the MTase may have resulted in the selection of compounds targeting an original viral activity, thus with a higher probability to be specific than compounds selected for MTase inhibition.

5. Conclusion

It is now established that capping of viral mRNA is a possible target for the development of molecules with antiviral effect as inhibitors of the capping would abrogate the translation of viral proteins and thereby impair viral replication. As the structure of the alphavirus nsP1 is not known, we developed an enzyme based assay to select among approved compounds small molecules inhibiting one of the step of the capping process using VEEV nsP1 as model. The development of the assay enabled the screening of 1220 compounds and the selection of 18

compounds followed by further characterization of two series of compounds. The analysis of the series suggest that they work through an original mechanism of action when compared to reference molecules, opening new opportunities for the development of novel anti-VEEV compounds.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.01.003.

References

Adams, A.P., Navarro-Lopez, R., Ramirez-Aguilar, F.J., Lopez-Gonzalez, I., Leal, G., Flores-Mayorga, J.M., Travassos da Rosa, A.P., Saxton-Shaw, K.D., Singh, A.J., Borland, E.M., Powers, A.M., Tesh, R.B., Weaver, S.C., Estrada-Franco, J.G., 2012. Venezuelan equine encephalitis virus activity in the Gulf Coast region of Mexico, 2003-2010. PLoS Neglected Trop. Dis. 6, e1875.
Aguilar, P.V., Estrada-Franco, J.G., Navarro-Lopez, R., Ferro, C., Haddow, A.D., Weaver, S.C., 2011. Endemic Venezuelan equine encephalitis in the Americas: hidden under the dengue umbrella. Future Virol. 6, 721–740.
Aouadi, W., Eydoux, C., Coutard, B., Martin, B., Debart, F., Vasseur, J.J., Contreras, J.M., Morice, C., Querat, G., Jung, M.L., Canard, B., Guillemot, J.C., Decroly, E., 2017. Toward the identification of viral cap-methyltransferase inhibitors by fluorescence screening assay. Antivir. Res. 144, 330–339.
Bullard-Feibelman, K.M., Fuller, B.P., Geiss, B.J., 2016. A Sensitive and Robust High-Throughput Screening Assay for Inhibitors of the Chikungunya Virus nsP1 Capping Enzyme. PLoS One 11, e0158923.
Charrel, R.N., Leparc-Goffart, I., Gallian, P., de Lamballerie, X., 2014. Globalization of Chikungunya: 10 years to invade the world. Clin. Microbiol. Infect. 20, 662–663.
Chung, D., Schroeder, C.E., Sotsky, J., Yao, T., Roy, S., Smith, R.A., Tower, N.A., Noah, J.W., McKellip, S., Sosa, M., Rasmussen, L., White, E.L., Aube, J., Golden, J.E., 2013. ML336: Development of Quinazolinone-Based Inhibitors against Venezuelan Equine Encephalitis Virus (VEEV). Probe Reports from the NIH Molecular Libraries Program.
Chung, D.H., Jonsson, C.B., Tower, N.A., Chu, Y.K., Sahin, E., Golden, J.E., Noah, J.W., Schroeder, C.E., Sotsky, J.B., Sosa, M.I., Cramer, D.E., McKellip, S.N., Rasmussen, L., White, E.L., Schmaljohn, C.S., Julander, J.G., Smith, J.M., Filone, C.M., Connor, J.H., Sakurai, Y., Davey, R.A., 2014. Discovery of a novel compound with anti-venezuelan equine encephalitis virus activity that targets the nonstructural protein 2. PLoS Pathog. 10, e1004213.
Coutard, B., Barral, K., Lichiere, J., Selisko, B., Martin, B., Aouadi, W., Lombardia, M.O., Debart, F., Vasseur, J.J., Guillemot, J.C., Canard, B., Decroly, E., 2017. Zika Virus Methyltransferase: structure and Functions for Drug Design Perspectives. J. Virol. 91, e02202.
Delang, L., Li, C., Tas, A., Querat, G., Albulescu, I.C., De Burghgraeve, T., Guerrero, N.A., Gigante, A., Piorkowski, G., Decroly, E., Jochmans, D., Canard, B., Snijder, E.J., Perez-Perez, M.J., van Hemert, M.J., Coutard, B., Leyssen, P., Neyts, J., 2016. The viral capping enzyme nsP1: a novel target for the inhibition of chikungunya virus infection. Sci. Rep. 6, 31819.
Delang, L., Segura Guerrero, N., Tas, A., Querat, G., Pastorino, B., Froeyen, M., Dallmeier, K., Jochmans, D., Herdewijn, P., Bello, F., Snijder, E.J., de Lamballerie, X., Martina, B., Neyts, J., van Hemert, M.J., Leyssen, P., 2014. Mutations in the chikungunya virus non-structural proteins cause resistance to favipiravir (T-705), a broad-spectrum antiviral. J. Antimicrob. Chemother. 69, 2770–2784.
Delekta, P.C., Raveh, A., Larsen, M.J., Schultz, P.J., Tamayo-Castillo, G., Sherman, D.H., Miller, D.J., 2015. The combined use of alphavirus replicons and pseudoinfectious particles for the discovery of antivirals derived from natural products. J. Biomol. Screen 20, 673–680.
Feibelman, K.M., Fuller, B.P., Li, L., LaBarbera, D.V., Geiss, B.J., 2018. Identification of small molecule inhibitors of the Chikungunya virus nsP1 RNA capping enzyme. Antivir. Res. 154, 124–131.
Furuta, Y., Gowen, B.B., Takahashi, K., Shiraki, K., Smee, D.F., Barnard, D.L., 2013. Favipiravir (T-705), a novel viral RNA polymerase inhibitor. Antivir. Res. 100, 446–454.
Gigante, A., Canela, M.D., Delang, L., Priego, E.M., Camarasa, M.J., Querat, G., Neyts, J., Leyssen, P., Perez-Perez, M.J., 2014. Identification of [1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones as novel inhibitors of Chikungunya virus replication. J. Med. Chem. 57, 4000–4008.
Gigante, A., Gomez-SanJuan, A., Delang, L., Li, C., Bueno, O., Gamo, A.M., Priego, E.M., Camarasa, M.J., Jochmans, D., Leyssen, P., Decroly, E., Coutard, B., Querat, G., Neyts,

J., Perez-Perez, M.J., 2017. Antiviral activity of [1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones against chikungunya virus targeting the viral capping nsP1. Antivir. Res. 144, 216–222.

Gomez-SanJuan, A., Gamo, A.M., Delang, L., Perez-Sanchez, A., Amrun, S.N., Abdelnabi, R., Jacobs, S., Priego, E.M., Camarasa, M.J., Jochmans, D., Leyssen, P., Ng, L.F.P., Querat, G., Neyts, J., Perez-Perez, M.J., 2018. Inhibition of the replication of different strains of chikungunya virus by 3-aryl-[1,2,3]triazolo[4,5- d]pyrimidin-7(6 H)-ones. ACS Infect. Dis. 4, 605–619.

Gould, E.A., Coutard, B., Malet, H., Morin, B., Jamal, S., Weaver, S., Gorbalenya, A., Moureau, G., Baronti, C., Delogu, I., Forrester, N., Khasnatinov, M., Gritsun, T., de Lamballerie, X., Canard, B., 2010. Understanding the alphaviruses: recent research on important emerging pathogens and progress towards their control. Antivir. Res. 87, 111–124.

Hyde, J.L., Gardner, C.L., Kimura, T., White, J.P., Liu, G., Trobaugh, D.W., Huang, C., Tonelli, M., Paessler, S., Takeda, K., Klimstra, W.B., Amarasinghe, G.K., Diamond, M.S., 2014. A viral RNA structural element alters host recognition of nonself RNA. Science 343, 783–787.

Johnson, K.M., Martin, D.H., 1974. Venezuelan equine encephalitis. Adv. Vet. Sci. Comp. Med. 18, 79–116.

Kallio, K., Hellstrom, K., Jokitalo, E., Ahola, T., 2016. RNA replication and membrane modification require the same functions of alphavirus nonstructural proteins. J. Virol. 90, 1687–1692.

Kaur, R., Mudgal, R., Narwal, M., Tomar, S., 2018. Development of an ELISA assay for screening inhibitors against divalent metal ion dependent alphavirus capping enzyme. Virus Res. 256, 209–218.

Li, C., Guillen, J., Rabah, N., Blanjoie, A., Debart, F., Vasseur, J.J., Canard, B., Decroly, E., Coutard, B., 2015. mRNA capping by Venezuelan equine encephalitis virus nsP1: functional characterization and implications for antiviral research. J. Virol. 89, 8292–8303.

Lim, S.P., Sonntag, L.S., Noble, C., Nilar, S.H., Ng, R.H., Zou, G., Monaghan, P., Chung, K.Y., Dong, H., Liu, B., Bodenreider, C., Lee, G., Ding, M., Chan, W.L., Wang, G., Jian, Y.L., Chao, A.T., Lescar, J., Yin, Z., Vedananda, T.R., Keller, T.H., Shi, P.Y., 2011. Small molecule inhibitors that selectively block dengue virus methyltransferase. J. Biol. Chem. 286, 6233–6240.

Lucas-Hourani, M., Munier-Lehmann, H., Helynck, O., Komarova, A., Despres, P., Tangy, F., Vidalain, P.O., 2014. High-throughput screening for broad-spectrum chemical inhibitors of RNA viruses. J. Vis. Exp.

Markland, W., McQuaid, T.J., Jain, J., Kwong, A.D., 2000. Broad-spectrum antiviral activity of the IMP dehydrogenase inhibitor VX-497: a comparison with ribavirin and demonstration of antiviral additivity with alpha interferon. Antimicrob. Agents Chemother. 44, 859–866.

Pisano, M.B., Oria, G., Beskow, G., Aguilar, J., Konigheim, B., Cacace, M.L., Aguirre, L., Stein, M., Contigiani, M.S., 2013. Venezuelan equine encephalitis viruses (VEEV) in Argentina: serological evidence of human infection. PLoS Neglected Trop. Dis. 7, e2551.

Pohjala, L., Utt, A., Varjak, M., Lulla, A., Merits, A., Ahola, T., Tammela, P., 2011. Inhibitors of alphavirus entry and replication identified with a stable Chikungunya replicon cell line and virus-based assays. PLoS One 6, e28923.

Ravichandran, R., Manian, M., 2008. Ribavirin therapy for Chikungunya arthritis. J. Infect. Dev. Ctries 2, 140–142.

Rivas, F., Diaz, L.A., Cardenas, V.M., Daza, E., Bruzon, L., Alcala, A., De la Hoz, O., Caceres, F.M., Aristizabal, G., Martinez, J.W., Revelo, D., De la Hoz, F., Boshell, J., Camacho, T., Calderon, L., Olano, V.A., Villarreal, L.I., Roselli, D., Alvarez, G., Ludwig, G., Tsai, T., 1997. Epidemic Venezuelan equine encephalitis in La Guajira, Colombia, 1995. J. Infect. Dis. 175, 828–832.

Seyedi, S.S., Shukri, M., Hassandarvish, P., Oo, A., Shankar, E.M., Abubakar, S., Zandi, K., 2016. Computational approach towards exploring potential anti-chikungunya activity of selected flavonoids. Sci. Rep. 6, 24027.

Spurgers, K.B., Hurt, C.R., Cohen, J.W., Eccelston, L.T., Lind, C.M., Lingappa, V.R., Glass, P.J., 2013. Validation of a cell-based ELISA as a screening tool identifying anti-alphavirus small-molecule inhibitors. J. Virol. Methods 193, 226–231.

Tomar, S., Narwal, M., Harms, E., Smith, J.L., Kuhn, R.J., 2011. Heterologous production, purification and characterization of enzymatically active Sindbis virus nonstructural protein nsP1. Protein Expr. Purif. 79, 277–284.

Urakova, N., Kuznetsova, V., Crossman, D.K., Sokratian, A., Guthrie, D.B., Kolykhalov, A.A., Lockwood, M.A., Natchus, M.G., Crowley, M.R., Painter, G.R., Frolova, E.I., Frolov, I., 2017. beta-D-N(4)-hydroxycytidine is a potent anti-alphavirus compound that induces high level of mutations in viral genome. J. Virol. 92, e01965.

Varghese, F.S., Kaukinen, P., Glasker, S., Bespalov, M., Hanski, L., Wennerberg, K., Kummerer, B.M., Ahola, T., 2016. Discovery of berberine, abamectin and ivermectin as antivirals against chikungunya and other alphaviruses. Antivir. Res. 126, 117–124.

Vasiljeva, L., Merits, A., Auvinen, P., Kaariainen, L., 2000. Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2. J. Biol. Chem. 275, 17281–17287.

Wada, Y., Orba, Y., Sasaki, M., Kobayashi, S., Carr, M.J., Nobori, H., Sato, A., Hall, W.W., Sawa, H., 2017. Discovery of a novel antiviral agent targeting the nonstructural protein 4 (nsP4) of chikungunya virus. Virology 505, 102–112.

Wang, H.L., O'Rear, J., Stollar, V., 1996. Mutagenesis of the Sindbis virus nsP1 protein: effects on methyltransferase activity and viral infectivity. Virology 217, 527–531.