Rational modifications on a benzylidene-acrylohydrazide antiviral scaffold, synthesis and evaluation of bioactivity against Chikungunya virus

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Highlights

- Rational modifications on a substituted benzylidene-acrylohydrazide scaffold with antiviral activity against CHIKV.
- Synthesis of different series of novel analogues with improved stability and drug-like properties.
- Different new inhibitors of the CHIKV replication identified.
- Molecular modelling studies on the CHIKV nsP2 protease.

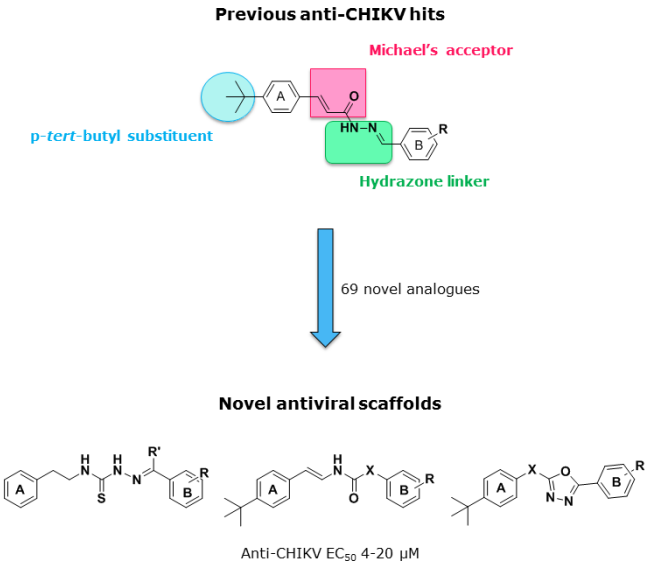
Keywords

Novel small-molecule antivirals; Chikungunya virus; structure-activity relationship studies; molecular modelling.

Abstract

Chikungunya virus is a re-emerging arbovirus transmitted to humans by *Aedes* mosquitoes, responsible for an acute febrile illness associated with painful and debilitating arthralgia, which can persist for several months or become chronic. Over the past few years, infection with this virus has spread worldwide with a previously unknown virulence. No specific antiviral treatments nor vaccines are currently available against this important pathogen. Starting from the structure of a class of selective anti-CHIKV agents previously identified in our research group, different modifications to this scaffold were rationally designed, and 69 novel small-molecule derivatives were synthesised and evaluated for their inhibition of Chikungunya virus replication in *Vero* cells. Further structure-activity relationships associated with this class of antiviral agents were elucidated for the original scaffolds, and novel antiviral compounds with EC_{50} values in the low micromolar range were identified. This work provides the foundation for further investigation of these new structures as antivirals against Chikungunya virus.

Graphical abstract



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1. Introduction

Chikungunya virus (CHIKV) is an Arbovirus, transmitted to humans by mosquito *Aedes Aegypti*, which belongs to the *Alphavirus* genus of the Togaviridae family, and it is associated with an acute pathology characterised by fever, rash and arthralgia. The last condition is often severe and has a tendency to persist for several months, becoming chronic in the 10% of infected individuals.[1] Since its first description in Tanzania in 1952, infection with this virus has had recurrent outbreaks in tropical regions and, from 2005, it has re-emerged with a previously unknown virulence in different parts of Africa, Indian Ocean islands, India and South-East Asia, also reaching Europe and the US.[2-3] The virus ability to adapt to a new vector, the mosquito *Aedes Albopictus*, has contributed to the worldwide spread of the infection.[4] Different clinically approved compounds such as chloroquine, alpha-interferon and ribavirin have been found to show some antiviral activity *in vitro*, but they demonstrated poor *in vivo* activity against CHIKV infection in animal models and, to date, no specific treatment is available, nor a vaccine is approved: the therapy is still limited to supportive treatment of the symptoms.[5-6] Even though research efforts on the identification and development of selective treatments against CHIKV have been rapidly increasing in the last few years, only few selective anti-CHIKV agents have been reported to date, and none of them has reached the clinical evaluation stage.[7]

As part of an ongoing project aiming to identify novel antiviral agents against Chikungunya virus, the structure of our previously reported hit **1** (**Figure 1**), designed and synthesised as inhibitor of the CHIKV life cycle following a docking-based virtual screening analysis of commercial compounds on the structure of the viral nsP2 protease,[8] has been further modified to improve its drug-like properties and gain further insights into the antiviral structure-activity relationships associated with its molecular scaffold. These novel structural modifications allowed a deeper understanding of the functional groups required for antiviral activity and the successful identification of novel anti-CHIKV agents with antiviral EC_{50} values in the low micromolar range and better drug-like characteristics.

2. Results and discussion

2.1 Rational design of novel analogues

The structure of hit **1** is characterised by the presence of two differently substituted phenyl rings, ring A and ring B, a hydrazone group directly attached to ring B, and an aliphatic spacer in the form of a trans double bond connecting ring A with the central hydrazide function. While different close analogues of **1** have been prepared and evaluated mainly to determine the role of the aromatic substituents and the presence of the trans double-bond and the hydrazone group,[9] the present study focuses on a series of 69 novel molecular derivatives, designed to replace the reactive Michael's acceptor in the structure of **1** and to susbtitute the hydrazone linker, expected to be poorly stable in aqueous environments, with more water-stable polar groups. As summarised in **Figure 1**, several new modifications to the original structures were carried out in order to: 1. explore different *para* hydrophobic aromatic substituents on phenyl ring A (**Series a**); 2. include the reactive Michael's acceptor trans double bond into a condensed aromatic or hetero-aromatic ring (**Series b**); 3. replace the Michael's acceptor trans double bond with a di-keto/enol function, thus also introducing an extra hydroxyl group in the molecule and increasing its polarity (**Series c**), or with a hydrazine-carboxamide or hydrazine-carbothioamide (**Series d**); 4. replace the hydrazone linker with either a urea function (**Series e**), or a double-hydrazide (**Series f**); 5. include the hydrazone linker into an oxadiazole ring (**Series g**), while also exploring three different spacers in the new molecular scaffold, the original trans double bond, a saturated ethylydene group (two-carbon spacer) or a shorter methylene function (one-carbon spacer); 6. include the hydrazone linker into a triazole ring (**Series h**); 7. condense the hydrazone linker and aromatic ring B in a terminal benzimidazole group (**Series i**), while maintaining the unsaturated double bond or reducing it to a saturated ethylic spacer.

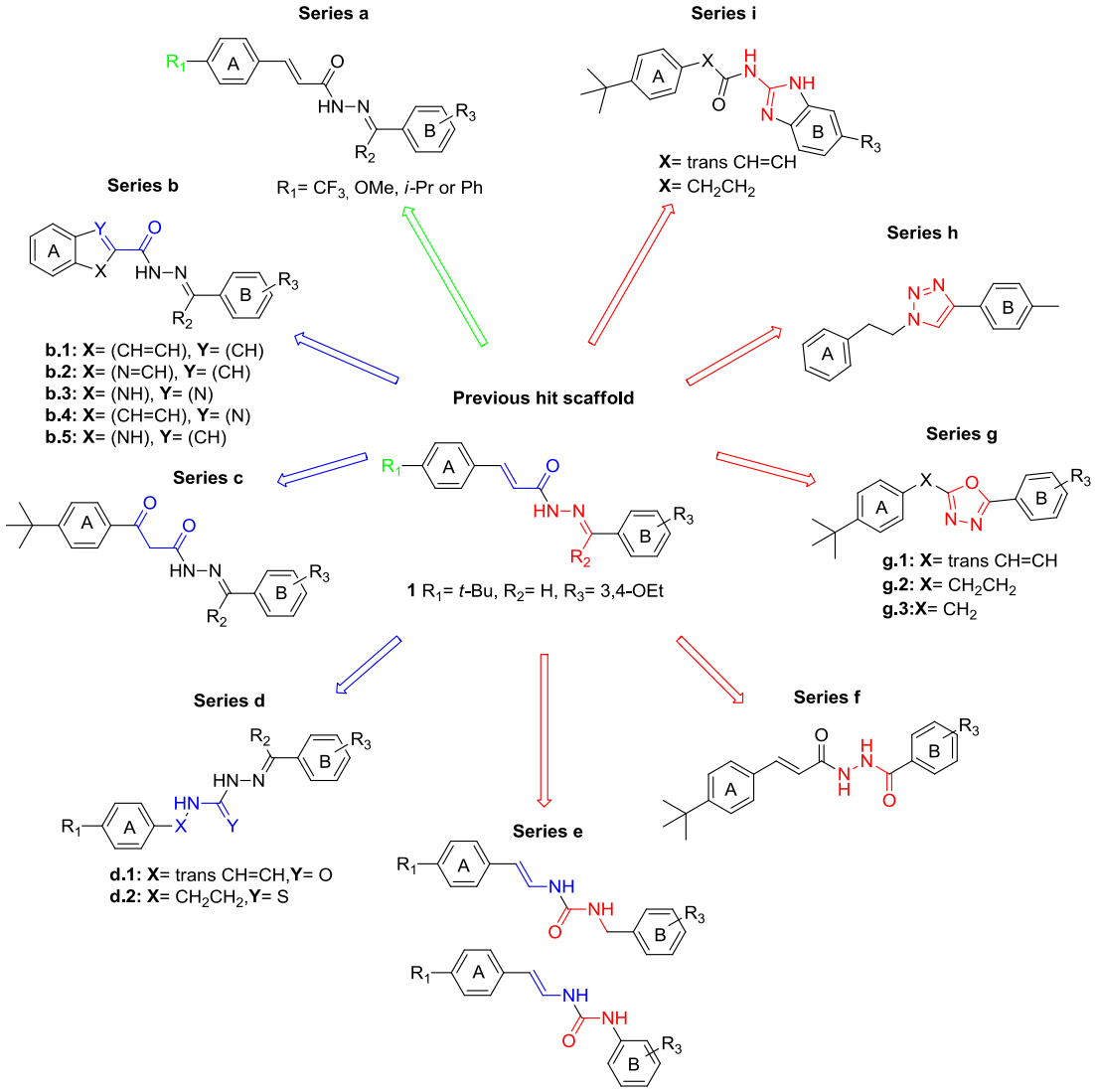


Figure 1. Structure of our previous antiviral hit and the different modifications carried out in the present study.

With the aim to take into account and to further confirm our previously reported structure-activity considerations,[8-9] **Series a** was designed to explore different hydrophobic substituents, other than a *tert*-butyl, a methyl or unsubstituted, in the *para* position of aromatic ring A, while for all the other series of derivatives either a *para tert*-butyl or an unsubstituted ring A was maintained. The substituents for ring B were instead chosen based on previous extensive modifications on this second phenyl group, maintaining those substitutions that were found to be associated with a better antiviral profile.[8-9] As depicted in **Figure 2**, **Series d.2** was envisaged in order to combine the structural features of our previous hit **1** with the left hand side portion of **2**, a structurally related in-house compound that, due to its similarities with **1**, has been chosen for evaluation as potential anti-CHIKV agent (**Table 1**). As reported in **Table 1** and highlighted in **Figure 2**, the antiviral effect of **2** seems to be mainly associated with a cytotoxic effect against the cells, possibly due to the presence of a rigid 2-tolyl-indole moiety on the right hand side of the structure.

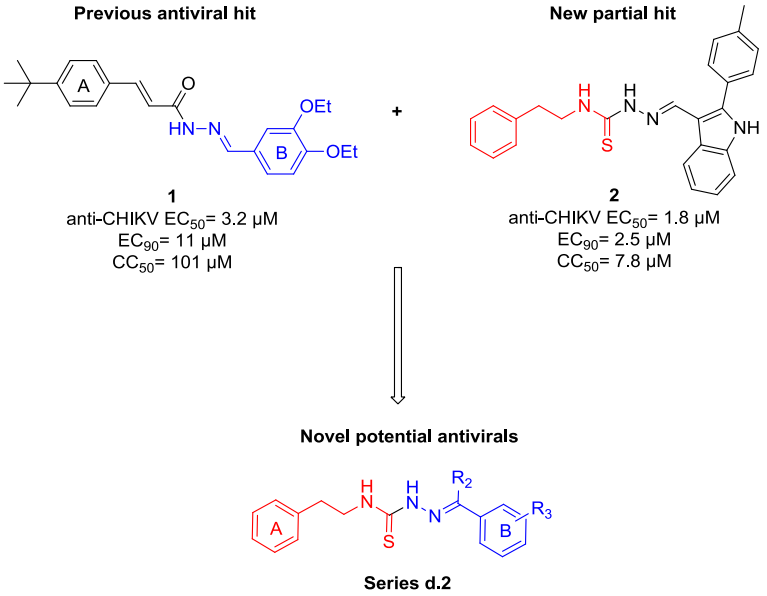
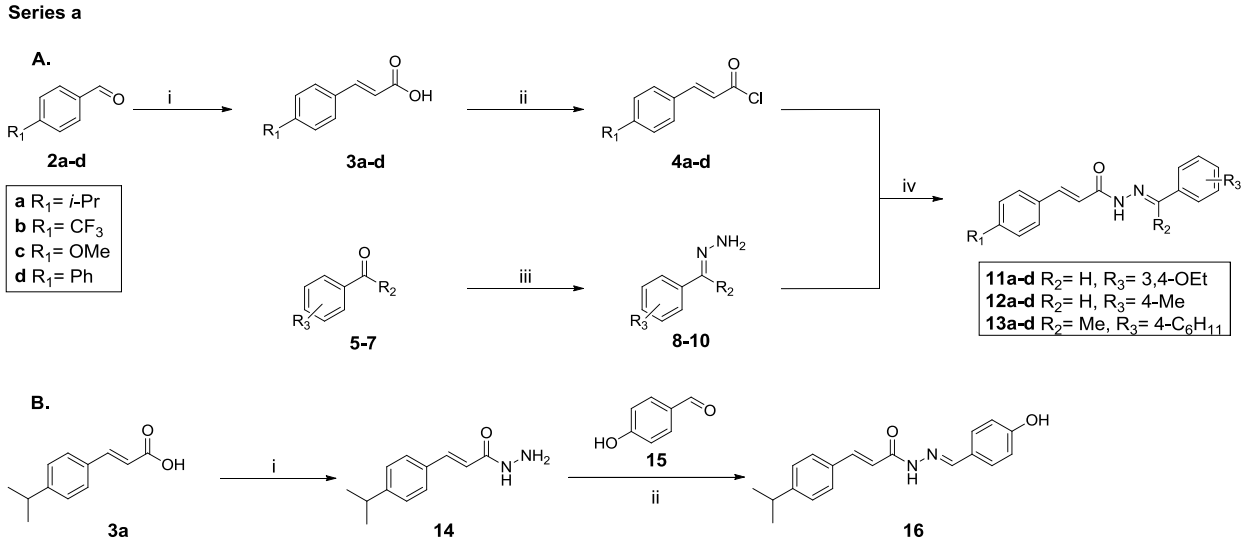


Figure 2. Rational design of novel potential antiviral agents belonging to **Series d.2**.

2.2 Chemistry

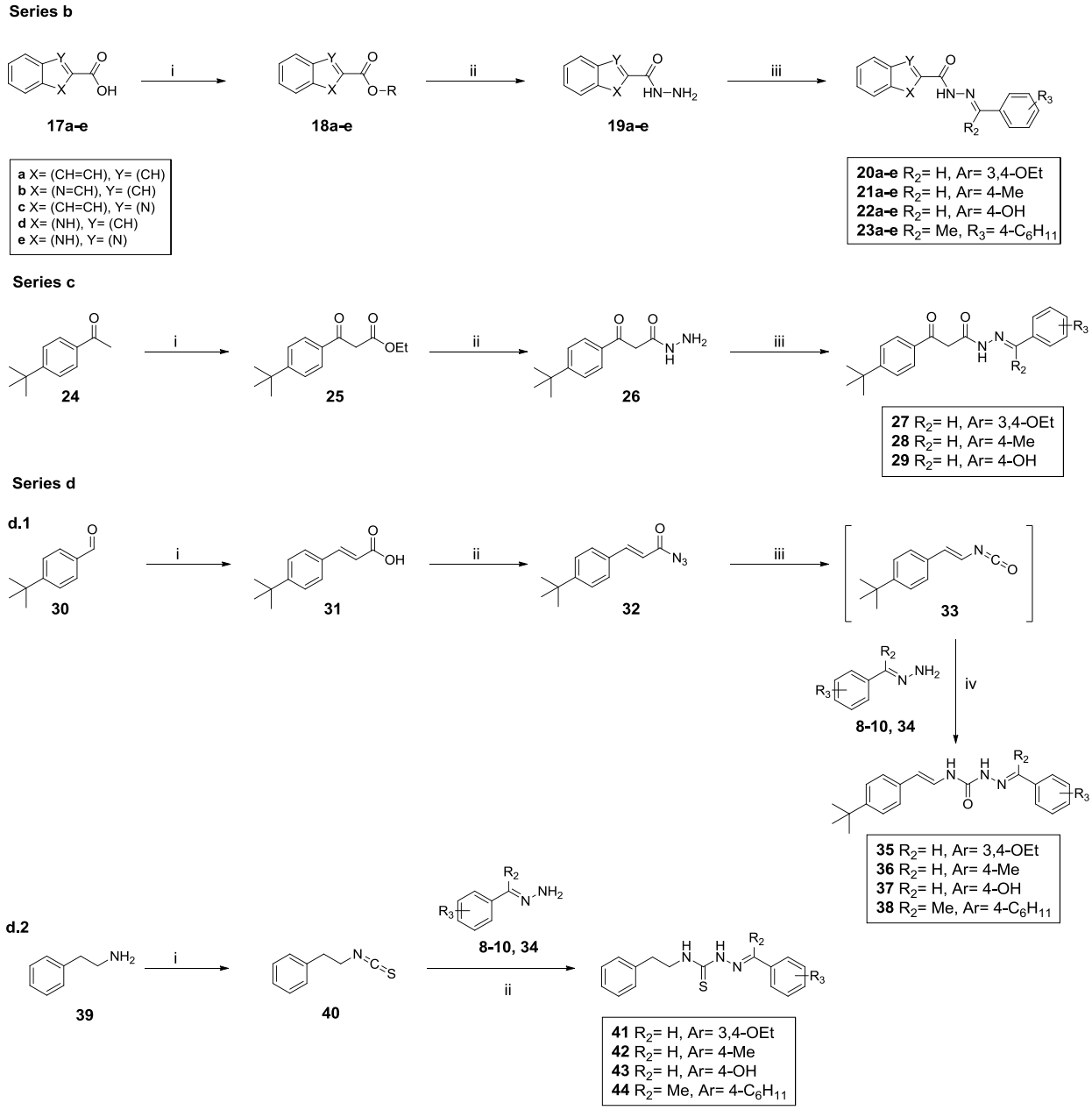
All target compounds were synthesised according to an optimised two to four-step synthetic pathway, as shown in **Schemes 1-5**.



Scheme 1: *Reagents and conditions*: A: i. malonic acid, cat. piperidine, pyridine, r.t., 3h (80-92%); ii. oxalyl chloride, Et_2O, r.t., 5 h (91-99%); iii. hydrazine monohydrate, EtOH, reflux, 3h (76-94%); iv. NEt_3, THF, r.t., o.n. (55-74%); B: i. (a) oxalyl chloride, Et_2O, r.t., 3 h, (b) hydrazine monohydrate, r.t., 2 h (35%); vi. EtOH, r.t., 72 h (42%).

A series of 12 novel benzylidene-acrylohydrazides was prepared to further explore the effect of the aromatic substituent in the *para* position of aromatic ring A, by replacing the original *tert*-butyl with an isopropyl, trifluoromethyl, methoxy and phenyl groups, respectively. Cinnamic acids **3a-d** were prepared following a Knoevenagel condensation- Doebner modification reaction between aldehydes **2a-d** and malonic acid, in the presence of catalytic amounts of piperidine and refluxing the mixture in pyridine.[10] Acid intermediates **3a-d** were then converted into the corresponding acyl chlorides **4a-d**, using oxalyl chloride.[11] Hydrazone intermediates **8-10** were prepared by refluxing aldehydes **5-7** with hydrazine monohydrate in ethanol, and these hydrazones were finally reacted with acyl chlorides **4a-d** at r.t. in THF, in the presence of triethylamine, thus yielding final desired hydrazones **11a-d**, **12a-d** and **13a-d**. This synthetic approach was also applied for the preparation of final hydrazones bearing a *para* hydroxyl in ring B, as this modification had previously been associated with an interesting antiviral profile in the original hit scaffold.[8] While the preparation of the intermediate hydrazine with the 4-OH group was successful (data not shown), the last step for the formation of the hydrazide bond did not work with this substituent in ring B, as a complex mixture of multiple by-products was obtained, from which it was not possible to isolate the desired final compound. This might be due to the presence of triethylamine in the reaction mixture, which might deprotonate the 4-hydroxyl group of the hydrazone intermediate, causing the formation of a nucleophilic centre that could react with the acryloyl chloride, thus preventing the desired reaction to take place. In order to obtain at least one final product carrying a 4-hydroxy group on aromatic ring B (**16**), an alternative synthetic strategy was applied. As we had previously reported for the synthesis of **1**,[8] cinnamic acid **3a** was converted into the corresponding chloride using oxalyl chloride in DCM, and then treated *in situ* with hydrazine monohydrate to give acrylo-hydrazide **14**,[12] which was finally reacted with 4-hydroxybenzaldehyde

15 to give the target compound **16**.



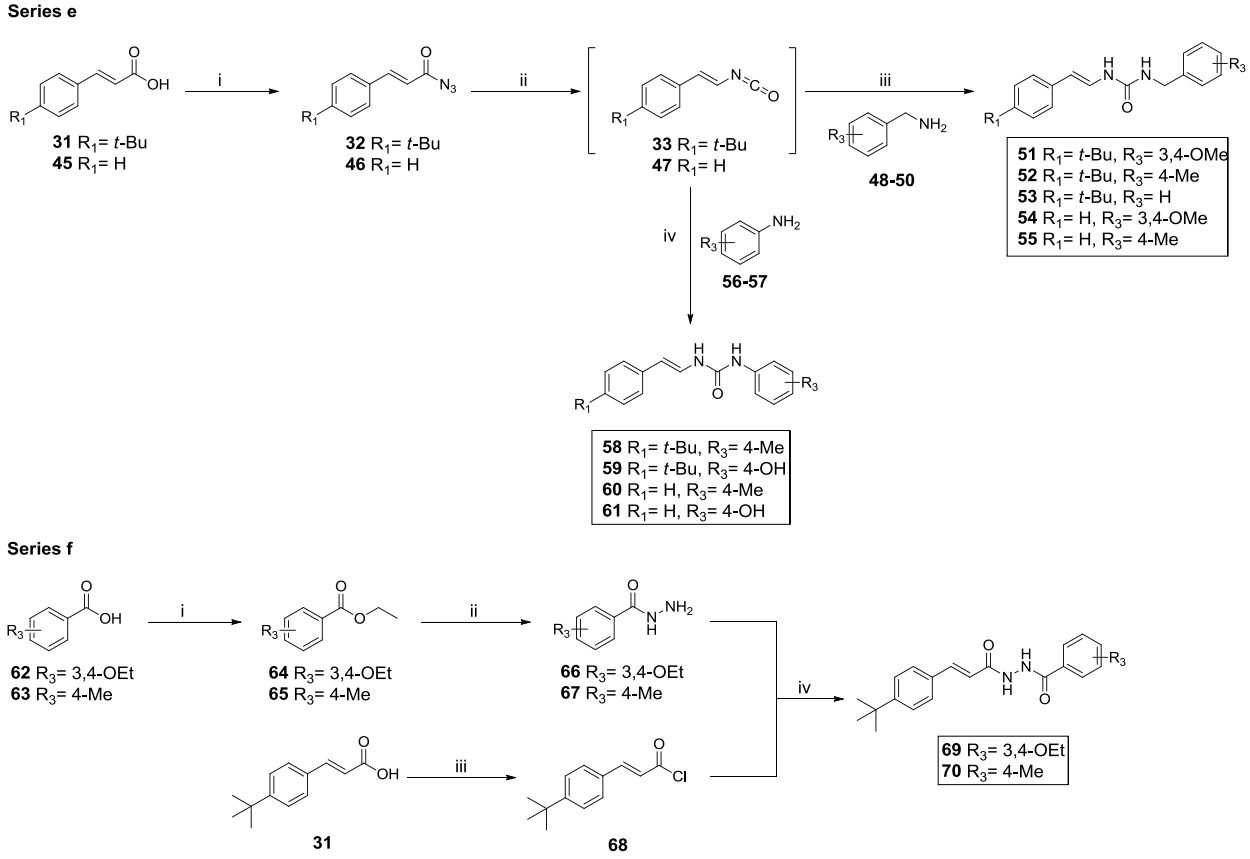
Scheme 2: *Reagents and conditions*: Series b: i. HCl, MeOH, reflux, o.n. (75-88%) or $SOCl_2$, EtOH, reflux, 24h (82-84%); ii. hydrazine monohydrate, EtOH, reflux, o.n. (79-87%); ii. differently substituted benzaldehyde, EtOH, reflux, 24 h (65-83%). Series c: i. diethyl oxalate, EtONa, EtOH, 0 °C, 30 min, r.t., 24 h (55%); ii. hydrazine monohydrate, EtOH, reflux, o.n. (56%); iii. differently substituted benzaldehyde, EtOH, reflux, 24 h (63-87%). Series d.1: i. malonic acid, cat. piperidine, pyridine, r.t., 3h (87%); ii. (a) ethyl chloroformate, Et_2O, 0 °C, 1h, (b) NaN_3, H_2O, 0 °C, 7h (95%); iii. PhMe, 120 °C, 4h; iv. r.t., o.n. (37-50%). Series d.2: i. $CSCl_2$, NEt_3, THF, 0 °C, 30 min, r.t., 30 min (100%); ii. PhMe, r.t., o.n. (35-44%).

A first attempt to modify the Michael's acceptor in the structure of **1** was its insertion into a condensed aromatic or hetero-aromatic ring (**Series b**). In particular, along with a naphthalene core, the hetero-aromatic moieties of 3- and 1-quinoline, 2-indole and benzimidazole were chosen as a means to partially increase the polarity and water solubility of the structure at the same time. A series of 20 final compounds was prepared with these modifications by converting starting acids **17a-e** into their methyl or ethyl ester counterparts, by reflux in 1.25M HCl in methanol (**18a-d**) or by reflux in EtOH in the presence of $SOCl_2$ (**18e**), and subsequently treating intermediate esters **18a-e** with hydrazine monohydrate in boiling EtOH to achieve the desired ester displacement in hydrazides **19a-e**.[13] Final compounds **20-23a-e** were obtained by reacting hydrazides **19a-e** with the appropriately substituted benzaldehyde or acetophenone in refluxing EtOH for 24 hours.

A small series of 3 final products (**Series c**) was envisaged to mask the trans double bond in **1** with an extra carbonyl group in β-position to the hydrazide carbonyl. As this β-di-keto group is expected to be in equilibrium with its enolic form, this modification should preserve the rigid geometry of the molecule, previously found important for the antiviral activity of **1**,[8] while increasing its polarity with the introduction of an extra hydroxyl group. To carry out this desired molecular alteration, acetophenone **24** was converted into di-keto ester **25** according to a Claisen condensation,[14] by treating it with diethyloxalate in EtOH, in the presence of sodium ethoxide. Ester **25** was then converted into the corresponding hydrazide **26** as described above for Series b, and this intermediate was reacted with the appropriate benzaldehyde to give final products **27-29**.

A further modification of the original linker of **1** was envisaged to insert an extra nitrogen between the trans double bond and the carbonyl group, thus obtaining an hydrazine-carboxamide spacer in the final products **35-38** (**Series d.1**). This alteration should alter the nature and the reactivity of the Michael's acceptor and increase the polarity of the final molecules; moreover, the linker length would be increased of one atomic position in comparison with **1** and, while the effect of shortening the spacer between the two phenylic portions of the molecule has already been explored,[9] the effect of an increased molecular size on the antiviral activity of the scaffold had not been evaluated so far. To obtain this modification, substituted cinnamic acid **31** was prepared through a Knoevenagel condensation between benzaldehyde **30** and malonic acid,[15] followed by conversion of this acid into the corresponding anhydride using ethyl chloroformate in the presence of triethylamine.[16] The anhydride was not isolated, but treated *in situ* with sodium azide in water, giving desired intermediate **32**. Isocyanate **33**, which was not isolated, was prepared through a Curtius rearrangement by refluxing azide **32** in toluene for 4 hours.[17] The reaction mixture was allowed to cool to r.t., and then the appropriate hydrazine **8-10** or **34** was added, thus giving the final desired products **35-38**.

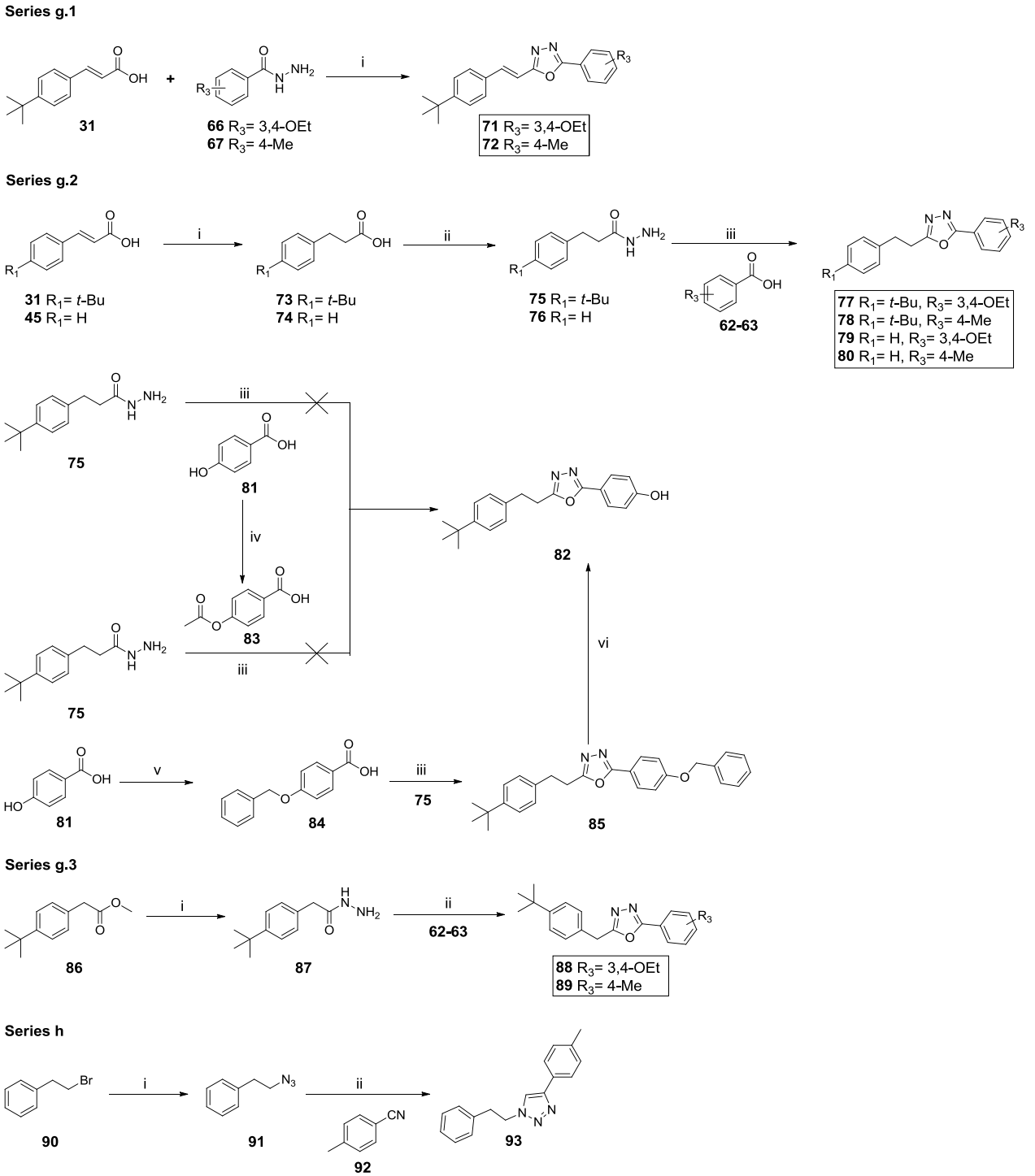
A final modification of the original Michael's acceptor group was designed, as described above, to combine the scaffold of **1** with hit **2**, a potential in-house antiviral candidate with toxicity issues possibly related with the presence of a tolyl-naphthyl substituent on its hydrazine-thioamide group (**Series d.2**). Final products **41-44** were obtained by converting phenylethylamine **39** into the corresponding isothiocyanate **40**, using thiophosgene in THF in the presence of triethylamine. Intermediate **40** was then treated with the appropriate benzaldehyde or acetophenone in toluene at r.t. to afford the target compounds **41-44** in moderate yields.



Scheme 3: *Reagents and conditions*: Series e: i. (a) ethyl chloroformate, Et_2O, 0 °C, 1h, (b) NaN_3, H_2O, 0 °C, 7h (70-95%); ii. PhMe, 120 °C, 4h; iii. r.t., o.n. (39-47%); iv. r.t., o.n. (42-55%). Series f: i. H_2SO_4, EtOH, reflux, o.n. (85-90%); ii. hydrazine monohydrate, EtOH, reflux, o.n. (78-86%); iii. oxalyl chloride, DCM, 0 °C to r.t., 5h. (99%); iv. NEt_3, THF, 0 °C to r.t., o.n. (25-50%).

A first attempt to replace the hydrazone bond in **1** with a more stable group in aqueous conditions was made by inserting a urea function as spacer between the trans double bond and aromatic ring B (**Series e**). This alteration was envisaged also as a means to mitigate the reactivity of the Michael's acceptor, and it was carried out both with a methylene spacer between the urea and ring B (**51-55**), thus maintaining the same overall length of the scaffold, and with the urea feature directly linked to ring B (**58-61**), to further assess the importance of the linker length for antiviral activity. The desired final compounds were obtained by reacting isocyanates **33** and **47**, not isolated but obtained *in situ* as described above for Scheme 2, with the corresponding benzylamine **48-50** or aniline **56-57**, at room temperature in toluene.

The hydrazone bond was also replaced with a more water stable double hydrazide in compounds **69-70**, while maintaining the original overall length of the spacer between the two aromatic portions of the molecule (**Series f**). This modification was achieved by reacting the appropriate hydrazide **66-67**, obtained by hydrazynolysis of the intermediate esters **64-65**, with acyl chloride **68**, isolated from the reaction of substituted cinnamic acid **31** with oxalyl chloride.

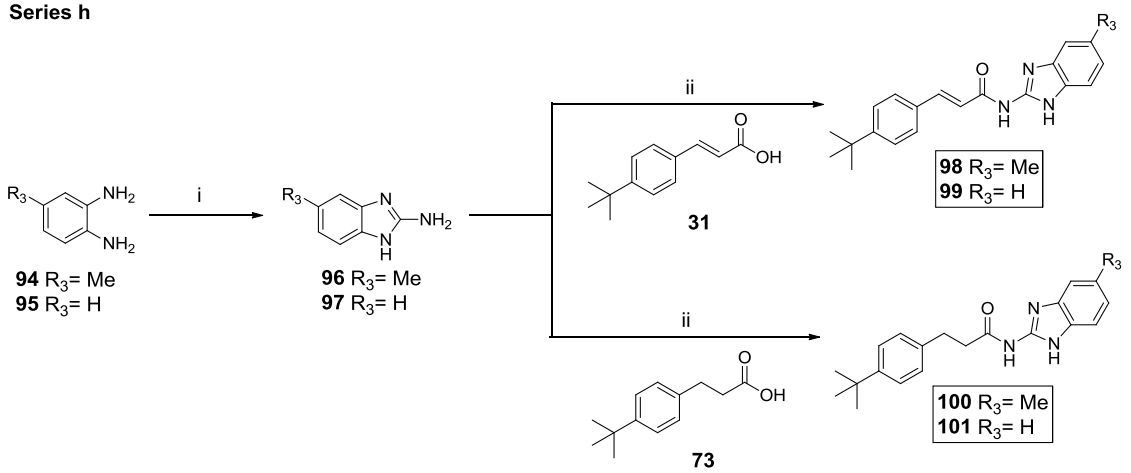


Scheme 4: *Reagents and conditions*: Series g.1: i. $POCl_3$, reflux, o.n. (52-69%). Series g.2: i. 10% Pd/C, H_2, THF, r.t., 4h (93-98%); ii. a) HCl, MeOH, reflux, o.n. (89-93%); b) hydrazine monohydrate, EtOH, reflux, o.n. (80-88%); iii. $POCl_3$, reflux, o.n. (63-75%); iv. acetic anhydride, H_2SO_4, 80 °C, 2h (95%); v. benzyl bromide, KOH, 9:1 EtOH: H_2O, reflux, 20h (99%); vi. 10% Pd/C, H_2, MeOH/THF, o.n. (51%). Series g.3: i. hydrazine monohydrate, EtOH, reflux, o.n. (88%); ii. $POCl_3$, reflux, o.n. (72-74%). Series h: i. NaN_3, DMF, 80°C, 24h (73%); ii. sodium ascorbate, $CuSO_4$, H_2O, DCM, r.t., 24h (37%).

The desired inclusion of the hydrazone bond of **1** in an oxadiazole ring was achieved by refluxing substituted cinnamic acid **31** with hydrazides **66-67** in $POCl_3$, obtaining final products **71-72** with a trans double bond between aromatic ring A and the newly introduced oxadiazole (**Series g.1**). As this modification is associated with a significant extra rigidity in the final structure, the contemporary reduction of the trans double bond was also envisaged in final products **77-80**, in which the extra rigidity of the oxadiazole ring is compensated with the increased flexibility of the aliphatic linker (**Series g.2**). This double modification was achieved by reducing cinnamic acids **31** and **45** under catalytic hydrogenation conditions. Reduced acids **73-74** were then converted to their methyl ester derivatives and then displaced with hydrazine monohydrate to give hydrazides **75-76**, which were finally reacted with acids **62-63** in refluxing phosphorus oxychloride to give the desired final products **77-80**. One final product in this series was designed with a 4-hydroxy function on aromatic ring A (**82**), however when refluxing **75** with **81** in phosphorus oxychloride, no trace of the desired product **82** could be observed, while a mixture of different impurities was formed. The reason for this undesired reactivity might rely on the interference of the free phenolic group with the reaction mechanism, via a potential

interaction with the carbonyl group of the hydrazide **75**. The free hydroxyl group of **81** was therefore acetylated into intermediate **83**, but also in this case the desired product was not obtained and the formation of a mixture of impurities was observed. The desired product **82** was finally obtained by protection of the free hydroxyl in **81** to benzyl ether in **84**, which was reacted with hydrazide **75** giving the desired intermediate **85** with moderate yield (52%). **85** was finally deprotected into the target molecule **82** by catalytic hydrogenation. A small series of two novel products, **88-89**, was obtained, following the same strategy, with a shorter aliphatic spacer of one methylene group between the oxadiazole and aromatic ring A, by converting methyl ester **86** into the corresponding hydrazide **87**, and then refluxing this hydrazide in $POCl_3$ in the presence of benzoic acid **62** or **63** (**Series g.3**).

Lastly, one final product with a triazole ring replacing the hydrazone bond (**93**, **Series h**) was obtained starting from 2-bromoethylbenzene **90**, which was treated with sodium azide to achieve the nucleophilic displacement of the bromide leaving group by the azide in intermediate **91**.[18] Intermediate azide **91** was then reacted with nitrile **92** according to a variation of Huisgen 1,3-dipolar cycloaddition, in which 1,4-disubstituted 1,2,3-triazole are formed in a region-specific manner by a copper(I)-catalysed reaction.[19-20] The catalyst is formed *in situ* by reduction of the Cu(II) salt ($CuSO_4$), using sodium ascorbate as the reducing agent.



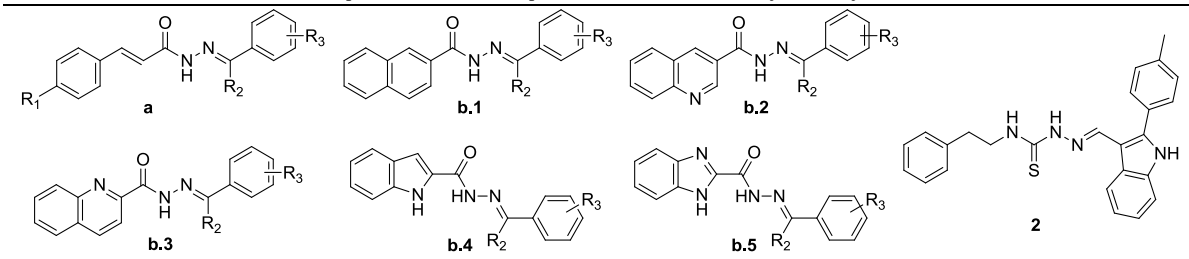
Scheme 5: *Reagents and conditions*: i. BrCN, 1:1 H_2O:MeOH, 50 °C, 1h (87-89%); ii. TBTU, DiPEA, THF, r.t., o.n. (35-53%).

One last attempt to replace the hydrazone bond in the original scaffold was made by condensing it with aromatic ring B in a benzimidazole heterocycle (**Series h**). This last modification was carried out both maintaining the trans double bond feature in the linker (**98-99**), and reducing it to a saturated ethylic spacer (**100-101**), in order to compensate for the higher rigidity of the new moiety in comparison with the original phenyl-hydrazone. A small series of 4 final products was obtained by reacting *ortho* phenylendiamines **94** and **95** with cyanogen bromide at 50 °C in a water-methanol mixture, thus isolating amino-benzimidazoles **96** and **97**. The final target products were then obtained with a coupling reaction between aromatic amines **96-97** and the appropriate acid **31** or **73**, using TBTU as coupling reagent in the presence of diisopropyethylamine.[21]

2.3 Antiviral and cytotoxicity studies

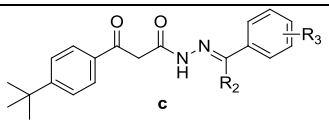
The newly prepared compounds were evaluated for their potential anti-CHIKV activity in Vero cells, investigating their ability to inhibit the cytopathogenic effect (CPE) induced by the virus, and for their potential cytotoxicity. The known anti-CHIKV agent Chloroquine was included as positive control.[22]

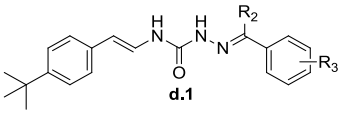
Table 1: Antiviral effect of the test compounds on CHIKV replication in Vero cells and cytotoxicity.

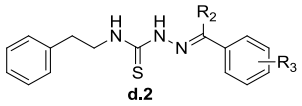


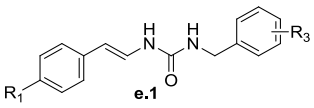
Comp.	Scaffold	R_1 (ring A)	R_2	R_3 (ring B)	EC_{50}(μM)[a,d]	EC_{90}(μM)[b,d]	CC_{50}(μM)[c,d]	SI[e]	Max. inhib.[f]	Max at [μM][f]
1[g]	a	*t*-Bu	H	3,4-OEt	3.2±1.8	11±4	101±50	32	100%	12.5
2	-	-	-	-	**1.8±0.9**	**2.5±0.8**	**7.8±0.3**	**4.4**	**100%**	**2.5**
11a	a	*i*-Pr	H	3,4-OEt	>29.2	>29.2	n.d.	-	30%	8
11b	a	CF_3	H	3,4-OEt	48.9±1.2	n.d.	84.1±16.8	1.7	75%	69
11c	a	OMe	H	3,4-OEt	>271	>271	n.d.	-	8%	45
11d	a	Ph	H	3,4-OEt	>80.2	>80.2	n.d.	-	47%	120
12a	a	*i*-Pr	H	4-Me	25.9	n.d.	25±5.9	1	50%	20

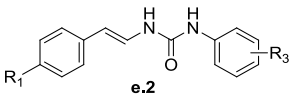
12b	a	CF_3	H	4-Me	96.3±16.1	>150	203±41	2.1	61%	99
12c	a	OMe	H	4-Me	>112	>112	n.d.	-	13%	112
12d	a	Ph	H	4-Me	230	>294	>294	>1.3	78%	294
13a	a	*i*-Pr	Me	4-C_6H_{11}	>193	>193	n.d.	-	13%	193
13b	a	CF_3	Me	4-C_6H_{11}	>241	>241	n.d.	-	12%	241
13c	a	OMe	Me	4-C_6H_{11}	>87.7	>87.7	n.d.	-	9%	66
13d	a	Ph	Me	4-C_6H_{11}	>29.6	>29.6	n.d.	-	15%	30
16	a	*i*-Pr	H	4-OH	>324	>324	n.d.	-	46%	36
20a	b.1	-	H	3,4-OEt	>276	>276	n.d.	-	24%	276
20b	b.2	-	H	3,4-OEt	>275	>275	n.d.	-	3%	275
20c	b.3	-	H	3,4-OEt	>275	>275	n.d.	-	8%	275
20d	b.4	-	H	3,4-OEt	>213	>213	n.d.	-	50%	213
20e	b.5	-	H	3,4-OEt	>284	>284	n.d.	-	6%	284
21a	b.1	-	H	4-Me	>347	>347	n.d.	-	10%	347
21b	b.2	-	H	4-Me	>346	>346	n.d.	-	27%	346
21c	b.3	-	H	4-Me	>346	>346	n.d.	-	3%	346
21d	b.4	-	H	4-Me	>361	>361	n.d.	-	45%	361
21e	b.5	-	H	4-Me	>120	>120	n.d.	-	14%	120
22a	b.1	-	H	4-OH	>344	>344	n.d.	-	2%	344
22b	b.2	-	H	4-OH	>343	>343	n.d.	-	<1%	343
22c	b.3	-	H	4-OH	>343	>343	n.d.	-	<1%	343
22d	b.4	-	H	4-OH	348	>358	n.d.	-	51%	358
22e	b.5	-	H	4-OH	>357	>357	n.d.	-	33%	357
23a	b.1	-	Me	4-C_6H_{11}	>270	>270	n.d.	-	14%	270
23b	b.2	-	Me	4-C_6H_{11}	>202	>202	n.d.	-	19%	202
23c	b.3	-	Me	4-C_6H_{11}	>269	>269	n.d.	-	13%	269
23d	b.4	-	Me	4-C_6H_{11}	>209	>209	n.d.	-	41%	209
23e	b.5	-	Me	4-C_6H_{11}	2.6	n.d.	6.2±0.04	2.4	83%	4.3

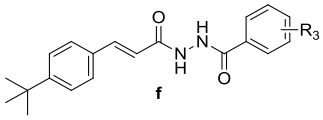




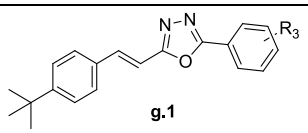




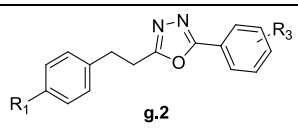




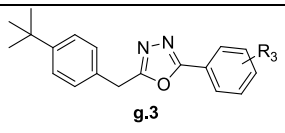
Comp.	Scaffold	R_1 (ring A)	R_2	R_3 (ring B)	EC_{50}(μM)[a,d]	EC_{90}(μM)[b,d]	CC_{50}(μM)[c,d]	SI[e]	Max. inhib.[f]	Max at [μM][f]
27	c	-	H	3,4-OEt	>244	>244	81±53	-	4%	244
28	c	-	H	4-Me	>74	>74	84±54	-	13%	74
29	c	-	H	4-OH	7.2±1.3	8	14.7±3.3	2.3	87%	7.9
35	d.1	-	H	3,4-OEt	>100	>100	n.d.	-	49%	100
36	d.1	-	H	4-Me	246±52.1	>298	192±38.7	0.8	50%	187
37	d.1	-	H	4-OH	5.6±0.4	7.5	10.4±2.3	1.9	89%	7.4
38	d.1	-	Me	C_6H_{11}	42.3±15.7	78.8	123±34.7	2.9	90%	79
41	d.2	-	H	3,4-OEt	**5.9±1.1**	**9.8±2.8**	**109±29.5**	**18**	**90%**	**9.3**
42	d.2	-	H	4-Me	>336	>336	n.d.	-	12%	56
43	d.2	-	H	4-OH	48.3±19.9	n.d.	87.8±44	1.8	67%	51
44	d.2	-	Me	C_6H_{11}	**4.3±0.5**	**5.9±0.5**	**52.9±11.6**	**12.2**	**90%**	**6**
51	e.1	*t*-Bu	-	3,4-OMe	**11.6**	**14.8**	**n.d.**	**-**	**100%**	**17**
52	e.1	*t*-Bu	-	4-Me	>103	>103	n.d.	-	10%	103
53	e.1	*t*-Bu	-	H	>107	>107	n.d.	-	8%	107
54	e.1	H	-	3,4-OMe	>320	>320	n.d.	-	13%	320
55	e.1	H	-	4-Me	>376	>376	n.d.	-	18%	376
58	e.2	*t*-Bu	-	4-Me	>61	>61	n.d.	-	16%	61
59	e.2	*t*-Bu	-	4-OH	**9.1**	**11.9**	**n.d.**	**-**	**100%**	**4.1**
60	e.2	H	-	4-Me	>396	>396	n.d.	-	21%	396
61	e.2	H	-	4-OH	>393	>393	n.d.	-	2%	197
69	f	-	-	3,4-OEt	>244	>244	n.d.	-	11%	6.3
70	f	-	-	4-Me	>99.1	>99.1	n.d.	-	9%	99.1



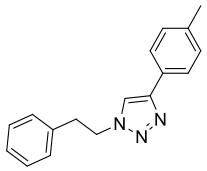
g.1



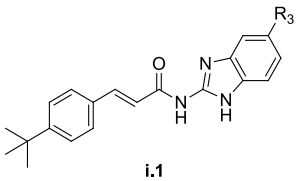
g.2



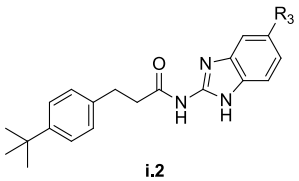
g.3



h (93)



i.1



i.2

Comp.	Scaffold	R_1 (ring A)	R_2	R_3 (ring B)	EC_{50}(μM)[a,d]	EC_{90}(μM)[b,d]	CC_{50}(μM)[c,d]	SI[e]	Max. inhib.[f]	Max at [μM][f]
71	g.1	-	-	3,4-OEt	**29**	**88.2**	**n.d.**	-	**100%**	**127.4**
72	g.1	-	-	4-Me	>157	>157	n.d.	-	8%	157
77	g.2	*t*-Bu	-	3,4-OEt	12.4±2.1	n.d.	15.5±3.1	1.2	61%	13.5
78	g.2	*t*-Bu	-	4-Me	**17.2**	**21.7**	**n.d.**	-	**100%**	**26**
79	g.2	H	-	3,4-OEt	>98.4	>98.4	n.d.	-	4%	73.9
80	g.2	H	-	4-Me	>378	>378	n.d.	-	2%	1
82	g.2	*t*-Bu	-	4-OH	108±31.7	>103	191±47.1	1.8	69%	103
88	g.3	-	-	3,4-OEt	39.7	n.d.	n.d.	-	15%	131.4
89	g.3	-	-	4-Me	**17.9**	**22.7**	**n.d.**	-	**100%**	**27.2**
93	h	-	-	-	122±15	n.d.	200±47.5	1.6	74%	163
98	i.1	-	-	Me	>300	>300	n.d.	-	47%	300
99	i.1	-	-	H	>157	>157	n.d.	-	38%	157
100	i.2	-	-	Me	>298	>298	n.d.	-	8%	1.6
101	i.2	-	-	H	>311	>311	n.d.	-	41%	311
Chloroquine	-	-	-	-	11±7	21±18	89±28	8.1	-	-

[a] EC_{50} = 50% effective concentration (concentration at which 50% inhibition of CPE is observed).

[b] EC_{90} = 90% effective concentration (concentration at which 50% inhibition of CPE is observed).

[c] CC_{50} = 50% cytostatic/cytotoxic concentration (concentration at which 50% adverse effect is observed on the host cell).

[d] The EC_{50}, EC_{90} and CC_{50} values are the mean of at least 3 independent experiments, with standard deviations of ±10% of the value quoted unless otherwise stated (mean value ± standard deviations).

[f] Maximum observed inhibition of the virus-induced CPE and concentration (μM) at which maximum inhibition is reached.

[g] Biological data as previously reported.[8]

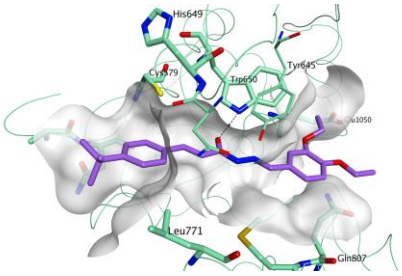
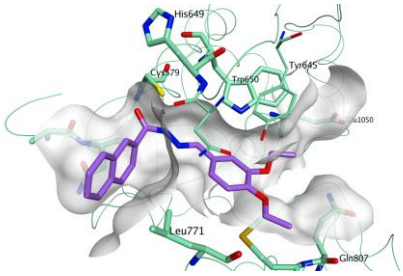
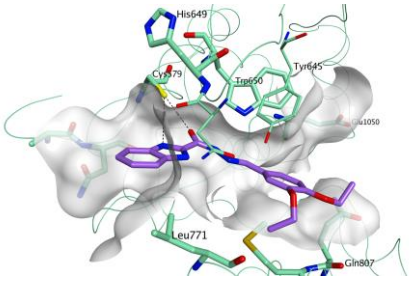
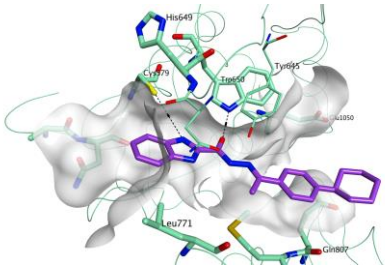
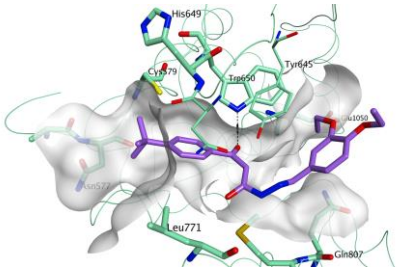
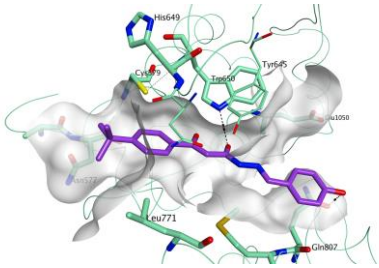
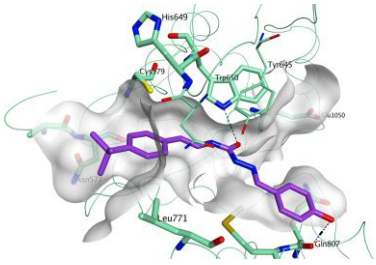
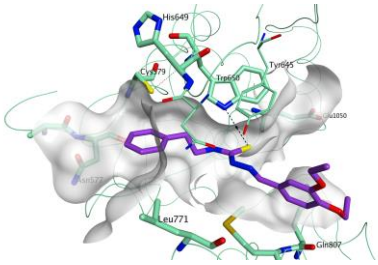
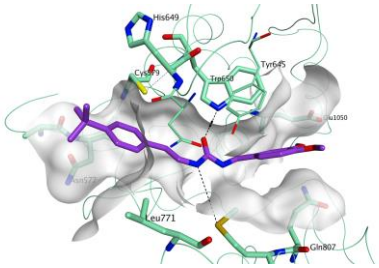
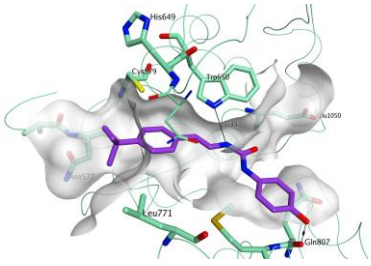
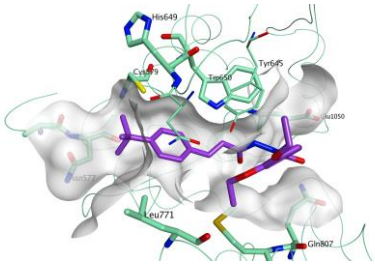
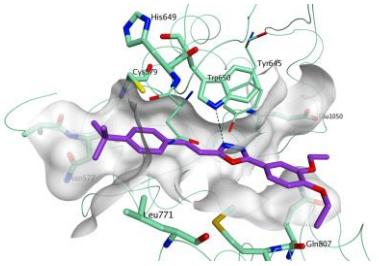
n.d.= value could not be calculated.

Considering together both antiviral activity results and cytotoxicity associated with the novel compounds, the presence of a para *tert*-butyl substituent in the original scaffold of **1**, as partially observed previously,[8] appears to be important for antiviral activity, as its replacement with different groups (*iso*-propyl, trifluoromethyl, methoxy or phenyl, **Series a**) in compounds **11-13a-d** and **16** is associated with a complete loss of activity. The only derivatives which retain some antiviral effect in the micromolar range are **11b**, **12a** and **12b**, but for these compounds the selectivity index SI is low, indicating that their antiviral activity is associated with a toxic effect on the cells. Also the attempted enclosure of the trans double bond of **1** into a condensed aromatic or heteroaromatic ring in **20-23a-e** (**Series b**) is associated with a complete abolishment of antiviral effect, even if these modifications are not associated with cytotoxicity. The only analogue in this series which still retains some activity is **23e**, but also in this case the antiviral effect of the molecule appears to be correlated to cytotoxicity. The attempted replacement of the Michael's acceptor with a β-di-carbonyl group in **27-29** (**Series c**) is associated with loss of antiviral activity and increased cytotoxicity for **27** and **28**, while **29** still retains some antiviral potential, but its toxic effect is increased in comparison with **1**, and its SI value low. Replacement of the hydrazide spacer with a hydrazine-carboxamide (**Series d.1**) is associated with a complete loss of activity for **35** and **36**, while **37** and, to less extent, **38** still maintain antiviral EC_{50} values in the low micromolar (**37**) or micromolar (**38**) range, but they are also associated with increased cytotoxicity. A more positive effect is observed for the combination of the structures of **1** and **2** (**Series d.2**), as analogues **41** and **44** in this series are associated with retained antiviral activity in comparison with **1**, even if with lower SI values, and reduced cytotoxicity in comparison with **2**. Replacement of the hydrazone spacer with a urea function while maintaining the original length of the molecule (**Series e.1**) is associated with antiviral activitity retention in analogue **51**, but this modification appears to be tolerated only with the concomitant presence of a 3,4-dimethoxy substituent on aromatic ring B, as the other analogues in this series (**52-53**) do not show any residual antiviral effect. In the case of the novel urea analogues with a shorter linker (**Series e.2**), derivative **59** is associated with activity retention in comparison with **1**, but also in this case, the other analogues belonging to this series (**58**, **60-61**) are associated with loss of antiviral activity. Conversion of the hydrazide linker to a more stable double hydrazide in **69** and **70** (**Series f**) is associated with complete loss of antiviral activity. Inclusion of the hydrazone bond in an oxadiazole ring (**Series g.1**, **g.2** and **g.3**) appears to be tolerated in all three sub-series of structures (with a trans-double bond, saturated ethylene and shorter methylene spacer, respectively), but with a different effect found for the substituent in aromatic ring B: in **Series g.1**, a 3,4-diethoxy feature is associated with activity retention (**71**), while the 4-methil analogue **72** is inactive; in **Series g.2**, while the presence of the *tert*-butyl substituent in ring A is further confirmed as important for activity retention, a 4-methyl group in ring B is associated with

antiviral activity retention (**78**), while the presence of a 3,4-diethoxy substituent is linked to increased cytotoxicity; in **Series g.3**, **89** (4-methyl group in ring B) retains the original antiviral activity without showing relevant cytotoxicity, while a 3,4-diethoxy feature in ring B is correlated with loss of antiviral effect. Finally, inclusion of the hydrazone group in a triazole ring in **93** (**Series h**) is associated with partial antiviral activity retention, even if at high concentrations, while the attempted condensation of the hydrazone group with ring B in a benzimidazole ring in **98-101** (**Series i.1** and **i.2**) is associated with a complete loss of antiviral activity.

2.4 Molecular docking studies on CHIKV nsP2 protease

As hit **1** was identified as selective antiviral agents against CHIKV following docking-based studies on the catalytic pocket of the CHIKV nsP2 protease, and a recent study has shown that three of our previously reported close analogues of **1** inhibit the protheolitical activity of CHIKV nsP2 *in vitro*,[23] the predicted binding to the nsP2 protease active site was investigated for the novel compounds prepared, aiming to identify a rational explanation for the differences in activity found for the new structures. The Glide SP docking algorithm was used.[24-25] The predicted binding to the nsP2 protease found for original hit **1** and representative novel analogues of each series explored in this work is shown in **Figure 3**.

1 	**20a** 	**20e** 
23e 	**27** 	**29** 
37 	**41** 	**51** 
59 	**69** 	**71** 
78	**89**	**93**

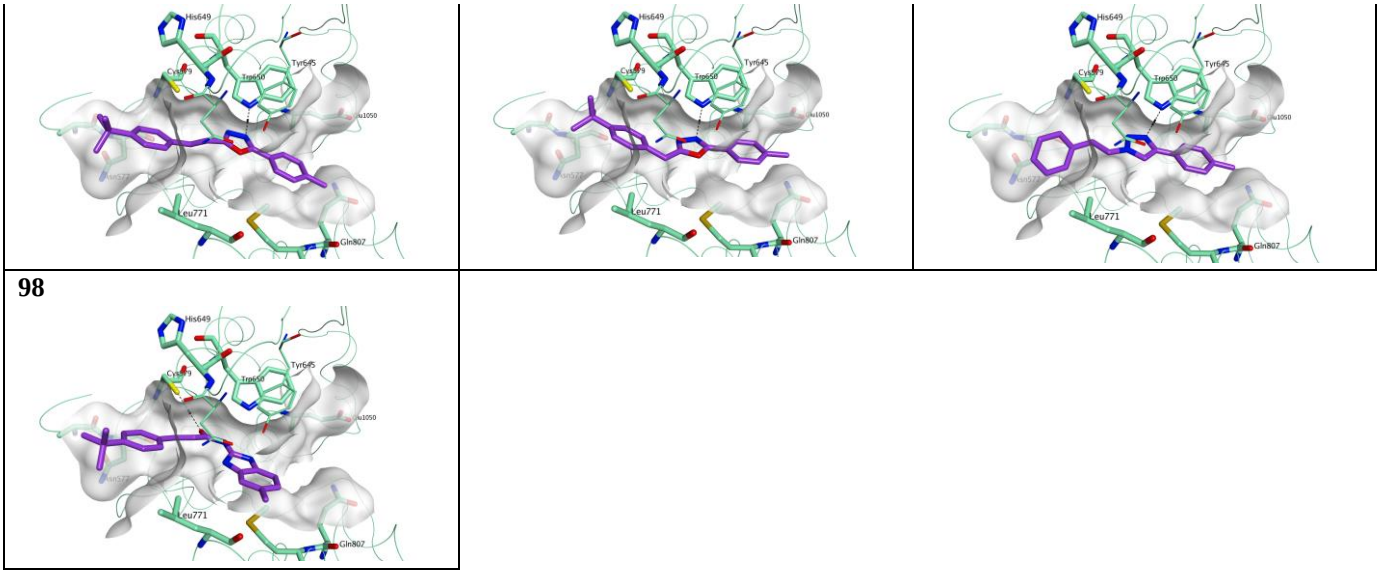


Figure 3. Predicted binding mode of **1a** and representative analogues of each series of novel compounds to the proteolitic site of CHIKV nsP2 (PDB ID 3TRK).

According to our molecular docking studies summarised in **Figure 3**, the hydrazone bond of **1** fits the central part of the proteolytic site of the enzyme and is involved, through its carbonyl oxygen, in a direct hydrogen bond with the side chain of Trp650, which forms the enzyme catalytic triad together with Cys579 and His649. The two aromatic portions, even though not involved in direct interactions with the surrounding enzyme residues, appear important to anchor the central part of the molecule in the region defined by the catalytic triad. Even if, as expected, a very similar predicted binding mode is shared by all the novel analogues of **Series a** (**11-13a-d**, **16**, data not shown), in which different *para* aromatic substituents have been explored for ring A, the presence of a *tert*-butyl in this position appears to be key to an optimal fitting of the catalytic pocket, being all the attempted modifications associated with loss of antiviral activity. The predicted binding for **Series b**, where the trans double bond of the Michael's acceptor is included in a condensed aromatic ring, reveals for most of the derivatives in this series, exemplified in **Figure 3** by **20a** and **20e**, an altered occupation of the target site, where the space optimally fitted by ring B of **1a** is not completely reached and the hydrogen bond with Trp650 is lost. These observations seem to be in line with the loss of antiviral activity found for this series. An exception to this trend is represented by **23e**, for which the presence of a p*ara*-cyclohexyl substituent on ring B combined with a condensed benzimidazole as ring A appears to be associated with a conserved overall fitting of the target site, and this observation might explain the residual activity found for this analogue (**Table 1**). As represented by the docking results found for **27**, the replacement of the Michael's acceptor with a β-dicarbonyl group in **Series c** is associated with a perturbed occupation of the catalytic pocket, with ring A shifted towards the catalytic triad and an incomplete fitting of the spatial portion on the left hand side of Cys579. This effect appears to be compensated by the presence of a 4-hydroxy substituent in ring B, which is expected to stabilise the same occupation of the target site found for **1a** by forming an extra hydrogen bond with the backbone of Gln807, thus providing a rational explanation to its residual antiviral effect. The same observation can be made for **Series d.1**, where the perturbed fitting of the pocket due to the presence of a longer linker is compensated for **37** by the formation of an extra hydrogen bond with Gln807. In **Series d.2**, the presence of the hydrazine-carbothioamide linker associated with a saturated ethylene spacer and an unsubstituted ring A appears to be associated with a much less consistent occupation of the target pocket, due to the extra flexibility in the structure, which can be compensated by a retained hydrogen bond with Trp650 and the presence of a bulky substituent (**41**) in ring B, which still allows to anchor the molecule on the sub-pocket on the right hand side of the catalytic triad, thus explaining the retention of antiviral activity. The same effect of a bulky ring B substituent can be observed in fact for **44** (data not shown). This same observation of the necessity of a bulky substituent in ring B for binding and activity retention, can be extended to **Series e.1**, where the concomitant presence of a 3,4-dimethoxy group in ring B and a 4-*tert*-butyl in ring A appears to be essential to anchor the molecule on the target site, thus preserving the potential for hydrogen bond formation with Trp650 and the antiviral activity (**51**). In **Series e.2**, this effect of stabilisation of the binding mode is achieved with the presence of a 4-hydroxy substituent (**59**), which is predicted to form an extra hydrogen bond with the lateral chain of Gln807. As exemplified by the docking results found for **69**, the presence of a double-hydrazide linker in **Series f** is associated with a distorted occupation of the target site and the loss of the hydrogen bond with Trp650, thus providing a reasonable explanation to the loss of antiviral activity found for this series. As can be observed for the predicted binding mode found for **71**, **78** and **89**, the replacement of the hydrazone linker with an oxadiazole ring in **Series g.1**, **g.2** and **g.3**, respectively, appears to be associated with a retained potential occupation of the target site, with the predicted maintenance of the hydrogen bond with Trp560 and with a similar fitting of the binding pocket for all three sub-series of structures, which are associated, at least for the three analogues shown in **Figure 3**, with antiviral activity retention, even if with higher EC_{50} values in comparison with **1a-b**. Analogue **93** (**Series h**) is expected to only partially fit the catalytic pocket of the protease, due to the absence of substituent in ring A and to the flexibility of its linker: also in this case, the docking results obtained

appear to support the experimental data, which highlight loss of antiviral activity for this compound. Finally, **Series i**, as shown in **Figure 3** for the predicted binding found for **98**, is not expected to allow an optimal, linear occupation of the target pocket, as the hydrazone bond is rigidly condensed with ring B into a benzimidazole group: this observation is consistent with the biological results, which demonstrate loss of antiviral activity for this series of novel structures. The correlation between the results obtained with docking simulations and the experimental data appears to represent a useful validation of the predicted binding mode of these agents to the CHIKV nsP2 protease, and such docking studies might now be used with relative confidence to make an estimation on the potential activity of novel analogues, possibly directing future synthetic efforts.

3. Conclusions

Starting from the structures of two anti-CHIKV hits, several series of novel analogues were designed and 69 new derivatives were synthesised to expand the structure-activity relationships associated with these antiviral agents, and to improve their drug-like and water-stability properties. Different structural elements were confirmed as essential for antiviral properties, such as the presence of a *tert*-butyl substituent on ring B, the successful replacement of the Michael's acceptor present in the original hit structures was achieved by the insertion of a hydrazine-carbothioamide (**41**, **44**), and a viable replacement of the original hydrazone linker has been achieved with a urea (**51**, **59**) or an oxadiazole linker (**71**, **78**, **89**). The differences in biological activities found for the various series of novel analogues were rationally explained and correlated with the predicted binding to the CHIKV nsP2 protease with a series of molecular docking studies, and these considerations might now be used to direct future synthetic efforts. Different novel antiviral agents with anti-CHIKV EC_{50} values in the low micromolar range were identified, and these findings provide the foundation for further investigation of these new structures as antivirals against Chikungunya virus. Further structural improvements towards the identification of preclinical antiviral candidates are the focus of present investigations and will be reported in due course, along with the confirmation of the mechanism of action of these compounds.

4. Experimental

4.1 Synthetic chemistry methods

All solvents used for chromatography were HPLC grade from Fisher Scientific (UK). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance DPX500 spectrometer operating at 500 and 125 MHz, with Me_4Si as internal standard. Mass spectra were determined with a Bruker microTOF spectrometer using electrospray ionization (ESI source). For mass spectra, solutions were made in HPLC grade methanol. Flash column chromatography was performed with silica gel 60 (230–400mesh) (Merck) and TLC was carried out on precoated silica plates (kiesel gel 60 F_{254}, BDH). Compounds were visualised by illumination under UV light (254 nm). Melting points were determined on an electrothermal instrument and are uncorrected. All solvents were dried prior to use and stored over 4 Å molecular sieves, under nitrogen. All final compounds were more than 95% pure.

Details for the preparation and full characterisation of the new target final compounds are described in the Supporting Information. All intermediates were generally prepared according to literature procedures, which are described in detail along with compound characterisation in the Supporting Information.

4.2 Virus cell-based CPE reduction assay

Chikungunya virus, Indian Ocean strain 899, isolated in 2006, was cultured on African green monkey kidney (Vero) cells (ATCC CCL-81) in minimum essential medium MEM Rega3 (Invitrogen, Belgium) supplemented with 10% Foetal Bovine Serum (FBS; Integro, The Netherlands), 1% L-glutamine and 1% sodium bicarbonate (Invitrogen). Antiviral assays were performed in MEM Rega-3 medium supplemented with 2% FBS.

Vero cells were seeded in 96-well tissue culture plates (Becton Dickinson Falcon 96-Well Cell Culture Plate) at a density of 2.5 x 104 cells/well in 100 µl assay medium and were allowed to adhere overnight in an incubator (37 °C, 5% CO2, 95-99% RH). Next, a compound dilution series was added after which the cultures were infected with 0.01 MOI of CHIKV 899 in 100 µl assay medium and returned to the incubator. Each assay was performed in multiplicate in the same test and assays were repeated independently. On day 5 post-infection (p.i.), the cell viability in each assay well was measured using the MTS/PMS method as described by the manufacturer (Promega, The Netherlands). Chloroquine was included in the assay as a reference compound.[25] The 50% effective concentration (EC_{50}) is defined as the compound concentration that is required to inhibit virus-induced cytopathogenic effect (CPE) by 50% and was determined using logarithmic interpolation employing a custom-designed database-coupled interface (Accelrys, United Kingdom). The anti-metabolic effect of the compounds was evaluated in uninfected, compound-treated cells, also by means of the MTS/PMS method. The 50% cytostatic/cytotoxic concentration (CC_{50}) is defined to be the concentration of compound that reduces the overall metabolic activity of the uninfected, compound-treated cells by 50%, and was calculated using logarithmic interpolation. All assay wells were checked microscopically for minor signs of virus-induced CPE or alterations of the cell or monolayer morphology.

4.3 Molecular Modelling

All molecular modelling studies were performed on a MacPro dual 2.66 GHz Xeon running Ubuntu 14.04. CHIKV nsP2 protease crystal structure was downloaded from the PDB data bank (http://www.rcsb.org/; PDB code 3TRK). Hydrogen atoms were added to the protein, using the Protonate 3D routine of the Molecular Operating Environment (MOE2015.10).[26] Ligand structures were built with MOE and minimized using the MMFF94x force field until a RMSD gradient of 0.05 kcal mol_1 was reached. The Maestro LigPrep tool[27] was used to prepare the ligands using the default settings, whereas the protein was prepared using the preparation wizard tool.[28] The docking simulations were performed using Maestro Glide SP using the default parameters.[24]

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Appendix A. Supplementary data
All experimental procedures and compound characterisation data for the reaction intermediates are reported and described in detail in the Supporting Information.

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