Discovery of Novel multi-target Indole-based Derivatives as Potent and Selective Inhibitors of Chikungunya Virus Replication

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Abstract

We recently identified indole derivatives (**IIIe** and **III f**) with anti-chikungunya virus (CHIKV) activities at lower micro molar concentrations and a selective index of inhibition higher than the lead compound Arbidol. Here we highlight new structural information for the optimization of the previously identified lead compounds that contain the indole chemical core. Based on the structural data, a series of indole derivatives was synthesized and tested for their antiviral activity against chikungunya virus in Vero cell culture by a CPE reduction assay.

Systematic optimization of the lead compounds resulted in tert-butyl-5-hydroxy-1-methyl-2-(2-trifluoromethylsulfonyl)methyl)-indole-3-carboxylate derivative **IIc** with a 10-fold improved anti-CHIKV inhibitory activity ($EC_{50} = 6.5 \pm 1 \mu M$) as compared to Arbidol demonstrating a potent, selective and specific inhibition of CHIKV replication with only a moderate cell protective effect against other related alphaviruses. The reported computational insights, together with the accessible synthetic procedure, pave the road towards the design of novel synthetic derivatives with enhanced anti-viral activities.

Introduction

Chikungunya virus (CHIKV) is a re-emerging arbovirus that belongs to the genus alphavirus of the *Togaviridae* family. An infection with this virus results in chikungunya fever (CHIKF), with symptoms starting generally 4–7 days after the mosquito bite. The infection is occurring in two phases, the first being acute, characterized by fever and joint pains, while the second stage is persistent (chronic), causing disabling polyarthritides.[1] Although during the mid twentieth century epidemiological emergency of CHIKV was limited to Africa, the virus spread to India and South-East Asia starting from 2004, causing large scale epidemics. After that, CHIKV transmission was also reported for the first time in Europe. In December 2013, the first local transmission was observed in the Americas, resulting in over 1,5 million infected patients. To date, despite its serious nature, no drugs have been approved for the treatment of CHIKV, but promising in vitro results have been obtained with Arbidol (ARB), a drug that already has been licensed for the treatment of influenza A and B virus infections in Russia and China.[2]

So far, Arbidol has shown a wide range of activity against many RNA, DNA, enveloped and non enveloped viruses[3] and recently also against CHIKV replication in immortalized Vero cells or primary human fibroblasts (MRC-5 lung cells) ($EC_{50} < 10 \mu\text{g/mL}$).[4] The exact mechanism of the anti-CHIKV activity of Arbidol is not entirely understood. A previously reported study demonstrated its involvement in blocking the early stages of the viral life cycle.[4] Moreover, a single mutation from glycine to arginine in the E2 protein (G407R) generated an Arbidol resistant-CHIKV mutant giving an important insight of the mechanism of action. Gly407 is localised in the “wings” insertion of domain A, a region that could be involved in the interaction with different receptors.[4]

Starting from Arbidol we identified several indole derivatives that inhibit CHIKV replication in phenotypic virus cell-based assays. [5] We demonstrated that the combined effect of an increased steric hindrance on the thiophenol ring and the replacement of an ethyl group in position 2 with a tert-butyl were necessary to improve the in vitro antiviral potency of this class of compounds. Moreover, oxidation of the sulfur atom in the linker led to a reduced cellular toxicity while preserving or increasing the antiviral activity. In particular, two new compounds **IIIe** and **IIIf** (**Chart 1**) had an antiviral activity higher than the other derivatives (EC_{50} 30 μ M-32 μ M) with a selective index of inhibition 13.2 and 14.6 respectively, which is higher than Arbidol.

Arbidol

IIIe

EC_{50} : 30 $\pm$ 4 CC_{50} : 397 $\pm$ 24

SI: 13.2

IIIf

EC_{50} : 32 $\pm$ 1.1 CC_{50} : > 468

SI: 14.6

Chart1

Here we describe the optimization of the previously identified indole derivatives based on novel structural information using the crystal structure of the CHIKV glycoprotein complex and the characterization of the mechanism of action of the anti-CHIKV activity of this series of compounds.

2. Results and discussion

2.1. Docking studies

In order to elucidate the potential binding pocket of Arbidol in the E2 viral protein and to suggest a hypothetical binding mode for the new indole derivatives, different molecular modelling studies were performed. The E2 unit of the crystal structure of the mature E3-E2-E1 glycoprotein complex (PDB ID: 3N42) was used in the simulations (Gly407 corresponds to Gly82 in the crystal structure). [6] Using the site finder tool in MOE 2015.4, two potential binding sites in proximity of Gly82 were identified (site 1 and site 2; **Figure 1**).

Figure 1

Docking studies of Arbidol on site 1, the closest site to Gly82, gave inconsistent results, indicating that this small hydrophilic and shallow pocket is probably not the binding site for Arbidol (**Figure 2b**). Site 2 consists of a more hydrophobic and well-defined pocket formed by Trp64, Arg80, Met97, Thr96 and Thr160 (**Figure 2a**). Gly82 is not directly involved in site 2 formation but it should be noted that this residue is located in a very flexible loop and it can be speculated that a substitution to arginine, a significantly bigger residue, could change the loop conformation affecting the overall architecture of the proposed site 2. Moreover, Arbidol has been reported to interact with tryptophan-rich molecules. [7]

Taking this into account, site 2 was considered the most relevant for our docking simulations. The results showed that Arbidol occupies the binding site inserting the ethyl ester group and the thiophenol deep in the pocket, whereas the dimethyl amino moiety and the hydroxyl group are more solvent exposed. Moreover, contacts between the ethyl ester and Trp64 and Arg80 are observed.

Figure 2a

Figure 2b

A different binding mode seems to be prevalent for the previously reported derivatives **IIIe** and **IIIf**. In this case, the presence of the bulkier t-butyl ester and the differently substituted thiophenols do not allow the same orientation as Arbidol. The indole ring of these derivatives sits deeper in the active site, in proximity of Trp64, while the differently substituted thiophenol occupies the more solvent exposed portion of the site, the same occupied by the indole ring of Arbidol. Finally, as shown in **Figure 3a and 3b**, the t-butyl group of compound **IIIe and IIIf** occupies a small hydrophobic sub-pocket, formed by Thr160 and Thr96, possibly increasing the binding affinity. This correlates well with the increased biological activity often observed with the analogues that contain this ester.

Figure 3a

Figure 3b

Based on the above results, the design and synthesis of indole based molecules as new potential entry inhibitors was continued. In particular, from a structural point of view, the synthesized molecules can be assigned to three main groups:

I) Indole t-butylester derivatives with the replacement of bromine in position 5 and phenol group in position 4 as the lead Arbidol,

II) The most interesting compounds of series **I** were converted again into corresponding sulfoxides to confirm oxidation of sulfide to lead a reduction in cellular toxicity while preserving or increasing antiviral properties.

III) Selected derivatives from the two previous series were analyzed regarding the simultaneously introduction of amine group and thiol oxidation.

We decided to use the same structural decorations in each subset of compounds in order to enable the identification of structural elements required to increase the antiviral effects and to confirm that the replacement of a hydroxyl group in position 5 is necessary to improve the in vitro antiviral potency of this class of compounds.

2.2. Chemistry

Indole derivatives **Ia–g** and **IIa–f** were synthesized according to **Scheme 1**. The synthesis of 5-hydroxy-indole derivatives started from commercially available tert-butyl acetoacetate **3**, which was easily converted into imine giving compound **4** in high yields. It was reacted with 1,4-benzoquinone in presence of catalytic amount of ZnI_2 as described to Nenitzescu reaction giving intermediate **5**. After protection of hydroxyl group using acetic anhydride and pyridine, N-alkylation with iodomethane was carried out by a modified Kikugawa's procedure [10] to afford compound **6** in quantitative yields. Treatment with 1 equiv of NBS allowed to obtain selective allylic brominated [11] compound **7** and subsequent treatment with several substituted

thiophenols in methanol provided esters **Ia–g**. The synthesis of sulfoxides **IIa–f** was obtained starting from **Ia–f** derivatives, that were oxidized using 77% meta chloroperbenzoic acid in CH_2Cl_2 at room temperature in very short time (5 min). Reaction with N,N,N',N' -Tetramethylethylenediamine and acetic acid yielded the final compounds **IV a-d**.

Scheme 1

2.3 Effect of Arbidol derivatives on the cytopathogenic effect (CPE) induced by CHIKV

All synthesized compounds (**Ia–g**, **IIa–f**, **IVa–d**) were evaluated for cytotoxicity and anti-CHIKV activity in Vero cells, more specifically for their ability to inhibit the cytopathogenic effect (CPE) induced by the virus. The results are summarized in **Tables 1–3**. Regarding the *t*-butylseries, (**Series I**) it is worth to note that the replacement of the methylhydroxyl group in position 5 with a hydroxyl group resulted in compounds with increased antiviral activity even though this was associated with significant cytotoxicity (EC_{50} values ranging from 2.9 to 4.8 μ M). As previously shown the presence of an electron-withdrawing substituent (such as fluorine, trifluoromethyl or chlorine) in the para position of the thiophenol ring yields molecules with an antiviral effect, more pronounced than that of Arbidol. On the other hand, when a trifluoromethyl group or chlorine was introduced in *orto* position, only a weak improvement in antiviral effect was observed. Analysis of the structure–activity relationships revealed that the bromine on the indole backbone is not essential for the inhibitory antiviral activity (**Id** vs **Ic** and **Ig** vs **Ib**). Derivatives with the lowest EC_{50} values were converted into their corresponding sulfoxides (**Series II**), keeping in mind that the oxidation of the sulfide is expected to reduce the cellular toxicity by reasons previously mentioned. The results are summarized in **Table 2**. The oxidation to sulfoxides significantly reduced the cytotoxicity of all compounds, with improved antiviral activity with only slightly higher EC_{50} values for **IIa**. Moreover, it can be observed that all amine compounds were less active even in the case of the corresponding bromide derivatives (**Table III**). Based on these data, compound **IIc** emerged as a very interesting compound, as it showed a better therapeutic index compared to Arbidol and the previous analogs with a 10-fold improved anti-CHIKV inhibitory activity ($EC_{50} = 6.5 \pm 1 \mu$ M). Hence, it was selected together with the

previous described trifluoromethyl derivative **III**f for further experiments to characterize the anti-CHIKV activity.

Series I

CHIKV inhibition					
Cpd	R ₁	R ₂	^a CC ₅₀ (μM)	^b EC ₅₀ (μM)	SI ^c
Ia	H	4-CF ₃	11	3.6	3.1
Ib	H	2,6-Cl	7.8	2.9	2.7
Ic	H	2-CF ₃	19	3.4	4.1
Id	Br	2-CF ₃	26	9.6	2.7
Ie	H	4-F	11	4.5	3.2
If	H	4-Cl	15	4.8	3.2
Ig	Br	2,6-Cl	13	5.7	2.3
Arbidol			161±18	35±8	4.6

Table 1

Series II

CHIKV inhibition					
Cpd	R ₁	R ₂	^a CC ₅₀ (μM)	^b EC ₅₀ (μM)	SI ^c
IIa	H	4-CF ₃	24	13	1.9
IIb	H	2,6-Cl	151	20	7.6
IIc	H	2-CF ₃	156	6.5±1	22
IId	Br	2-CF ₃	26	9.6	2.7
IIf	H	4-F	>100	17 ±3	>5.7
IIe	H	4-Cl	93	8.2 ±6	11
Arbidol			161±18	35±8	4.6

Table 2

Series IV

CHIKV inhibition						
Cpd	R ₁	R ₂	X	^a CC ₅₀ (μM)	^b EC ₅₀ (μM)	SI ^c
IVa	Br	2,6-Cl	-S	17	≥17	-
IVb	H	2,6-Cl	-S=O	9.2	4.1	2.2
IVc	H	2-CF ₃	-S=O	66	22	3
IVd	Br	2-CF ₃	-S=O	56	12	4.6
Arbidol				161±18	35±8	4.6

Table 3

2.4 Virus yield assay

To evaluate the direct inhibitory effect of **IIc** on CHIKV replication, a virus yield assay was performed and compared with the most active compound of the precedent library **III f**. Interestingly, **IIc** reduced in a dose-dependent manner both viral RNA levels as well as the production of infectious virus particles (**Figure 4, Table 4**) and thus can be considered as a selective inhibitor of CHIKV replication and not only a cell-protective compound. On the other hand, the compound showed only moderate antiviral activity against related alphaviruses Semliki Forest virus and Sindbis virus.

Compound	CC ₅₀ ± SD (μM)	EC ₅₀ ± SD (μM)		
		CHIKV	SFV	SINV
IIc	156 ± 4	6.5 ± 1 ^a 1 ± 0.2 ^b 1 ± 0.1 ^c	33 ± 2 ^a	20 ± 1 ^a
III f	> 468 ⁶	28 ± 4 ^a 9 ± 3 ^b 10 ± 2 ^c	128 ± 35 ^a	96 ± 19 ^a
Arbidol	161 ± 18 ⁶	35 ± 7 ^a	ND	ND

a) CPE reduction assay, b) RT-qPCR or c) titration assay. ND: not determined

Table 4

Fig.4

2.5. CHIKVpp assay

The activity of **IIc** and **IIIb** was further evaluated in an entry assay using CHIKV pseudoparticles (CHIKVpp). CHIKVpp are lentiviral vectors carrying CHIKV glycoproteins on the exterior and a luciferase reporter gene inside the vesicles and are thus ideally suited to study CHIKV entry independently from post entry steps in the viral life cycle. BGM cells were treated with the compound after which they were exposed to the appropriate dilution of CHIKVpp. After 3 days, the intracellular luciferase signal was measured, which is proportionate to the amount of CHIKVpp that were able to enter the cells in the presence of the compound. Surprisingly, **IIc** was ~6-fold less potent than Arbidol in the inhibition of CHIKVpp entry into BGM cells even though **IIc** is a more potent inhibitor of CHIKV replication than Arbidol (**Figure 5a, Table 5**), suggesting that **IIc** may have a different mechanism of action.

Compound	CC ₅₀ ±SD (μM)	EC ₅₀ ±SD (μM)
IIc	50 ± 5	27 ± 1.6
IIIb	> 60	36 ± 0.9
Arbidol	> 60	4.4 ± 1.2

Table 5

2.6 Time of addition assay

In a time of addition assay, the compound was added at different time points before and after infection to determine at which time point in the viral life cycle it loses its activity and this in parallel with reference compounds with known mechanism of action. **IIc** inhibited CHIKV replication at a post-entry step as it showed good inhibition of CHIKV replication even when added to cells up to 8h post infection. In contrast, Arbidol is only able to inhibit CHIKV replication with more than 50% when added before or 2h after infection (**Figure 5b**).

A **B** **Fig.5**

2.7 Docking studies II

Following our previous molecular docking simulations, the same studies on series I and II were performed. The newly synthesized compounds, as expected, showed a binding mode similar to the one observed for derivatives **IIIe** and **IIIf**, with the t-butyl ester inserted in to the lateral sub-pocket, the indole core occupying the deepest part of the site and the thiophenol ring positioned in the more solvent exposed portion of the pocket. **Figure 6** reports compound **IIc** in the active site.

Fig.6

Based on the CHIKVpp data and the docking results, it could be speculated that the different binding to E2 observed for the new derivative **IIc** and for compound **IIIb** (as consequence of the structural modifications) could be responsible for the decrease in the inhibition of CHIKVpp entry into BGM cells, whereas Arbidol occupation of the binding pocket is resulting in a much larger inhibition of CHIKVpp entry.

3. Conclusion

Here we highlight the progress towards the discovery of CHIKV inhibitors with an indole core structure. From our structure activity relationship analysis Tert-butyl-5-hydroxy-1-methyl-2-(2-trifluoromethylsulfonyl)methyl)-indole-3-carboxylate (**IIc**) emerged as a new inhibitor of CHIKV replication at a post-entry step of the viral life cycle, besides a modest effect on viral entry, suggesting that additional inhibitory mechanisms may be present that contribute to its potent antiviral activity.

4. Experimental section

4 Chemistry

All reagents were analytical grade and purchased from Sigma–Aldrich (Milano, Italy). Flash chromatography was performed on Carlo Erba silica gel 60 (230–400 mesh; CarloErba, Milan, Italy). TLC was carried out using plates coated with silica gel 60F 254 nm purchased from Merck (Darmstadt, Germany). ^1H and ^{13}C NMR spectra were registered on a Bruker AC 300. Chemical shifts are reported in ppm. Yields are given for isolated products showing one spot on a TLC plate and no impurities detectable in the NMR spectrum. The abbreviations used are follows: s, singlet; d, doublet; dd double doublet; bs, broad signal. Mass spectral analyses were carried out using an electrospray spectrometer, Finnigan LCQ Deca. The purity (95% or higher) of all final products that were evaluated for bioactivity was assessed by HPLC. The analytical HPLC analyses were carried out on Beckman Coulter 125 S, equipped with two high pressure binary gradient delivery systems, a System Gold 166 variable-wavelength UV e vis detector and a Rheodyne 7725i injector (Rheodyne, Inc., Cotati, CA, USA) with a 20-mL stainless steel loop. For the analytical tests, compounds were prepared dissolving in methanol (0.5 mg/mL). Each solution (20 mL) was injected in a Jupiter Phenomenex RP 18 (4.5 _ 250 mm) analytical column. The mobile phase was a combination mixture of H_2O + 0.1% TFA (solvent A) and CH_3CN + 0.1% TFA (solvent B). The elution was made in gradient from 5% of B to 71% in 35 min, the flow rate was 1.0 mL/min.

4.1 tert-butyl 3-iminobutanoate (4)

A mixture of tert-butyl acetoacetate (3) (2.0g, 12.389mmol), aqueous ammonium hydroxyde solution (25% w/v, 19.642ml, 185.835mmol) in methanol (20.0ml) was stirred for an hour. The mixture was partitioned between water (20 mL) and ethyl acetate (3 x 30 mL). The combined extracts were washed with brine (15 mL), dried over Na_2SO_4 and evaporated under vacuum to

give the compound **4** as a pale yellow oil. Yield: 80%. Rf: 0.60 (hexane/ethyl acetate 9/1). ¹H NMR (300 MHz, CDCl₃): δ 4.31(s, 1H), 1.79 (s, 3H), 1.4(s, 9H).

4.2 Tert-butyl 5-hydroxy-2-methyl-1H-indole-3-carboxylate (5)

Compound **4** (1.6g, 9.991mmol) was added drop wise to a solution of a 1,4-benzoquinone (1.1g, 9.991mmol) and zinc iodide (0.632g, 1.982mmol) refluxed in dry DCM (18ml) for 2h and then cooled at 0°C for 30'. The reaction mixture was diluted with ethyl acetate and washed with water (7mL). The layers were separated and the organic phase dried over Na₂SO₄ and concentrated. The product was purified by flash chromatography using hexane/ethyl acetate 8:2. Brown powder. Yield 74%. Rf: 0.22 (hexane/ethyl acetate 8/2). ¹H NMR (300 MHz, CDCl₃): δ 7.25 (s, 1H), 7.10 (d, 1H), 6.67 (d, 1H), 2.52 (s, 3H), 1.51 (s, 9H)

4.3 Procedures for synthesis of compound (6)

4.3.1 Tert-butyl 5-acetoxy-2-methyl-1H-indole-3-carboxylate

A mixture of tert-butyl 5-hydroxy-2-methyl-1H-indole-3-carboxylate (**5**) (1.8g, 7.33mmol), acetic anhydride (13.83 ml, 146.67 mmol) and pyridine (1.78ml, 21.99 mmol) was refluxed for 1h. The cooled reaction mixture was then washed with saturated sodium bicarbonate solution. After extraction with ethyl acetate, the combined organic layer were washed with a solution of citric acid 10%, dried over Na₂SO₄ and concentrated. The crude product was used directly without further purification. Yield 93% Rf: 0.61 (hexane/ethyl acetate 6/4). ¹H NMR (300 MHz, CDCl₃): δ 7.78 (s, 1H), 7.22 (d, 1H), 6.98 (d, 1H), 2.72(s, 3H), 2.34 (s, 3H), 1.65 (s, 9H).

4.3.2 Tert-butyl -5- acetoxy-1,2-dimethyl-indole-3-carboxylate (6)

A mixture of tert-butyl 5-acetoxy-2-methyl-1H-indole-3-carboxylate (1.9g, 6.789mmol), anhydrous DMF (22ml) and NaH 95% (0.24g, 10.183mmol) was stirred at RT until clear, then iodomethane (1.3ml, 20.367mmol) were added. After 1h the reaction was quenched with H₂O

and extracted with DCM. The organic phase was washed with a solution of citric acid 10% for two times, then dried over Na₂SO₄, filtrated and evaporated. The purification by silica column was not necessary. Yield 81%; Rf: 0.50 (hexane/ethyl acetate 7/3). ¹H NMR (300MHz, CDCl₃): δ 7.81(s,1H), 7.10 (d,1H), 6.89(d,1H), 3.43(s,3H), 2.61(s,3H), 1.67 (s,9H).

4.4 General procedures for the compounds 7a and 7b

A solution of **6** (1.7g, 5.499mmol) in CCl₄ (30ml) was brought to reflux: then N-bromosuccinimide (0.979g, 5.499mmol) and dibenzoyl peroxide (0.093g, 0.385mmol) were added. After 2 h the solution was cooled to RT and water was added.

The reaction mixture was washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. The product was purified by flash chromatography using hexane/ethyl acetate 8/2 as eluent. From this purification we recovered tert-butyl-5-acetoxy-2-(bromomethyl)-1-methyl-indole-3-carboxilate (**7a**) and tert-butyl-5-acetoxy-6-bromo-2-(bromomethyl)-1-methyl –indole-3-carboxilate (**7b**) in yield 60 and 30% respectively.

4.4.1 Tert-butyl 5-acetoxy-2-(bromomethyl)-1-methyl-indole-3-carboxilate (7a)

Yield 60% Rf: 0.55 (hexane/ethyl acetate 7/3). ¹H NMR (300MHz, CDCl₃) : δ 7.87(s,1H); 7,26 (d,1H); 7,02 (d,1H); 5,05 (s,2H); 3,71 (s,3H); 2,34 (s,3H); 1,68 (s,9H).

4.4.2 Tert-butyl-6-bromo-5-acetoxy-2-(bromomethyl)-1-methyl-indole-3-carboxilate (7b)

Yield 30%. Rf: 0.66 (hexane/ethyl acetate 7/3). ¹H NMR (300MHz, CDCl₃): δ 7.93 (s,1H); 7,60 (s,1H); 5,10 (s,2H); 3,78 (s,3H); 2,40 (s,3H); 1,67 (s,9H).

4.5 General procedures for the compounds I a–g

To a stirred solution of thiophenol (1mmol) and KOH (0.168g, 3mmol) in water (3 mL), after 15 min 1 eq. of compound 7a or 7b was added. The reaction mixture was stirred at RT for 3 h. After neutralization with acetic acid. The solution was washed with water and extracted with

DCM for two times. The organic layer were dried and evaporated. The product was purified by flash chromatography using Hexane/ethyl acetate 7/3 as eluent.

4.5.1 Tert-butyl-5-hydroxy-1-methyl-2-(4-trifluoromethylphenylthio)methyl-indole-3-carboxilate Ia

Yield 42% Rf: 0.30 (hexane/ethyl acetate 7/3). ¹H NMR (300MHz, CDCl₃): δ 7,55 (d,1H); δ 7,48 (m,4H); δ 7,21 (d,1H); δ 6,88 (dd,1H); δ 4,83 (2H); δ 3,76 (s,3H); δ 1,60 (s,9H). ¹³C NMR (300 MHz, CDCl₃): δ = 28.7, 30.1, 80.3, 106.7, 110.2, 112.5, 125.6, 130.3, 132.25, 141.4, 151.3, 164.8. ESI(m/z): 438.16 [M⁺ + 1].

4.5.2 Tert-butyl-5-hydroxy-1-methyl-2-(2,6-dichlorophenylthio)methyl-indole-3-carboxilate Ib

Yield 64%. Rf: 0.55 (hexane/ethyl acetate 7/3). ¹H NMR (300MHz, CDCl₃): δ 7.50 (d,1H); 7,33 (d, 2H); 7,18 (d,1H); 7,16 (d,1H); 6,86 (dd,1H); 4,72 (s,2H); 3,75 (s,3H); 1,60 (s,9H). ¹³C NMR (300 MHz, CDCl₃): δ = 28.6, 30.1, 81.3, 109.5, 115.0, 126.2, 131.3, 133.1, 154.3, 165.7. ESI(m/z): 460.16 [M⁺ + 23].

4.5.3 Tert-butyl-5-hydroxy-1-methyl-2-(2-trifluoromethylphenylthio)methyl-indole-3-carboxilate Ic

Yield 51%. Rf: 0.51 (hexane/ethyl acetate 7/3). ¹H NMR (300MHz, CDCl₃): δ 7,66 (d,1H); 7,58 (s,1H); 7,52 (d,1H); 7,43-7,31 (m,2H); 7,18 (d,1H); 6,87 (d,1H); 4,80 (s, 2H); 3,68 (s,3H); 1,60 (s,9H). ¹³C NMR (300 MHz, CDCl₃): δ = 28.5, 29.6, 80.2, 106.7, 110.1, 112.5, 126.7, 127.2, 131.9, 132.3, 134.6, 141.4, 151.3, 164.8. ESI(m/z): 438.14 [M⁺ + 1].

4.5.4 Tert-butyl-6-bromo-5-hydroxy-1-methyl-2-(2-trifluoromethylphenylthio)methyl-indole-3-carboxylate Id

Yield 50%. Rf: 0.79 (hexane/ethyl acetate 7/3). ^1H NMR (300MHz, CDCl_3) δ = 7,89 (s,1H); 7,67 (d,1H); 7,56 (s,1H); 7,50 (d,1H); 7,41-7,35 (m,2H); 4,79 (s,2H); 3,69 (s,3H); 1,58 (s,9H). ^{13}C NMR (300 MHz, CDCl_3): δ = 20.9, 28.4, 30.3, 80.6, 110.9, 113.6, 116.1, 126.8, 135.2, 142.8, 164.0, 169.5. ESI(m/z): 538.06 [M^+ + 23].

4.5.5 Tert-butyl-5-hydroxy-1-methyl-2-(4-fluorophenylthio)methyl-indole-3-carboxylate Ie

Yield 51%. Rf: 0.38 (hexane/ethyl acetate 7/3). ^1H NMR (300MHz, CDCl_3): δ 7,45 (d,1H); 7,32-7,24 (m,3H); 6,99-6,93 (m,2H); 6,86 (dd, 1H); 4,66 (s,2H); 3,67 (s,3H); 1,60 (s,9H). ^{13}C NMR (300 MHz, CDCl_3): δ = 28.5, 30.1, 80.2, 106.7, 110.0, 112.2, 115.7, 126.5, 127.2, 132.2, 135.6, 142.6, 151.2, 164.4. ESI(m/z): 410.2 [M^+ + 23].

4.5.6 Tert-butyl-5-hydroxy-1-methyl-2-(4-chlorophenylthio)methyl-indole-3-carboxylate If

Yield 59%. Rf: 0.41 (hexane/ethyl acetate 7/3). ^1H NMR (300MHz, CDCl_3): δ 7,55 (d,1H); 7,27-7,18 (m,5H); 6,82 (dd,1H); 4,70 (s,2H); 3,70 (s,3H); 1,58 (s,9H). ^{13}C NMR (300 MHz, CDCl_3): δ = 29.4, 30.0, 80.2, 106.7, 110.0, 112.3, 127.1, 128.9, 133.8, 142.2, 151.3, 164.7. ESI(m/z): 404.1 [M^+ + 1].

4.5.7 Tert-butyl-6-bromo-5-hydroxy-1-methyl-2-(2,6-dichlorophenylthio)methyl-indole-3-carboxylate Ig

Yield 39%. Rf: 0.62 (hexane/ethyl acetate 7/3). ^1H NMR (300MHz, CDCl_3) δ 7,67 (s,1H); 7,43 (s,1H); 7,31 (d,1H); 7,30 (s,1H); 7,16 (dd,1H); 5,30 (s,1H); 4,70 (s,2H); 3,72 (s,3H); 1,52 (s,9H). ^{13}C NMR (300 MHz, CDCl_3): δ = 28.6, 30.1, 80.1, 107.4, 112.2, 128.4, 130.5, 142.4, 142.9, 147.3, 164.0. ESI(m/z): 517.97 [M^+ + 1].

4.6 General procedure for the compounds II a-f.

To a solution containing 1 equiv of **I a-f** in CH₂Cl₂ (1.5 mL), 1.3 equiv of MCPBA 77% were added and the reaction mixture was stirred for 5 min. Then Na₂SO₃ was added and the reaction mixture was extracted with CHCl₃. The organic layer was dried over sodium sulfate and evaporated under vacuum to yield a red oil. The crude product was precipitated by addition of a mixture hexane/ethyl acetate.

4.6.1 **Tert-butyl-5-hydroxy-1-methyl-2-(4-trifluoromethylsulfynyl)methyl-indole-3-carboxylate IIa**

Yield 55%. Rf: 0.60 (hexane/ethyl acetate 7/3) ¹H NMR (300MHz, CDCl₃) δ 7,76 (m,4H); 7,50 (s,1H); 7,11 (d,1H); 6,85 (dd,1H); 5,02 (d,1H); 4,65 (d,1H); 3,70 (s,3H); 1,60 (s,9H). ¹³C NMR (300 MHz, CDCl₃): δ = 28.5, 30.6, 54.7, 80.6, 106.5, 110.6, 113.1, 124.8, 125.9, 126.7, 132.5, 136.1, 151.6, 164.8. ESI(m/z): 476.13 [M⁺ + 23].

4.6.2 **Tert-Butyl-5-hydroxy-1-methyl-2-((2,6-dichlorophenyl)sulfynyl)methyl-indol-3-carboxylate IIb**

Yield 40%. Rf: 0.83 (hexane/ethyl acetate 8/2) ¹H NMR (300MHz, CDCl₃) δ 7,50 (d,1H); 7,35 (s,3H); 7,22 (d,1H); 6,85 (dd,1H); 5,57 (d,1H) ; 5,25 (d,1H); 3,82 (s,3H); 1,61 (s,9H). ¹³C NMR (300 MHz, CDCl₃): δ = 28.1, 31.6, 53.7, 80.5, 105.8, 110.0, 113.8, 125.0, 125.9, 126.7, 133.5, 136.1, 154.6, 165.0. ESI(m/z): 455.05 [M⁺ + 1].

4.6.3 **Tert-butyl-5-hydroxy-1-methyl-2-(2-trifluoromethylsulfynyl)methyl-indole-3-carboxylate IIc**

Yield 82%. Rf: 0.78 (hexane/ethyl acetate 7/3) ¹H NMR (300MHz, CDCl₃) δ 7,69 (d,1H); 7,63-7,53 (m,3H); 7,41 (d,1H); 7,23 (d,1H); 6,85 (dd,1H); 5,20 (d,1H); 4,90 (d,1H); 3,81 (s,3H); 1,49

(s,9H). ^{13}C NMR (300 MHz, CDCl_3): δ = 28.8, 31.2, 50.7, 80.3, 106.6, 110.5, 112.8, 126.2, 131.2, 132.1, 134.8, 151.2, 164.0 ESI(m/z): 476.15 [M^+ + 23].

4.6.4 Tert-butyl-6-bromo-5- hydroxy -1-methyl-2-(2-trifluoromethylsulfonyl)methyl)-indole-3-carboxylate II d

Yield 60%. Rf: 0,22 (hexane/ethyl acetate 7/3) ^1H NMR (300MHz, CDCl_3) : δ 7,78 (s,1H); 7,70 (d,1H); 7,62 (s,1H); 7,52 (m,3H); 5,18 (d,1H); 4,85 (d,1H); 3,8 (s,3H); 1,58 (s,9H). ^{13}C NMR (300 MHz, CDCl_3): δ = 29.9, 37.4, 40.4, 59.2, 89.6, 120.4, 123.0, 125.1, 135.0, 140.4, 144.9, 152.0, 172.6 ESI(m/z): 556.12 [M^+ + 23].

4.6.5 Tert-butyl-5-hydroxy-1-methyl-2-((4-fluorophenylsulfonyl)methyl)-indole-3-carboxylate II e

Yield 50% Rf: 0.14 (hexane/ethyl acetate 7/3) ^1H NMR (300MHz, CDCl_3): δ 7,81 (d,1H); 7,57-7,52 (m,2H); 7,29 (d,1H); 7,17-7,12 (m,2H); 7,06 (dd,1H); 4,85 (d,1H); 4,72 (d,1H); 3,63 (s,3H); 1,62 (s,9H). ^{13}C NMR (300 MHz, CDCl_3): δ = 21.2, 28.5, 30.6, 54.4, 80.7, 110.3, 114.3, 116.4, 117.6, 126.5, 135.0, 136.9, 138.5, 146.1, 162.8, 166.2, 170.1. ESI(m/z): 426.17 [M^+ + 23].

4.6.6 Tert-butyl-5-hydroxy-1-methyl-2-((4-chlorophenylsulfonyl)methyl)-indole-3-carboxylate II f

Yield 97% Rf: 0.13 (hexane/ethyl acetate 7/3) . ^1H NMR (300MHz, CDCl_3): δ 7,44 (m,4H); 7,30 (s, 1H); 7,03 (d, 1H); 6,81 (d, 1H); 4,80 (dd, 2H); 3,61 (s,3H); 1,63 (s,9H). ^{13}C NMR (300 MHz, CDCl_3): δ = 28.6, 30.5, 54.8, 80.5, 106.5, 110.5, 113.1, 125.8, 129.4, 132.4, 135.9, 137.6, 141.4, 151.9, 164.8. ESI(m/z): 421.08 [M^+ + 1].

4.7 General procedures for the compounds IV a-d

A solution of **IIb-d** (1mmol), N,N,N',N'-Tetramethylethylenediamine (2.04 mL, 15 mmol) and acetic acid (0.184 mL) was refluxed for 2h. Then the solvent was evaporated under vacuum and water was added. The resultant mixture was adjusted to pH 10 with sodium hydroxide and

extracted with methylene chloride. The resulting organic layer was dried over Na₂SO₄, filtered and evaporated under vacuum. The product precipitates in diethyl ether.

4.7.1. Tert-butyl 2-((2,6-dichlorophenylthio)methyl)-6-bromo-4-((dimethylamino)methyl)-5-hydroxy-1-methyl-1H-indole-3-carboxylate IVa

Yield 59%. Rf: 0.62 (hexane/ethyl acetate 8/2 + 0.1eq di TEA) ¹H NMR (300MHz, CDCl₃): δ 7.42 (d,1H); 7.40 (s,1H); 7.23 (dd,1H); δ 4.49 (s,2H); 4.16 (s, 2H); 3.62 (s,3H); 2.37 (s,6H); 1.62 (s,9H). ¹³C NMR (300 MHz, CDCl₃): δ = 27.3, 29.0, 29.4, 42.2, 57.6, 81.2, 113.9, 128.6, 131.3, 141.6, 151.8, 165.7 ESI(m/z): 575.08 [M⁺ + 1].

4.7.2 Tert-butyl-2-((2,6-dichlorophenylsulfinyl)methyl)-4-((dimethylamino)methyl)-5-hydroxy-1-methyl-1H-indole-3-carboxylate IVb

Yield 86%. Rf: 0.67 (hexane/ethyl acetate 8/2+ 0.1eq di TEA) ¹H NMR (300MHz, MeOD): δ 7.46 (d,1h); 7.31-7.23 (m,3H); 6.79 (dd,1H); 4.68 (s,2H); 3.67 (s,3H); 3.26 (s,6H); 1.60 (s,9H). ¹³C NMR (300 MHz, MeOD): δ = 27.4, 29.0, 29.9, 42.2, 57.2, 81.0, 108.3, 114.0, 128.6, 131.0, 141.7, 142.5, 155.6, 165.9. ESI (m/z): 511.2 [M⁺ + 1].

4.7.3 Tert-butyl-2-((2-(trifluoromethyl)phenylsulfinyl)methyl)-4-((dimethylamino)methyl)-5-hydroxy-1-methyl-1H-indole-3-carboxylate IVc

White solid. Yield 70%. Rf: 0.43 (hexane/ethyl acetate 8/2+ 0.1eq di TEA)

¹H NMR (300MHz, CDCl₃): δ 8.05 (d,1H); 7.77-7.63 (m,3H); 7.15 (d,1H); 6.90 (d,1H); 4.15 (d,2H); 3.56 (s,3H); 2.38 (s,6H); 1.63 (s,9H). ¹³C NMR (300 MHz, CDCl₃): δ = 27.4, 29.3, 43.0, 59.1, 81.2, 110.0, 114.2, 125.8, 126.3, 132.0, 133.2, 142.4, 154.0, 165.9. ESI(m/z): 533.2 [M⁺ + 23].

4.7.4 Tert-butyl-2-((2-(trifluoromethyl)phenylsulfinyl)methyl)-6-bromo-4-((dimethylamino)methyl)-5-hydroxy-1-methyl-1H-indole-3-carboxylate IVd

Yield 70% Rf: 0.38 (hexane/ethyl acetate 8/2+ 0.1eq di TEA) ^1H NMR (300MHz, CDCl_3): δ 7,90-7,85 (m,2H); 7,83-7,78 (m,2H); 7,57 (s,1H); 4,34 (s,2H); 3,40 (s,3H); 2,51 (s,6H); 1,68 (s,9H). ^{13}C NMR (300 MHz, CDCl_3): δ = 27.4, 29.4, 42.4, 59.1, 81.4, 113.5, 115.3, 125.8, 126.3, 132.2, 133.2, 142.2, 154.0, 165.5 ESI(m/z): 590.0 [$\text{M}^+ + 1$].

5. Molecular Modeling

All molecular modeling studies were performed on a MacPro dual 2.66 GHz Xeon running Ubuntu 14.04. The mature E3-E2-E1 glycoprotein complex was downloaded from the PDB data bank (<http://www.rcsb.org/>; PDB code 3N42). Hydrogen atoms were added to the protein, using the Protonate 3D routine of the Molecular Operating Environment (MOE2015.10). [13] Ligand structures were built with MOE and minimized using the MMFF94x force field until a RMSD gradient of $0.05 \text{ kcal mol}^{-1}$ was reached. The Maestro LigPrep tool [14] was used to prepare the ligands using the default settings, whereas the protein was prepared using the preparation wizard tool. [15] The docking simulations were performed using Maestro Glide XP using the default parameters. [16]

6. Biological assays

6.1 CPE reduction assay

Vero cells were seeded in 96-well tissue culture plates (BD Falcon 96-Well Cell Culture Plate) at a density of 2.5×10^4 cells/well and were allowed to adhere overnight. Next, a compound dilution series was prepared in the medium on top of the cells after which the cultures were infected with 0.01 MOI of CHIKV 899. Each assay was performed in triplicate in the same test and assays were repeated independently. On day 5 post-infection (p.i.), the cell viability was quantified

using the MTS/PMS method as described by the manufacturer (Promega, The Netherlands). The 50% effective concentration (EC_{50}), which is defined as the compound concentration that is required to inhibit virus-induced cytopathogenic effect (CPE) by 50%, was determined using logarithmic interpolation. The overall anti-metabolic effect of the compounds, which is representative for a combined cytostatic, cytotoxic and anti-metabolic effect, was evaluated in uninfected cells, also using the MTS/PMS method. The 50% cytostatic/cytotoxic concentration (CC_{50} ; i.e., the concentration of compound that reduces the overall metabolic activity of the cells by 50%) was calculated using logarithmic interpolation. All assay wells were checked microscopically for minor signs of virus-induced CPE or alterations of the cell or monolayer morphology.

6.2 Virus yield assay

Vero A cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/well. After 24h of incubation, cells were treated with a serial dilution of the compound followed by infection with CHIKV 899 (MOI 0.001). After 2h, cells were washed twice with assay medium to remove non-adsorbed virus. The cells were then treated again with the same serial dilutions of compound and incubated for 48h. At the end of the incubation period, culture supernatants were collected for viral RNA isolation using Macherey Nagel NucleoSpin 96 virus kit and viral RNA was quantified by real-time qRT-PCR, while the amount of infectious virus particles was determined by end-point titration assay as described before. [12]

6.3 CHIKV pseudoparticle entry assay

CHIKV pseudoparticles (CHIKVpp) were prepared as previously described. [17] BGM cells were seeded in white 96-well tissue culture plates (ViewPlate-96, PerkinElmer) at a density of 2.5×10^4 cells/well and left to adhere overnight. The next day, serial dilutions of the compounds were added to the cells followed by infection with the appropriate dilution of CHIKVpp. Arbidol

was used as reference compound for entry inhibition. After 72h, the medium was aspirated from all wells and cells were lysed using Glo lysis buffer (Promega). The firefly luciferase activity in the cell lysate was then detected using the Luciferase Assay System kit (Promega).

6.4 Time of addition assay

Vero A cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/well and left to adhere overnight. The next day, the compounds were added to the cells either 2h prior, at, or after 2, 4, 6 and 8h of infection with 1 MOI of CHIKV 899. Following 24 of incubation, cells were lysed by adding Cells-to-cDNA™ lysis buffer (Life Technologies) and the intracellular viral RNA was quantified by qRT-PCR as described before. [13]

Acknowledgments

This work was supported by the grant PON 03 PE_00060_07 (MIUR) Sviluppo pre-clinico di nuove terapie e di strategie innovative per la produzione di molecole ad azione farmacologica. We would like to acknowledge the support of the Life Science Research Network Wales grant no. NRNPGSep14008, an initiative funded through the Welsh Government's Ser Cymru program. L.D. is funded by the Research Foundation of Flanders (FWO). We thank Caroline Collard and Nick Verstraeten for their excellent technical assistance.

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Fig.1 Two potential Arbidol binding sites in proximity of Gly82 (red circle) in the E2 glycoprotein.

Fig.2 Docking of Arbidol in the two potential binding sites. A) Site 1 is a shallow hydrophilic (turquoise surface) pocket for Arbidol binding. B) Site 2 is a more hydrophobic (green surface) and well-defined pocket that could allow Arbidol binding. The E2 protein is shown as turquoise ribbon.

Fig.3 Docking of **IIIe** (A, carbon atoms in gold) and **IIIf** (B, carbon atoms in purple). Both compounds show a different binding mode from Arbidol.

Fig.4 The indole derivatives **IIc** and **IIIf** act as specific and selective inhibitors of CHIKV replication. A) Dose-response effect of **IIc** and **IIIf** on CHIKV-induced CPE in Vero A cells as quantified by MTS/PMS method. B) Dose-response effect of **IIc** and **IIIf** on virus yield of CHIKV-899 in Vero A cells as determined by qRT-PCR (RNA) and C) titration for infectious progeny virus particles (TCID₅₀/ml). Data presented are mean values $\pm$ SD of at least three independent experiments.

Fig.5 The effect of indole derivatives (**IIc** and **IIIf**) on the CHIKV replication cycle. A) The dose-response effect of indole derivatives on the entry of CHIKV pseudoparticles (CHIKVpp) in BGM cells. After 72h of infection, cells were lysed and the intracellular firefly luciferase signal was quantified. B) Time-of-addition assay. Vero A cells were treated with the selected compound prior to (-2h), at the time of (0h), or after infection (2, 4 and 6 h) with MOI=1 of CHIKV strain 899. After 24h of infection, the intracellular viral RNA was quantified by qRT-PCR. Arbidol and T-705 were included in these assays as reference compounds for early and late stage inhibition, respectively. Data are average $\pm$ SD of at least three independent experiments.

Fig.6 Docking of **IIc** (carbon atoms in lilac). The compound shows a binding mode similar to the one observed for **IIIe** and **IIIf**.

Scheme 1

Reagents and conditions: a) NH_4OH , CH_3OH , rt, 1h; b) 1,4-benzoquinone, ZnI_2 , dry DCM, reflux, 1h, 0°C , 30'; c) acetic anhydride, pyridine, reflux, 1h d) CH_3I , 60% NaH, DMF, rt, 1 h; e) N-Bromosuccinimide, dibenzoylperoxide, CCl_4 , reflux, 3 h; f) R_1PhSH , KOH, CH_3OH , rt, 1h; g) MCPBA, dry DCM, rt, 5'; h-i) TMEDA, acetic acid, DCM, reflux, 2h.

Table 1

^a CC_{50} : 50% cytostatic/cytotoxic concentration (concentration at which 50% adverse effect is observed on the host cell). ^b EC_{50} : 50% Effective concentration (concentration at which 50% inhibition of CPE is observed in Vero cells).^c SI: Selectivity index ($\text{CC}_{50}/\text{EC}_{50}$).

Table 2

^a CC_{50} : 50% cytostatic/cytotoxic concentration (concentration at which 50% adverse effect is observed on the host cell). ^b EC_{50} : 50% Effective concentration (concentration at which 50% inhibition of CPE is observed in Vero cells).^c SI: Selectivity index ($\text{CC}_{50}/\text{EC}_{50}$).

Table 3

^a CC_{50} : 50% cytostatic/cytotoxic concentration (concentration at which 50% adverse effect is observed on the host cell). ^b EC_{50} : 50% Effective concentration (concentration at which 50% inhibition of CPE is observed in Vero cells).^c SI: Selectivity index ($\text{CC}_{50}/\text{EC}_{50}$).

Table 4

Antiviral activity of indole derivatives against selected alphaviruses in Vero A cells. SFV = Semliki Forest virus, SINV = Sindbis virus.

Table 5:

The inhibitory effect of indole derivatives on the entry of CHIKV pseudoparticles (CHIKVpp) in BGM cells.

Supporting Information

Discovery of novel multi-target indole-based derivative as potent and selective inhibitor of Chikungunya Virus replication

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Tert-butyl-5-hydroxy-1-methyl-2-(4-trifluoromethylphenylthio)methyl-indole-3-carboxylate (1a)

Tert-butyl-5-hydroxy-1-methyl-2-(2,6-dichlorophenylthio)methyl-indole-3-carboxylate (1b)

Tert-butyl-5-hydroxy-1-methyl-2-(2,6-dichlorophenylthio)methyl-indole-3-carboxylate (1b)

Tert-butyl-5-hydroxy-1-methyl-2-(2-(trifluoromethylphenylthio)methyl)-indole-3-carboxylate (Ic)

Tert-butyl-5-hydroxy-1-methyl-2-(2-(trifluoromethylphenylthio)methyl)-indole-3-carboxylate (Ic)

Tert-butyl-5-hydroxy-1-methyl-2-(4-chlorophenylthio)methyl-indole-3-carboxylate (If)

Tert-butyl-5-hydroxy-1-methyl-2-(4-chlorophenylthio)methyl-indole-3-carboxylate (If)

Tert-butyl-6-bromo-5-hydroxy-1-methyl-2-(2,6-dichlorophenylthio)methyl-indole-3-carboxylate (Ig)

Tert-butyl-6-bromo-5-hydroxy-1-methyl-2-(2,6-dichlorophenylthio)methyl)-indole-3-carboxylate (lg)

Tert-butyl-5-hydroxy-1-methyl-2-(4-(trifluoromethylsulfonyl)methyl)-indole-3-carboxylate (IIa)

Tert-butyl-5-hydroxy-1-methyl-2-(4-(trifluoromethylsulfonyl)methyl)-indole-3-carboxylate (IIa)

Tert-butyl-5-hydroxy-1-methyl-2-(2-trifluoromethylsulfonyl)methyl-indole-3-carboxylate IIc

Tert-butyl-5-hydroxy-1-methyl-2-(2-trifluoromethylsulfonyl)methyl-indole-3-carboxylate (IIc)

Tert-butyl-5-hydroxy-1-methyl-2-(4-fluorophenylsulfonyl)methyl-indole-3-carboxylate IIe

Tert-butyl-5-hydroxy-1-methyl-2-(4-fluorophenylsulfonylmethyl)-indole-3-carboxylate IIe

Tert-butyl-5-hydroxy-1-methyl-2-(4-chlorophenylsulfonyl)methyl-indole-3-carboxylate II f

Tert-butyl-5-hydroxy-1-methyl-2-(4-chlorophenylsulfonyl)methyl-indole-3-carboxylate II f

tert-butyl-2-((2,6-dichlorophenylsulfinyl)methyl)-6-bromo-4-((dimethylamino) methyl) -5-hydroxy -1-methyl-1H-indole-3-carboxylate IVa

Tert-butyl-2-((2,6-dichlorophenylsulfinyl)methyl)-4-((dimethylamino)methyl)-5-hydroxy-1-methyl-1H-indole-3-carboxylate IVb