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Computer-aided identification, design and synthesis of a novel series of compounds with selective antiviral activity against chikungunya virus.

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Highlights:

- *A homology model for the CHIKV nsP2 protease was constructed and validated.*
- *A new class of inhibitors of CHIKV replication was identified by virtual screening.*
- *SARs of the compound class were explored.*
- *Several analogues with low μM activity in the cell-based assay were identified*

ABSTRACT

Chikungunya virus (CHIKV) is an Arbovirus that is transmitted to humans primarily by the mosquito species *Aedes aegypti*. Infection with this pathogen is often associated with fever, rash and arthralgia. Neither a vaccine nor an antiviral drug is available for the prevention or treatment of this disease. Albeit considered a tropical pathogen, adaptation of the virus to the mosquito species *Aedes albopictus*, which is also very common in temperate zones, has resulted in recent outbreaks in Europe and the US. In the present study, we report on the discovery of a novel series of compounds that inhibit CHIKV replication in the low µM range. In particular, we have initially performed a virtual screening simulation of ~5 million compounds on the CHIKV nsP2, a viral protease, and then we have investigated the Structure-Activity Relationships of the hit identified *in silico*. Overall, a series of 26 compounds, including the original hit, was evaluated in a virus-cell-based CPE reduction assay. The study of such selective inhibitors will contribute to a better understanding of the CHIKV replication cycle and represents also a first step towards the development of a clinical candidate drug for the treatment of this disease.

1. Introduction

Chikungunya virus is an Arbovirus that belongs to the genus *Alphavirus*, family of the *Togaviridae*. It is transmitted to humans primarily by the mosquito species *Aedes aegypti* (Sourisseau et al., 2007) and the infection is associated with an acute pathology characterized by fever, rash and arthralgia (Vanlandingham et al., 2005). In particular, the arthralgia symptoms, often severe and debilitating, may persist for several months and become chronic in 10% of the infected individuals (Sissoko et al., 2009).

CHIKV infection was first described in Tanzania in 1952 (Ross, 1956) and small outbreaks have been reported from time to time. However, since 2005, a re-emergence with a previously unknown virulence was observed in large geographic areas around the Indian Ocean, extending from Africa, India to South-East Asia (Arankalle et al., 2007), even reaching Europe (Hochedez et al., 2007) and the US (CDC, 2007). The ability of the virus to adapt to a new vector, the mosquito species *Aedes albopictus* (Tsetsarkin et al., 2009), may have significantly contributed to a rapid and worldwide spread of this viral pathogen.

Clinically approved drugs such as chloroquine, alpha-interferon and ribavirin showed some antiviral effect *in vitro* but did not prove to be effective against CHIKV infection *in vivo* (De

Lamballerie et al., 2008) (Khan et al., 2010). In the last few years, an increasing number of research groups have focused their attention in identify novel anti-CHKV compounds. As a result, different natural products such as terpenoid compounds (Bourjot et al., 2012) and 5,7-dihydroxyflavones (Pohjala et al., 2011), clinically approved drugs such as phenothiazinyl compounds (Pohjala et al., 2011), arbidol (Delogu et al., 2011) and mycophenolic acid (Khan et al., 2011), along with the synthetic IFN inducer Poly (I:C) (new-5) have shown to impair CHIKV replication in cell-based systems (Li et al., 2012). Singh Kh et al. have also used a virtual screening approach to identify possible new inhibitors of the CHKV protease, but they did not evaluate experimentally the molecules selected *in silico* (Singh Kh et al., 2012). Despite all these efforts, neither a selective antiviral drug nor a vaccine has been approved for use in the clinical setting to date; care for CHIKV-infected patients is still limited to supportive treatment aiming at alleviating the infection-induced symptoms (Solignat et al., 2009).

CHIKV is an enveloped virus with an 11.8 kb single-stranded positive-sense RNA genome. It contains two open reading frames and encodes four non-structural proteins (nsP1, nsP2, nsP3, nsP4), three structural proteins (capsid, E1, E2) and two small polypeptides (E3, 6K) (Strauss and Strauss, 1994).

The four non-structural proteins possess enzymatic properties essential for virus replication and therefore represent interesting targets for the identification of selective antiviral inhibitors. Among them, the nsP2 protein plays a crucial role: its cysteine-protease activity is required for the proteolytic cleavage of the non-structural polyprotein precursor into the four mature nsPs (Perri et al., 2000). Three different cleavage sites, i.e. nsP1-2, nsP2-3, and nsP3-4, are the substrate for nsP2 proteolytic processing with a remarkable preference for nsP3-4>nsP1-2>>nsP2-3 (Russo et al., 2010).

The present study aims to explore nsP2 as a target for the discovery and development of selective inhibitors of CHIKV replication. In particular, we here report on the results obtained from a virtual screening campaign of a library of commercially available compounds against an optimized homology model of the CHIKV nsP2 protease. Furthermore, exploration of the structure-activity relationship and initial chemical optimisation of the hit compound has led to the identification of novel compounds, which are able to prevent virus-induced cell death at low µM concentrations.

2. Materials and methods

A detailed description of the Materials and Methods is available in the Supporting Information file.

3. Results and Discussion

3.1 Homology model and validation

At the time this project was initiated, the crystal structure of the CHIKV nsP2 was not yet resolved. Therefore, we decided to use a comparative modelling approach to build a model of the CHIKV protease. A homologous protein was identified by performing a similarity search between the sequence of CHIKV nsP2 and the sequences of the 3D structures stored in the Protein Data Bank (http://www.rcsb.org/pdb) through a PSI-BLAST search (Altschul et al., 1997). The Venezuelan equine encephalitis virus (VEEV) nsP2 protease (PDB ID: 2HWK) (Russo et al., 2006) yielded the best alignment score with a 40% sequence identity with the CHIKV sequence, thus representing a promising template to start building the homology model from. The sequences were aligned by MOE (Molecular Operating Environment, version 2009.10, Chemical Computing Group Inc.) with some constraints as reported in detail in the Material and Methods section in the Supplemental Information file. MOE was also used to construct the homology model which subsequently was validated in terms of stereochemical quality through Ramachandran plots (Lovell et al., 2003) on the Cambridge RAMPAGE server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) and in terms of amino acids environment using Verify 3D (Luthy et al., 1992) and Errat (Colovos and Yeates, 1993). The results demonstrated that we constructed a fairly reliable model, both in terms of main-chain stereochemistry as well as amino acid environment (Table 1). In the Ramachandran plot, 98.7% (favourable region: 94.7% plus allowed region: 4%) of the residues occupied the desired space.

Recent publication of the CHIKV nsP2 crystal structure (PDB ID: 3TRK) has retrospectively confirmed the reliability of our homology model: the backbone Cα superposition between the crystal structure and the homology model has a RMSD value of 1.78 Å (Fig. S1). Moreover, the RMSD value for the superposition of the binding site (19 residues within 4.5Å of the natural ligand) is significantly lower (0.82 Å), confirming the accuracy of the model in reproducing the active site characteristics. The overall structural overlap between the model and the template became more evident when comparing the architecture of the active site with the bound natural substrate (Fig. 2).

3.2 Virtual screening

The active site of the CHIKV nsP2 model was used to perform a structure-based virtual screening study with commercially available compounds. The starting point was a database of ~5 million structures, which was filtered using the pharmacophore model based on the protease binding site (Fig. 1). This process resulted in an enriched library of 12121 structures that possessed the required structural features. Molecular docking of these compounds was then performed using two different software packages: PLANTS (version 1.1) (Korb et al., 2009) and LeadIT-FlexX(version 1.2) (Rarey et al., 1996).

The docking results were subsequently re-scored using a multi-step consensus score. The first step encompassed a "rank sum" strategy that is widely used in docking experiments (Wang and Wang, 2001). The idea of the "rank sum" strategy is to rank all molecules according to each individual scoring function and to use the sum of the rank positions of the poses as a score. This resulted in two different hit lists, one based on the LeadIT-FlexX binding mode prediction, the other based on the PLANTS software. The second step comprised a voting strategy. The idea of the "vote rank" is that each scoring function votes for a pose to be a hit if the pose obtained a score in the top 25% of the score value range for all poses of a molecule. The number of votes for each pose finally serves as the consensus score to prioritize the compound structures.

A final selection of 15 derivatives was made by visual inspection; 9 of these compounds could be purchased and their potential antiviral activity on *in vitro* chikungunya virus replication was assessed. The molecules were subsequently submitted for evaluation of selective antiviral activity in a virus-cell-based assay for chikungunya virus.

3.3 Lead compound identification and biological validation.

Of these 9 hits obtained from the virtual screening, compound **1** selectively inhibited CHIKV-induced with an EC_{50} value of 5.0 µM in the cytopathic effect (CPE) reduction assay (Table 3, Fig. 3A). The proposed binding configuration of **1** in the active site of CHIKV nsP2 is shown in Fig. 4. The compound is predicted to fit the central portion of the nsP2 protease active site, with its hydrazone group placed in the region defined by the catalytic dyad, Cys579 and His649, and also in close proximity of Trp650. The cyclopropane moiety is positioned in the space previously occupied by the second glycine residue of the nsP3-4 junction peptide. The most relevant interactions observed for **1**

are a hydrophobic contact between the 3,4-diethoxyphenyl ring and the lateral chain of Trp650, two hydrogen-bond connections between the hydrazone function and the backbone amido groups of Tyr613 and Asn648 and another hydrophobic interaction between the *t*-butylic group of **1** and His649 (Fig 4, Fig. S2). It is interesting to note that docking **1** in the crystal structure of the CHKV nsP2 produced a virtually identical binding pose (Fig. S3).

Biological validation of the selective antiviral effect of Compound **1** on the replication of chikungunya virus was obtained by performing a virus yield assay in Vero cells. This assay allows quantification of the dose-response effect of the compound at two levels: (1) at the level of viral RNA production by quantification of the amount of viral RNA that is released in the supernatant (encapsidated in progeny virions; by RT-qPCR) and (2) at the level of infectious virus particle production by quantification of the amount of infectious progeny virions that are released by virus-infected compound-treated cells (by titration for infectious virus content). EC_{50} values of respectively 4.3 and 4.9 µM were derived from the dose-response curves obtained by both respective methods (Fig. 3B), of which is very similar to the EC_{50} obtained from the CPE reduction assay (Fig. 3A). Of importance, however, is to note that inhibition of virus replication is observed at concentrations that do not cause an adverse effect on the host cells (Fig. 3A), thus validating the compound as a selective inhibitor of virus replication.

Chloroquine was included as reference compound. It is one of the few compounds that have been reported in the literature to significantly inhibit CHIKV replication. Chloroquine a-specifically inhibits CHIKV replication by interfering with the protonation of endocytotic vesicles, a step that is essential to allow the virus to release its RNA into the cell cytoplasm. Chloroquine was found to be about 2-fold less potent in the virus-cell-based CPE reduction assay as compared to compound **1** (10 ± 1.4 µM vs 5 ± 0.2 µM). The antiviral effect of both compounds in the virus yield assay was quite similar and in agreement with the earlier reported *in vitro* antiviral activity of chloroquine (Khan et al., 2010).

3.4 Chemical validation and initial Structure-Activity Relationships analysis

To explore the potential of this molecular scaffold as selective inhibitors of CHIKV replication, a series of 23 structural analogues of compound **1** (Table 2, compounds **2-24**) was acquired from the SPECS library (http://www.specs.net). These compounds were identified by performing a chemical similarity search in the SPECS library, using compound **1** as the query. This initial series of

compounds was evaluated for selective antiviral activity in the CHIKV virus-cell-based assay (Table 3). Structurally, the compounds could be divided in two groups. Compounds **2-15** present two aromatic moieties and a cyclopropane ring, as observed in **1**. The second group (compounds **16-24**) is more diverse and, although these analogues still include the common carbonyl hydrazone linker, they do not contain a cyclopropane moiety in the alpha position to the carbonyl carbon. In most of the structures, the central core is asymmetrically substituted with two hydrophobic groups, with most of them carrying different substituents in diverse positions.

According to the activity data obtained for this initial series (Table 3), the cyclopropylic moiety seems to be important for the anti-CHIKV effect as compounds **16-24** did not show significant biological activity. A SAR analysis performed on **1-15** did not allow drawing definite conclusions, but it seems that the presence of an aliphatic group in *para* position of the benzylidene group is beneficial for antiviral activity (e.g. compounds **8** and **13**). It should be noted that the purchased compounds were evaluated by ^{1}H-NMR for purity. Furthermore, for chemical validation purposes, compound **1**, **6** and **8** were synthesised in our lab (Scheme 1A) and the spectroscopic data confirmed that the molecules were identical to the purchased compounds.

3.5 Design and synthesis of new derivatives

The biological data provided us with a starting point for further optimisation of these compounds. The next objective was to identify a suitable replacement of the cyclopropane ring, as the two chiral centres on this group could present a challenge in the preparation and purification of new analogues. Furthermore, replacement of the hydrazone group would be beneficial to overcome possible chemical instability issues (susceptible to hydrolysis). Compound **25** and **26** were designed to explore such modifications. In both structures, the cyclopropylic group was replaced with a *trans*-ethenylic function, which allows maintaining length and geometry of the original linker while avoiding the presence of any chiral centre in the molecule. Compound **25** differs from **1** only for this moiety, while in compound **26**, a first attempt was made to also replace the hydrazone moiety with an amide linker. Both compounds were able to dock in the nsP2 binding site in a similar binding pose as **1** (Fig S4)**.** Biological evaluation of **25** and **26** provided a clear outcome: the loss of activity of the latter indicates that further structural optimisation studies are required to identify a suitable alternative for the hydrazone group; however, **25**

showed a slightly improved antiviral activity profile compared to **1** (Table 3), indicating that the proposed replacement of the cyclopropane is acceptable (Fig. 3).

4. Conclusions

The work presented in this study underlines the usefulness of using a molecular modelling methodology that includes different *in silico* techniques in combination with a more classical medicinal chemistry approach, in the identification for novel and selective antiviral compounds. In this case, by using homology modelling and virtual screening, we identified a novel class of inhibitors of *in vitro* chikungunya virus replication. Starting from the original *in silico* hit (**1**), we were able to further expand the series of active compounds by using a more classic structure-activity relationships approach. In particular, compound **25** has a very promising activity profile and a simplified molecular structure compared to **1**, which will allow a more rapid and efficient optimization of this class of compounds by using a more accessible synthetic route. It is important to underline that despite the results of the molecular modeling studies, further experimental studies (currently ongoing) are required to prove that these compounds are indeed nsP2 inhibitors. However, notwithstanding the mode of action of these novel inhibitors, the promising results reported here could represent an initial step towards the discovery of a clinical candidate for the treatment of CHKV infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/.....

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Table 1

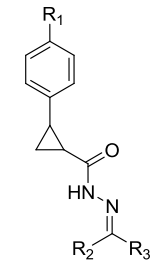
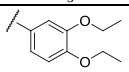
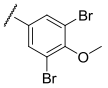
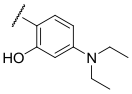
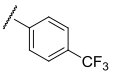
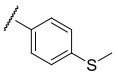
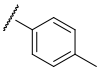
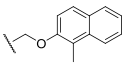
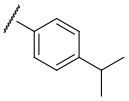
Validation results for the best scored CHIKV nsP2 model and the 3D structural template.

	Ramachandran plot[a] (%)	Errat (%)	Verify 3D[b] (total score)
Model CHIKV nsP2	94.7 – 4%	80	125
Template VEEV nsP2	96.2 – 3.1%	91	145

[a]Percentage of residues with phi, psi conformation in the most favoured region and allowed region of the Ramachandran plot.
[b]Verify 3D total score was obtained as a sum of all the individual values for each residue.

Table 2

Chemical structure of compounds **1**-**26**.

Struct.	Comp.	R_1	R_2	R_3	R_4	R_5
	**1**	*t*-but	H		-	-
	2	*t*-but	H		-	-
	3	*t*-but	H		-	-
	4	*t*-but	H		-	-
	5	*t*-but	H		-	-
	6	*t*-but	H		-	-
	7	*t*-but	H		-	-
	8	*t*-but	H		-	-
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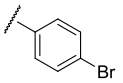
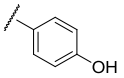
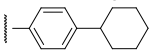
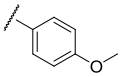
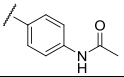
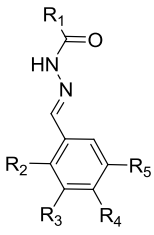
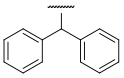
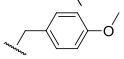
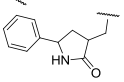
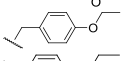
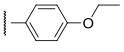
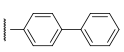
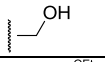
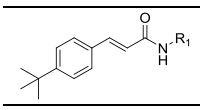
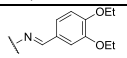
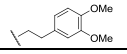
	10	H	H		-	-
	11	H	H		-	-
	12	H	H		-	-
	13	H	CH_3		-	-
	14	H	CH_3		-	-
	15	H	CH_3		-	-
	**16**		H	OCH_3	OEt	H
	17		H	-	OEt	H
	18		H	OCH_3	OCH_3	H
	19		H	OCH_3	OH	OCH_3
	20		H	OCH_3	OEt	H
	21		CH_3	H	H	H
	22		H	H	SCH_3	H
	23		H	H	OEt	H
	24		H	OH	OCH_3	H
	**25**		-	-	-	-
	26		-	-	-	-

Table 3

Effect of compounds **1-26 on chihuyanya virus-induced CPE formation in Vero cells**.

Compound	EC_{50} (µM)	EC_{90} (µM)	CC_{50} (µM)	SI[a]
1	5.0 ± 0.2	6.4 ± 0.5	72 ± 20	14
2	4.0 ± 0.3	7.6 ± 1.8	20 ± 2	5
3	NA	NA	4.1 ± 0.5	-
4	14 ± 6	19 ± 10	30 ± 5	2.1
5	NA	NA	3.3 ± 0.4	-
6	NA	NA	3.1 ± 0.3	-
7	NA	NA	>242	-
8	3.6 ± 0.9	4.8 ± 1.0	6.1 ± 2.3	1.7
9	NA	NA	>342	-
10	24 ± 3	39 ± 5	66 ± 3	2.7
11	6.4 ± 0.1	8.7 ± 0.2	15 ± 1	2.3
12	32 ± 1	44 ± 1	101 ± 1	3.1
13	5.6 ± 2.0	12 ± 6	72 ± 2	13
14	NA	NA	278 ± 13	-
15	NA	NA	216	-
16	NA	NA	> 303	-
17	NA	NA	> 279	-
18	NA	NA	> 379	-
19	NA	NA	> 329	-
20	NA	NA	>262	-
21	NA	NA	>355	-
22	NA	NA	>318	-
23	NA	NA	>291	-
24	NA	NA	>446	-
25	3.2 ± 1.8	11 ± 4	101 ± 50	32
26	NA	NA	54	-

[a]The selectivity index SI is calculated as the ratio of CC_{50}/EC_{50}.

Data are mean values ±SD for at least 3 independent experiments

NA = no activity could be observed

- = value could not be calculated

Fig. 1. Pharmacophore model used to filter the small molecule databases for the virtual screening studies on the model active site. The model consists of a hydrophobic/aromatic interaction with the residues Asn577, Cys579 and Ala612 (yellow - F1), one hydrogen bond acceptor point to Trp650 (cyan), an aromatic interaction with residues Tyr613, Met804 and Met808 (yellow – F3) and one hydrogen bond donor to Tyr645 (pink). Exclusion volumes are not shown for clarity.

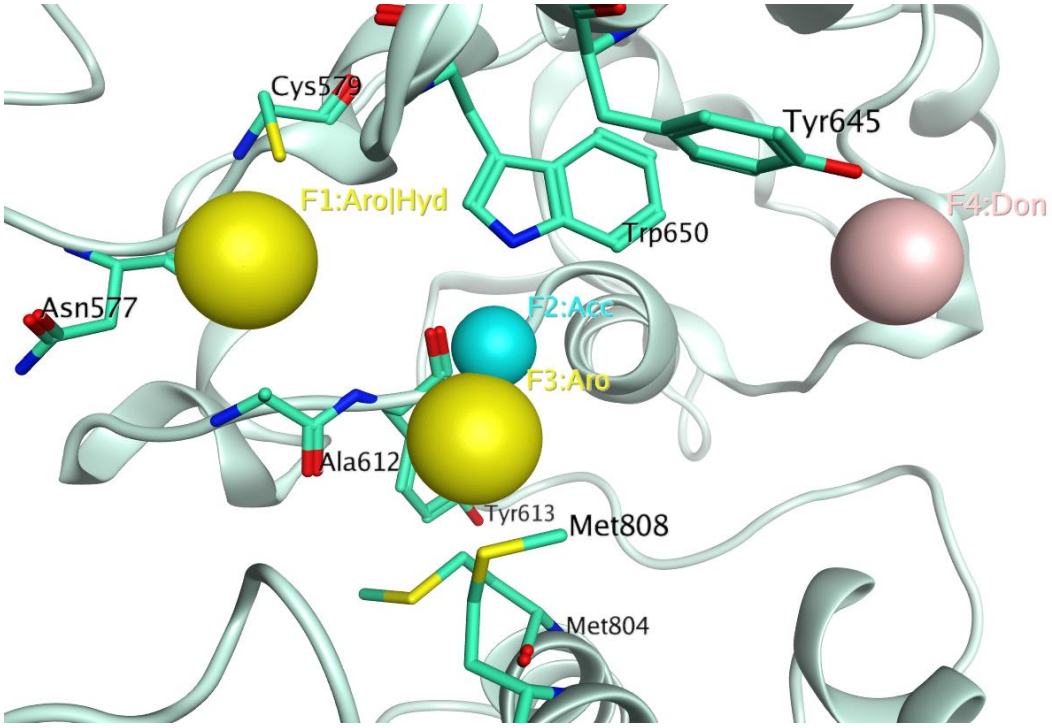


Fig. 2. Superposition between the crystal structure (in pink) and the model (in green) active sites and their relative position in reference to the nsP3-4 junction peptide (in white) optimised within the model structure.

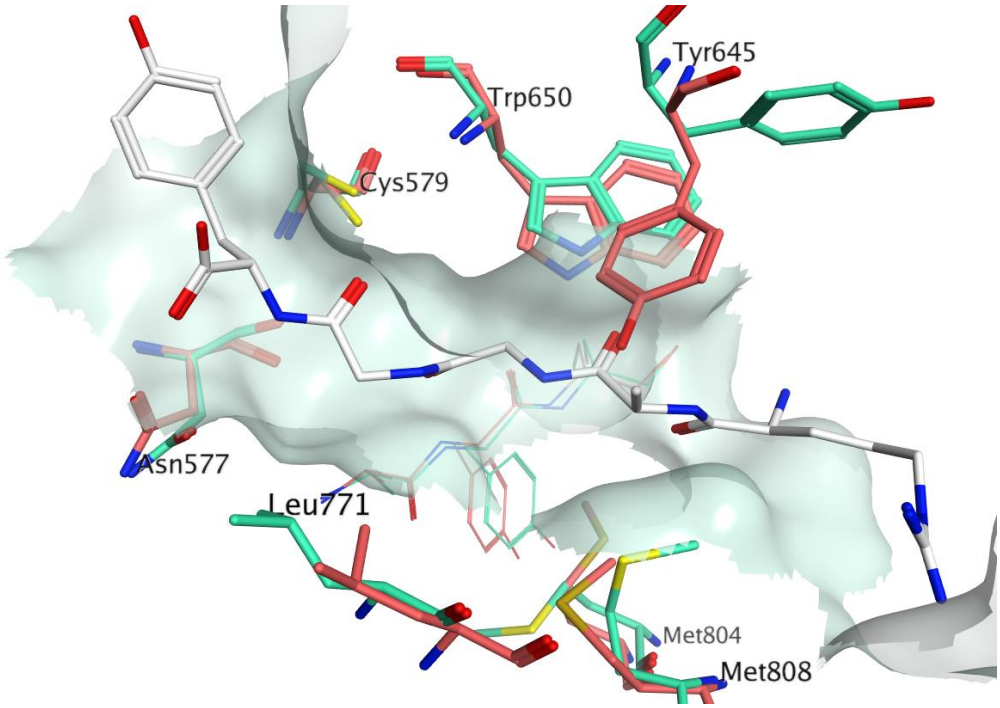


Fig. 3. Chloroquine (●), Compound **1** (○) and Compound **25 (●)** inhibit CHIKV replication *in vitro*. (**A**) Dose-response effect on uninfected host cells as measured by cell survival/viability; (**B**) Dose-response effect on virus replication as measured by cell survival (inhibition of virus-induced cytopathic effects); (C) Dose-response effect on virus replication as measured by inhibition of RNA replication (real-time quantitative RT-PCR readout); (D) Dose-response effect on virus replication as measured by inhibition of infectious progeny virion production (quantified by titration for infectious virus content). Results shown are the average + SD of at least 4 independent experiments.

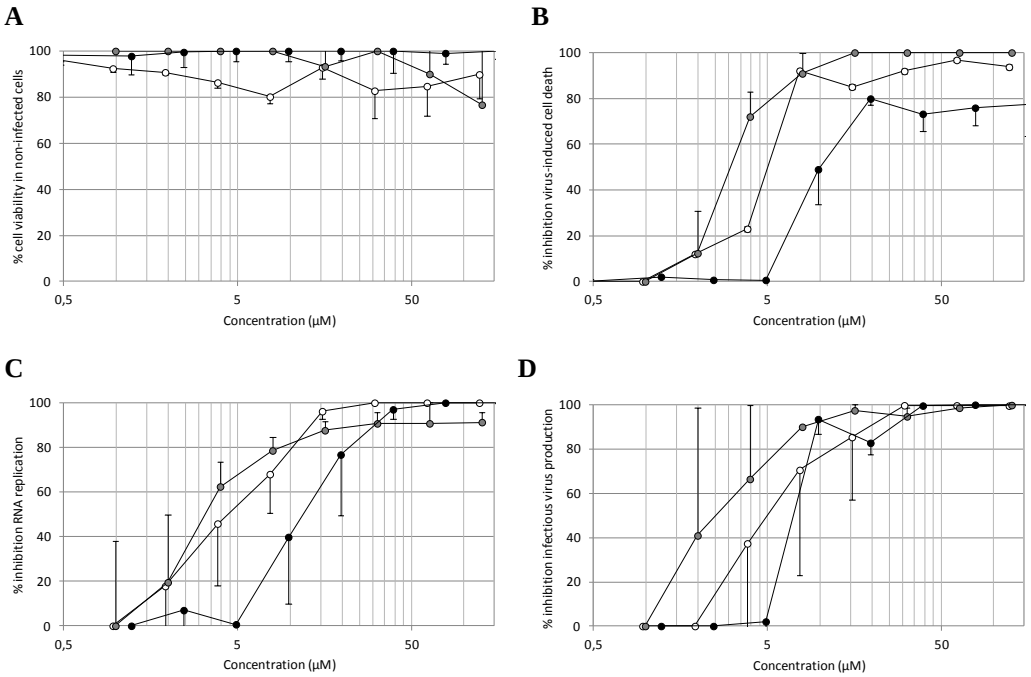
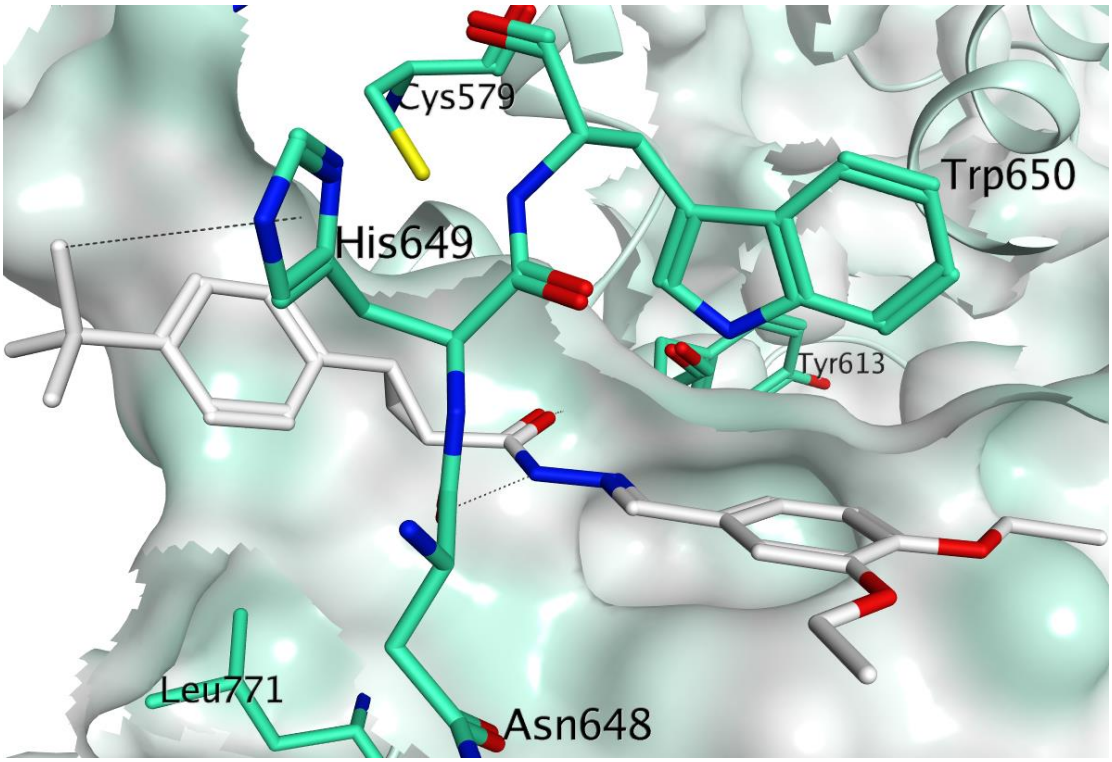
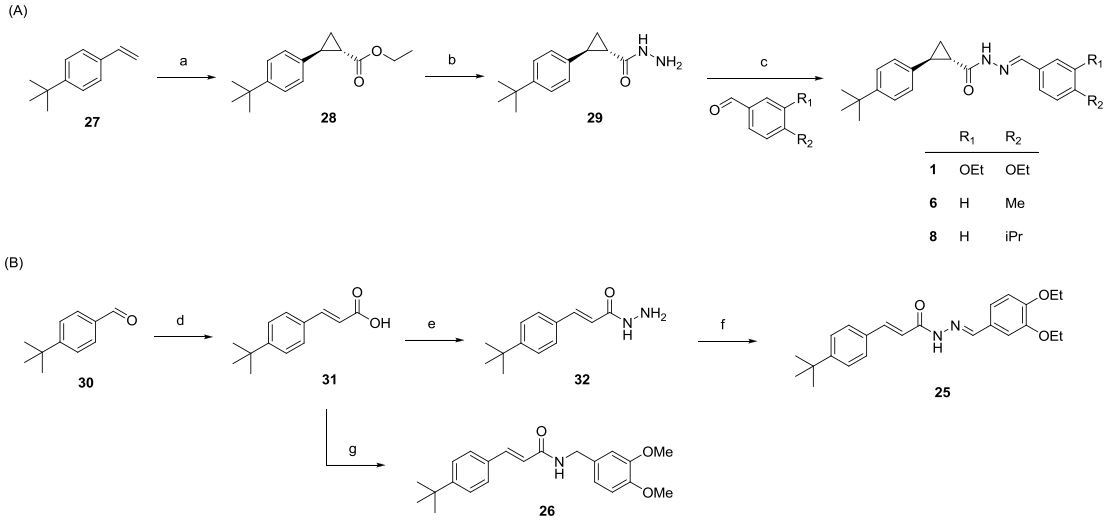


Fig. 4. Docking pose of **1** in the model of the CHKV nsP2 binding pocket.



Scheme 1. Synthesis of compounds **1**, **6**, **8**, **25** and **26**.[a]



[a]Reagents and reaction conditions: (a) ethyldiazoacetate, tert-buthylstyrene, 125 °C, 4 h, nitrogen atmosphere, R.T. overnight, yield 60%; (b) hydrazine monohydrate (60%), methanol, reflux, 12 h, yield 80%; (c) EtOH, reflux, 16 h, yield 20-90%; (d) malonic acid, catalytic piperidine, pyridine, R.T., 3 h, yield 95%; (e) i. oxalyl chloride, diethyl ether, R.T., 3 h, nitrogen atmosphere; ii. hydrazine monohydrate (60%), R.T., 2 h, yield 40%; (f) 3,4-diethoxybenzaldehyde, EtOH, reflux, 16 h, yield 25%; (g) 3,4-dimethoxybenzylamine, TBTU, diisopropylethylamine, anhydrous THF, R.T., 4 h, yield 96%.