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Broad spectrum anti-flavivirus pyridobenzothiazolones leading to less infective virions

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ABSTRACT

We report the design, synthesis, and biological evaluation of a class of 1*H*-pyrido[2,1-*b*][1,3]benzothiazol-1-ones originated from compound **1**, previously identified as anti-flavivirus agent. Some of the new compounds showed activity in low μM range with reasonable selectivity against Dengue 2, Yellow fever (Bolivia strain), and West Nile viruses. One of the most interesting molecules, compound **16,** showed broad antiviral activity against additional flaviviruses such as Dengue 1, 3 and 4, Zika, Japanese encephalitis, several strains of Yellow fever, and tick-borne encephalitis viruses. Compound **16** did not exert any effect on alphaviruses and phleboviruses and its activity was maintained in YFV infected cells from different species. The activity of **16** appears specific for flavivirus with respect to other virus families, suggesting, but not proving, that it might be targeting a viral factor. We demonstrated that the antiviral effect of **16** is not related to reduced viral RNA synthesis or virion release. On the contrary, viral particles grown in the presence of **16** showed reduced infectivity, being unable to perform a second round of infection. The chemical class herein presented thus emerges as suitable to provide pan-flavivirus inhibitors.

1. Introduction

Belonging to the *Flaviviridae* family, the Flavivirus genus consists of arthropod-borne RNA viruses which include important human pathogens like Dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Yellow fever virus (YFV), Zika virus (ZKV), and tick-borne encephalitis virus (TBEV) (Wilder-Smith et al., 2017).

Depending on the infective agent, flavivirus infections cause from mild flu-like symptoms to hemorrhagic fevers, hepatitis, and neuropathies such as encephalopathy, meningitis, and microcephaly in human embryos (Daep et al., 2014; Guzman et al., 2016; Monath and Vasconcelos, 2015; Petersen et al., 2016). Vaccines are available against YFV, JEV, TBEV, and only very recently against DENV, but the coverage is far from being complete (Guy et al., 2016; Ishikawa et al.,

Abbreviations: NS, non-structural; DENV, Dengue virus; WNV, West Nile virus; JEV, Japanese encephalitis virus; YFV, Yellow fever virus; ZKV, Zika virus; RdRp, RNA-dependent RNA polymerase; SAR, structure-activity relationship; PBTZs, 1*H*-pyrido[2,1-*b*][1,3]benzothiazol-1-ones; SI, selectivity index; Cp, cyclopentyl; TBTU, O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate; DIPEA, diisopropylethylamine; DIAD, diisopropylazadicarboxylate; MOI, multiplicity of infectivity; TCID, tissue culture infective dose; IC RNA, intracellular RNA; SPN RNA, supernatant RNA; FCS, fetal calf serum; HBSS, Hank's Balanced Salt Solution; p.i., post infection

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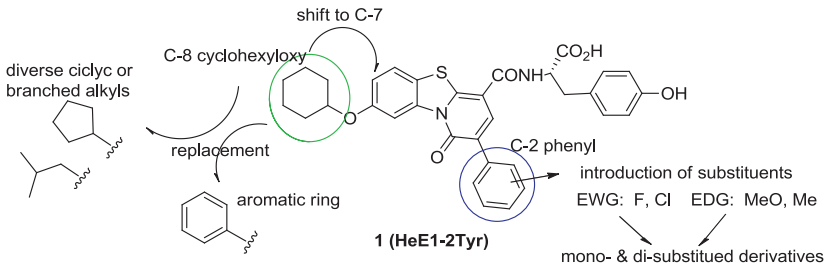


Fig. 1. Design concept around hit compound **1**: the C-8-cyclohexyloxy was replaced by other (cyclo)alkyls, the aromatic counterpart, or shifted to the C-7 position; the C-2 phenyl was mono- or di-functionalized at different positions with electron-withdrawing fluorine (EWG) as well as the electron-donating (EDG) groups.

2014), and the lack of effective therapies further worsens the scenario. The opportunities for broad-spectrum agents that are active against multiple flaviviruses are particularly desirable to prepare for the next flaviviral epidemic, which could emerge from yet unknown or neglected viruses (Boldescu et al., 2017).

We have recently identified compound **1** (Fig. 1) as a new flaviviral RNA-dependent RNA polymerase (RdRp) inhibitor able to reduce the replication of different flaviviruses in cells with discrete potency (EC_{50} between 2.1 and 10.0 μM) and reasonable selectivity (Tarantino et al., 2016).

Here, we describe a ligand-based structure-activity relationship (SAR) study around compound **1** by designing a series of different 1*H*-pyrido[2,1-*b*][1,3]benzothiazol-1-ones (hereafter called PBTZs) modified at the C-7/C-8 and C-2 positions, while retaining the Tyr moiety at the C-4 amide side chain because we found that some C-4 carboxylate analogues did not show any antiviral activity in cells (Tarantino et al., 2016) (Fig. 1). Most of the synthesized compounds displayed a selective antiviral activity against WNV, DENV2, and YFV. One of the best analogues, *i.e.* compound **16**, was evaluated expanding the flavivirus panel to DENV serotypes 1, 3 and 4, ZIKV, different strains of YFV, JEV, and TBEV thereby demonstrating the broad anti-flavivirus activity of this compound class. Interestingly, the antiviral effect of compound **16** did not affect viral RNA synthesis or virions release but rather was based on the production of viral particles with reduced infectivity.

2. Materials and methods

2.1. Chemical synthesis

Experimental procedures for the preparation of all the targets compounds **2–16** together with the experimental protocols of all the intermediates are reported in the Supplementary material.

2.2. Cell lines and viral strains

Simian Vero (E6 sub-clone), human HEK 293, human SW13, hamster BHK-21 and mosquito C6/36 cells were purchased from ATCC. P96 wells plate were seeded the day before so as to be 80% confluent at the start of experiments.

DENV-1 Djibouti (strain D1/H/IMTSSA/98/606) (de Micco et al., 2001); DENV-2 Martinique (strain H/IMTSSA-MART/98-703) (Tolou et al., 2000); DENV-3 Bolivia (strain 4025) (Roca et al., 2009); DENV-4 Dakar (strain HD34460, WRCEVA); WNV Uganda (956 D117, WRCEVA); YFV Bolivia (88–99); YFV Sudan (M 90-5 TVP 3230, WRCEVA), YFV Uganda (MR896 TVP 3236, WRCEVA); YFV Nigeria (A-Adeoye TVP 3223, WRCEVA); JEV Laos (CNS769-Laos 2009); ZKV PF: French Polynesia (H/PF/2013); Chikungunya Venturini (Bologna Italy 2008); Toscana (CE, France 2010, EVAg ref 001V-02442); TBEV Neudoerfl strain.

2.3. Antiviral assays

2.3.1. Antiviral activity against WNV, DENV, YFV, JEV, ZKV, Chikungunya, and Toscana viruses

The amount of each virus in the assay has been calibrated so that the replication growth has not yet plateaued at readout time. Approximate MOI varies from 0.2 to 0.001 depending on the virus strain.

Compounds were solubilized in DMSO (20 mM or 50 mM). One day prior to infection cells were seeded in 100 μl of medium (with 2.5% fetal calf serum (FCS)) of a 96-well cell culture plate. The next day, 8 two-fold serial dilutions of the compounds in duplicates or triplicates, were added to the cells (25 μl/well). For each virus, 4 control wells were supplemented with 25 μl of medium. Fifteen minutes later, 25 μl per well of medium containing the appropriate amount of diluted viral stock were added to the 96-well plates.

Cells were cultivated for 2–4 days after which 100 μl of the supernatant were collected for viral RNA purification using the Cador Pathogen 96 QIAcube HT kit ran on QIAcube HT automat according to the manufacturer protocol.

2.3.2. Antiviral activity against TBEV replicating in Vero E6 cells

TBEV Neudoerfl strain was produced as previously described (Albornoz et al., 2014). Vero E6 cells were infected with TBEV (MOI = 1) in the presence of different concentrations of compound **1** or **16**. 24 h p.i. the supernatant (SPN) was collected and titrated on Vero cells by the plaque assay to measure infectivity. EC_{50} was estimated by plotting values from triplicate independent experiments using GraphPad (non-linear fit, log inhibitor vs normalized response).

2.4. Quantification of viral RNA

Viral RNAs were quantified by real time RT-PCR along with four 2 log dilutions of an appropriate T7-generated RNA containing known quantities of RNA (100 copies to 100 million copies) to calibrate the quantification of each strains and serotypes.

Mean inhibition of virus yield in the triplicates or duplicates were plotted using Kaleidagraph software (Synergy Software) and the best fitting curve (Inhibition, Y is given by $Y = 100/(m0/m1)^{m2}$) was used to determine the EC_{50}.

2.5. Cytotoxicity assay

The assays were done using exactly the same settings as the antiviral assay. Readout was done on day 2 or 3, the supernatant was removed and replaced with 70 μl of medium containing CellTiter-Blue reagent (Promega) and further incubated for 90 min at 37 °C. Fluorescence of the plates were read on a TECAN Infinite M 200 Pro reader. The cell viabilities in percent were calculated as 100 x (mean value of X-Background without cells)/(Cell control – background).

2.6. Infectivity of YFV growth in the presence of 16

Vero E6 cells were seeded in P24 wells plate the day before. Cells were either left untreated or treated with **16** in duplicates and infected for 2 h with YFV Bolivia strain at a MOI of 50. Cells were then washed 3 times in Hank's Balanced Salt Solution (HBSS) medium and replenished with medium containing the same concentration of compound. Two untreated but infected wells were used to collect RNA from supernatant and cellular lysate to provide input quantities of viral RNA. Cells were cultivated for 18 h (or 30 h in a second independent experiment) before analysis. Cell supernatant were collected and 100 µl were used for RNA extraction (Qiagen EZ1 virus mini kit) and qRT-PCR quantification, 100 µl, after 1:100, dilution were used for $TCID_{50}$ titration (infected wells of the titration plate were assessed by qRT-PCR of cell supernatant at day 6 post infection) and 100 µl were used for analysis of viral RNA replication during the first hour of the next round of infection. Cells were lysed by addition of RLT buffer (Qiagen EZ1 or Qiacube HT RNA cell mini kit) and intracellular RNA were extracted using either EZ1 or QiaCube HT automats. Equal volume of viral supernatant recovered from a first round of replication in absence or presence of **16** after 25 time dilution were used to infect Vero cells in duplicate, seeded the day before in a 96 wells plate, in a total volume of 75 µl. After 2 h in incubator at 37 °C, cells were washed three times with 150 µl of HBSS and either lysed immediately (2 h) or replenished with fresh medium and further incubated for 7 h or 24 h before lysis. Cell monolayer were lysed by addition of 140 µl of RLT buffer and processed for intracellular RNA extraction using QIAcube HT automat and appropriate Qiagen protocols (RNeasy 96 QIAcube HT total RNA). Viral RNAs were quantified by real-time one step RT-PCR. Mean total copy numbers of each duplicate wells (copy #) and percent of mean copy numbers (Fig. 4) relative to the mean 2H time points (% of input) are indicated.

The viral inoculum was diluted, beginning 100 times and further by one log dilutions. We therefore think that the residual amount of compounds would have been negligible in the titration studies. Regarding the second round of infection, the conditioned stock of virus containing the residual compound issued from the first round of infection was diluted 25 times for this second round. Moreover, as stated in the text, the inoculum was left for 2 h and removed and the cells were washed three times with 150 µl of saline solution ensuring that the residual compound was virtually eliminated. Therefore we think that the second round of infection was allowed to proceed without substantial inhibitory compound.

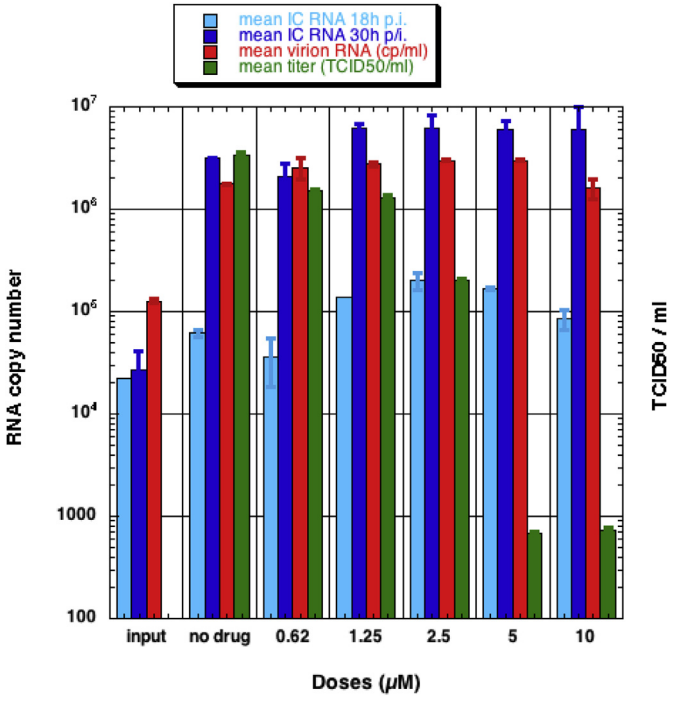
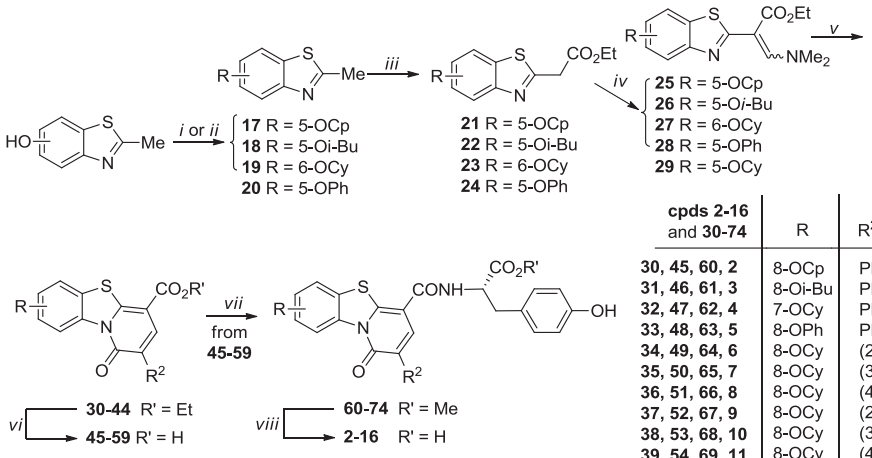


Fig. 3. Treatment with compound **16** did not affect YFV replication but affected the infectivity of released virions. IC RNA was collected at 18 (cyan bar) and 30 (blue bar) hours p.i.; SPN RNA (red bar) were collected at 30 h p.i. Infectious virus titers of released virions at 30 h p.i. was assessed by $TCID_{50}$ determination (green bar).

2.7. Benzonase digestion of SPN RNA

Cell supernatant were either left untreated or incubated for 2 h at 37 °C in presence of 125 units of Benzonase endonuclease (Merck) according to supplier instructions, before being added to VXL lysis buffer (Qiagen). For free RNA samples, cell culture medium was beforehand spiked with purified YFV RNA. RNAs were extracted using Qiagen Cador pathogen kit and viral RNA were quantified by real-time RT-PCR. Ratio of viral RNA copy numbers in benzonase-digested samples, versus untreated ones are plotted.



cpds 2-16 and 30-74	R	R^2
30, 45, 60, 2	8-OCp	Ph
31, 46, 61, 3	8-Oi-Bu	Ph
32, 47, 62, 4	7-OCy	Ph
33, 48, 63, 5	8-OPh	Ph
34, 49, 64, 6	8-OCy	(2-F)Ph
35, 50, 65, 7	8-OCy	(3-F)Ph
36, 51, 66, 8	8-OCy	(4-F)Ph
37, 52, 67, 9	8-OCy	(2-OMe)Ph
38, 53, 68, 10	8-OCy	(3-OMe)Ph
39, 54, 69, 11	8-OCy	(4-OMe)Ph
40, 55, 70, 12	8-OCy	(4-Cl)Ph
41, 56, 71, 13	8-OCy	(4-Me)Ph
42, 57, 72, 14	8-OCy	(3,4-diF)Ph
43, 58, 73, 15	8-OCy	(3,4-diOMe)Ph
44, 59, 74, 16	8-OCy	(3-F,4-OMe)Ph

Fig. 2. Synthesis of 1H-pyrido[2,1-b][1,3] benzothiazol-1-ones. Reagents and conditions: *i*) ROH, PPh_3, DIAD, dry THF, ultrasounds, rt; *ii*) 1-iodo-2-methylpropane, Cs_2CO_3, dry DMF, 80 °C; *iii*) diethyl carbonate, 60% NaH, dry THF, reflux; *iv*) $POCl_3$, DMF, 90 °C; *v*) arylacetic anhydrides (**75–79** (Morrill et al., 2014) and **80–86** in Supplementary material), 110 °C, neat; *vi*) aq. 10% NaOH/MeOH (1:5), 75 °C; *vii)* Tyr methyl ester HCl, TBTU, DIPEA, DMSO, rt; *viii)* aq 1N LiOH, 1,4-dioxane, rt.

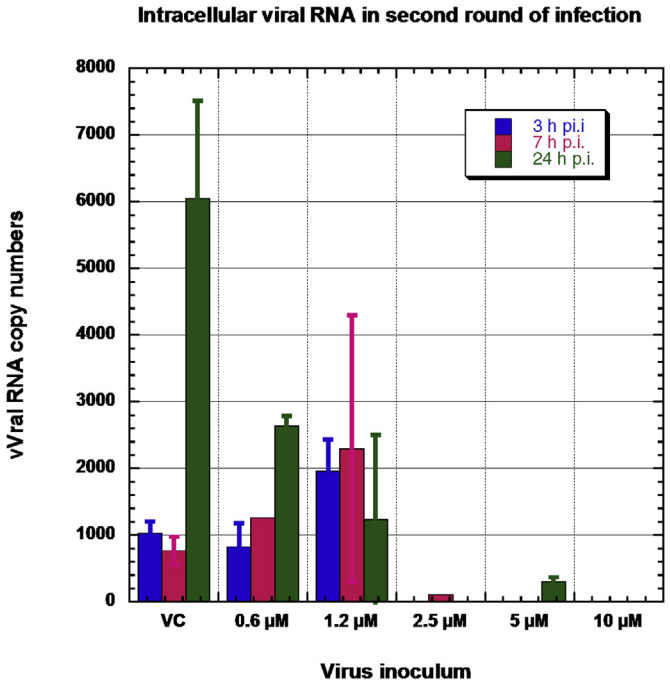


Fig. 4. Quantification of YFV IC RNA during a second round of replication of re-inoculum of virions, derived from compound **16** treatment at increasing concentration and compared to untreated control (VC) (single cycle of replication, 30 h p-i-), in fresh Vero cells. Mean total copy numbers and errors bars of each duplicate wells are indicated.

3. Results and discussion

3.1. Chemical synthesis of new compounds

All the target compounds were obtained following the synthetic scheme depicted in Fig. 2. The commercially available 2-methyl-1,3-benzothiazol-5-ol was reacted with cyclopentanol or 1-iodo-2-methyl-propane employing Mitsunobu reaction or nucleophilic substitution to give **17** and **18**, respectively. Similarly to **17**, compound **19** was obtained starting from commercial 2-methyl-1,3-benzothiazol-6-ol and using cyclohexanol. Intermediates **17–19** and the known benzothiazole **20** (Ueda et al., 2012) were functionalized at the C-2 methyl group by reaction with diethyl carbonate in presence of NaH to give the acetate derivatives **21–24**, in moderate to good yields. Subsequently, they were reacted with Vilsmeier reagent, prepared in situ, to afford almost quantitatively acrylate derivatives **25–28**. These latter as well as **29** (Manfroni et al., 2012) were condensed in neat conditions with the appropriate arylacetic anhydrides (**75–79**) (Morrill et al., 2014) and **80–86** prepared as reported in (Fig. S1, Supplementary material) to afford PBTZ esters **30–44**, in good yields. All the esters were then hydrolyzed under basic conditions into the corresponding acids **45–59**, which were submitted to the amidation step with Tyr methyl ester hydrochloride (**60–74**) and successively hydrolyzed under mild conditions to give target compounds **2–16**.

3.2. Antiviral activity and SAR analysis

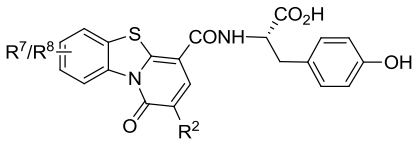
The new synthesized compounds **2–16** were tested against WNV (Uganda), DENV2 (Martinique) replicating in simian Vero E6 cells and YFV (Bolivia) replicating in human HEK293 cells. Antiviral activity (EC_{50}) was evaluated as the reduction of viral RNA in the supernatant after 3 or 4 days and cytotoxicity (CC_{50}) was assessed using the CellTiter Blue assay. Moreover, we tested also the enantiomeric counterpart of compound **1**, called (D)-**1** (Table 1) whose synthesis was previously described by us and having D-Tyr residue at the amide side chain instead of the canonical L amino acid (Tarantino et al., 2016). Results are reported in Table 1 together with data previously obtained for compound **1**, by using the same experimental protocols (Tarantino et al., 2016). Overall, we observed similar SAR trends for the tested compounds against the viruses employed as well as the cell lines.

In particular, the replacement of L-Tyr with its D counterpart ((D)-**1**) at C-4 amide side chain did not significantly influence the potency against the three viruses even if a slight increase in toxicity was observed (Table 1), thus indicating the canonical L-Tyr moiety as an important feature in obtaining selective anti-flavivirus activity. The shift of C-8-cyclohexyloxy moiety to the C-7 (**4**) position as well as its replacement with the smaller cyclopentyloxy (**2**), the branched isobutyloxy (**3**), or with the aromatic phenoxy counterpart (**5**) provided almost equal potent but more toxic compounds. These results indicated the C-8 cyclohexyloxy as an important chemical moiety for conferring selective antiviral activity in all the considered viruses. Differently, functionalization of the C-2 phenyl ring of the PBTZ scaffold generated the most interesting compounds (**6–16**) that, although showing similar anti-WNV activity to **1**, displayed significant differences against DENV2 and YFV. The insertion of an electron-withdrawing *meta*-fluorine (**7**) appeared as one of the best modifications within the mono-substituted compound set. With respect to parent **1**, derivative **7** was 2-fold more potent against DENV2 and YFV while maintaining a similar degree of cytotoxicity in both Vero E6 and HEK293 cells. Instead, the presence of an electron-donating group such as the *meta*-methoxy (**10**) produced reasonable antiviral activity but increased toxicity respect to both derivatives **1** and **7**. The presence of a fluorine atom (**6**) or a methoxy group (**9**) at *ortho* position of the C-2 phenyl did not significantly influence the biological profile respect to the parent compound **1**. With exception of the *para*-methoxy (**11**), *para* functionalization either with electron-withdrawing atoms (*i.e.* fluorine **8** and chlorine **12**) or electron-donating substituent (*i.e.* methyl **13**) produced more toxic compounds with respect to the starting hit. Interestingly, different combinations of fluorine and methoxy substituents at *meta* and *para* positions of the C-2 phenyl ring gave the di-substituted compounds (**14–16**) that resulted equally or slightly more potent against WNV and YFV and showed a 2-fold increase in anti-DENV2 activity. Compounds **7** and **14–16** emerged as the best ones, being characterized by potent and selective inhibitory activity against the three viruses (SI > 10). Compound **16** was selected as representative molecule within the series and used to investigate its activity against other flaviviruses: clinical strains of the remaining DENV serotypes (1, 3, 4), various genotypes of African YFV, clinical strains of JEV (Laos), a French Polynesian strain of ZKV, and the Neudoerlf strain of TBEV. The compound showed good activity against all the flaviviruses employed although with lower potency against JEV and ZKV (Table 2). We noticed that compounds **1** and **16** were more potent against TBEV than against other viruses by using the plaque assay while maintaining the same degree of cytotoxicity (data not shown). In comparison with compound **1** (Tarantino et al., 2016), compound **16** displayed comparable or slightly better activity when tested against DENV 1, 3, and 4, the YFV strains used, and TBEV thus highlighting its broad spectrum activity against different relevant flaviviruses.

3.3. Selectivity toward flavivirus genus

In order to explore the virus specificity of **16**, we decided to extend the cell-based assays to RNA viruses belonging to other families such as phlebovirus and alphavirus using clinical strains of Toscana virus (Phlebovirus, (−)-*ss*-RNA) and Chikungunya (Alphavirus; (+)-*ss*-RNA virus) replicating in Vero E6 cells (Table S1). NITD-008, a known reference compound specific for flavivirus polymerases (Yin et al., 2009), and favipiravir (T-705), an inhibitor of viral polymerases of numerous RNA viruses (Delang et al., 2014; Furuta et al., 2013), were used as positive controls (Table S1). Similarly to NITD-008, **16** was inactive against the Chikungunya and Toscana viruses (up to 20 µM).

Table 1
Cell-based antiviral assays for compounds (D)-**1** (Tarantino et al., 2016), and **2–16** against WNV (Uganda strain), DENV2 (Martinique strain), and YFV (Bolivian strain) compared to data for compound **1** (Tarantino et al., 2016).



cpds	R^2	R^7/R^8	Vero E6 cells					HEK 293 cells		
			EC_{50}[a] (μM)		CC_{50}[b](μM)	SI[c]		EC_{50}[a](μM)	CC_{50}[b](μM)	SI[c]
			WNV	DENV2		WNV	DENV2	YFV		
1[d]	Ph	H/CyO	2.1 ± 0.7	15 ± 5	115 ± 35	55	8	3.9 ± 1.4	50 ± 10	13
(D)-**1**	Ph	H/CyO	3.6	14	75	21	5	3.0	35 ± 10	12
2	Ph	H/CpO	7.0 ± 0.4	18.7	80	11	4	1.1	47	43
3	Ph	H/*i*BuO	4.1	18	75	18	4	3	40.5 ± 4	13
4	Ph	CyO/H	2.0	14	37	19	3	1.6	18 ± 2	11
5	Ph	H/PhO	4.9	20	80	16	4	2.9	48 ± 12	17
6	(2-F)Ph	H/CyO	7.7 ± 1.7	11	100	13	9	7.6	50	7
7	(3-F)Ph	H/CyO	3.6 ± 1.3	8.0 ± 1.0	100 ± 20	28	13	1.7 ± 1.3	55 ± 5	32
8	(4-F)Ph	H/CyO	3.9 ± 1.0	8.0 ± 3.0	58.0 ± 7.0	15	7	1.4 ± 1.0	41 ± 4	29
9	(2-OMe)Ph	H/CyO	3.2 ± 0.2	15	130	41	9	9.8	70	7
10	(3-OMe)Ph	H/CyO	4.1	12.0 ± 4.0	75	18	6	3.6 ± 2.6	38.0 ± 1.0	11
11	(4-OMe)Ph	H/CyO	4.1 ± 1.2	10.0 ± 4.0	80.0 ± 5.0	19	8	1.6 ± 0.4	38.0 ± 1.0	24
12	(4-Cl)Ph	H/CyO	1.6 ± 0.7	4.5	40	25	9	1.3	17 ± 1	13
13	(4-Me)Ph	H/CyO	2.1 ± 0.3	4.8	35	17	7	1.7	20 ± 3	12
14	(3,4-diF)Ph	H/CyO	2.0	6.8	75.0	38	11	2.4	26.5 ± 9	11
15	(3,4-diOMe)Ph	H/CyO	3.9	8.7	160	41	18	2.6	37 ± 1	14
16	(3-F,4-OMe)Ph	H/CyO	2.5	7.0 ± 1.0	92 ± 2	37	13	1.3 ± 0.5	28 ± 10	21

[a] EC_{50}: effective conc. that inhibits 50% virus replication as quantified by RT-PCR. The reported values with SD derived from two independent experiments performed in triplicate or from a single experiment.
[b] CC_{50}: cytotoxic conc. that reduces the redox conversion of resazulin into the fluorescent resorufin by 50%. The reported values with SD derived from two independent experiments performed in triplicate or from a single experiment.
[c] SI: selectivity index = CC_{50}/EC_{50}.
[d] Data for compound **1** are from ref. (Tarantino et al., 2016).

Conversely, the aspecific polymerase inhibitor favipiravir was active against all the flaviviruses and also the other viruses employed (Table S1). These results indicate that the activity is specific for a target present only in flaviviruses or for a host target essential only for their replication.

3.4. Anti-YFV activity in cell lines from different species

In order to check the possible involvement of a host factor in the mode of action, we evaluated the antiflavivirus activity of compound **16** in different cells infected with YFV (hamster BHK-21, mosquito C6/36, simian Vero E6, and human SW13). Compound **16** inhibited YFV replication with good SIs in all the distantly related cell species (Table 3). The activity of **16** was specific for flavivirus with respect to

Table 2
Antiviral activity of compound **16** against additional flaviviruses compared to **1**.

cpds	Vero E6 cells						HEK 293 cells		
	EC_{50} (μM) [a]								
	DENV1[b]	DENV3[c]	DENV4[d]	JEV[e]	ZKV[f]	TBEV[g]	YFV[h]	YFV[i]	YFV[j]
1	6.8[k]	10 ± 0.6[k]	7.2 ± 2.2[k]	NT[l]	NT[l]	0.6 ± 0.2[m]	8.3	12.0	3.1
16	5.1 ± 3.3	3.5 ± 1.0	2.9 ± 0.1	14.0 ± 4.0	10.7	0.7 ± 0.3[m]	1.7 ± 0.8	3.3 ± 1.6	2.0 ± 0.3

[a] EC_{50} = effective conc. that inhibits 50% virus replication as quantified by RT-PCR. The reported values represent the mean ± SD of from two or three independent experiments.
[b] DENV1 Djibouti strain.
[c] DENV3 Bolivia strain.
[d] DENV4 Dakar strain.
[e] JEV Laos strain.
[f] ZKV French Polynesia.
[g] TBEV Neudoerlf strain. [h]YFV Uganda strain.
[i] YFV Nigeria strain.
[j] YFV Sudan strain.
[k] Data for compound **1** are from ref. (Tarantino et al., 2016).
[l] Not tested.
[m] EC_{50} determined by plaque assay.

Table 3
Antiviral activity of compound **16** against YFV (Bolivia) replicating in cell lines from different species.

Cell Species	Mosquito C6/36	Hamster BHK21	Simian Vero E6	Human SW13
CC_{50} (μM)[a]	155.0 ± 25.0	77.0 ± 3.0	135.0	70.0
EC_{50} (μM)[b]	4.5 ± 3.5	3.5 ± 0	3.0 ± 0.1	0.9

[a] EC_{50} = effective conc. that inhibits 50% virus replication as quantified by RT-PCR. The reported values represent the mean ± SD of from two or three independent experiments.

[b] CC_{50}: cytotoxic conc. that reduce the redox conversion of resazulin into the fluorescent resorufin by 50%. The reported values represent the mean ± SD of data derived from two or three independent experiments.

other viral families (i.e. alphaviruses and phleboviruses) and was independent from the cell species. Although these data are suggestive of a viral target, we cannot exclude that a host target, highly conserved in mammalian and mosquito cells, could be involved and further experiments in this direction will be carry out in the next future.

3.5. Viral RNA replication and infectivity of released virions

Assuming that the compound's target was a viral protein, we next tried to select for escape mutants of YFV (Bolivia strain) replicating in Vero cells. Although multiple passages with doses < EC_{50} of the compound is an efficient method to select escape mutants, we chose another proven method (Delang et al., 2014). Thus we used a sub-optimal dose of compound **16** (i.e. 10 μM) that is 3 times the EC_{50} (3 μM) and allowed a 30% of viral replication compared to untreated virus culture. We failed to select for any resistant mutants (not shown), maybe due to because of the method of selection we chose and/or because the treated viruses proved to be non-infectious in the second round of replication, even in absence of compound **16**. We thus set up an experiment to monitor the viral replication in a single round of infection.

Vero E6 cells were treated in duplicates with **16** (10–0.6 μM), infected at high MOI with YFV and, after 2 h and extensive washing to remove the incoming virus, the infection was allowed to proceed in presence of **16** for a single round of replication (18 h in a first and 30 h in a second experiment). Both intracellular (IC) and supernatant (SPN) RNA were collected and quantified by RT-PCR and the infectivity of the recovered viruses (30 h p.i.) was assessed by tissue culture infective dose (TCID) titration (Fig. 3). The amount of viral IC RNA at 18 h and 30 h p.i. (cyan and blue bars in Fig. 3) as well as the level of SPN RNA at the end of a first replicative cycle (red bars) were not significantly affected by the presence of **16**. Therefore **16** is not able to block RNA synthesis or virion release during a single replication cycle. In contrast, the infectivity of the virions generated in presence of **16** was strongly diminished in a dose-response relationship (green bars in Fig. 3). In fact, **16** at 0.62 μM halved the TCID (from 3×10^6 to 1.5×10^6) and higher dosages (5 μM and 10 μM) reduced YFV infectivity more than 3 log. It is noteworthy that the half effective dose inhibition of TBEV infectious titer (see Table 2) is very close to what is reported for YFV in TCDI determination. This experiment showed that the antiviral effect of **16** is not related to a reduction of viral RNA synthesis or virion release, but to the strong impairment of virion infectivity.

To further investigate such loss of infectivity, a new experiment was carried out by checking the fate of viral RNA upon a second round of replication in absence of **16**. Virions collected from the previous single round of treatment were used to infect fresh Vero E6 cells for 3, 7 and 24 h. Viral IC RNA level at 3 h p.i. (Fig. 4) was similar in cells infected by viruses released from the previous round of infection either untreated or treated with 0.6 and 1.25 μM of **16**. On the contrary, virus

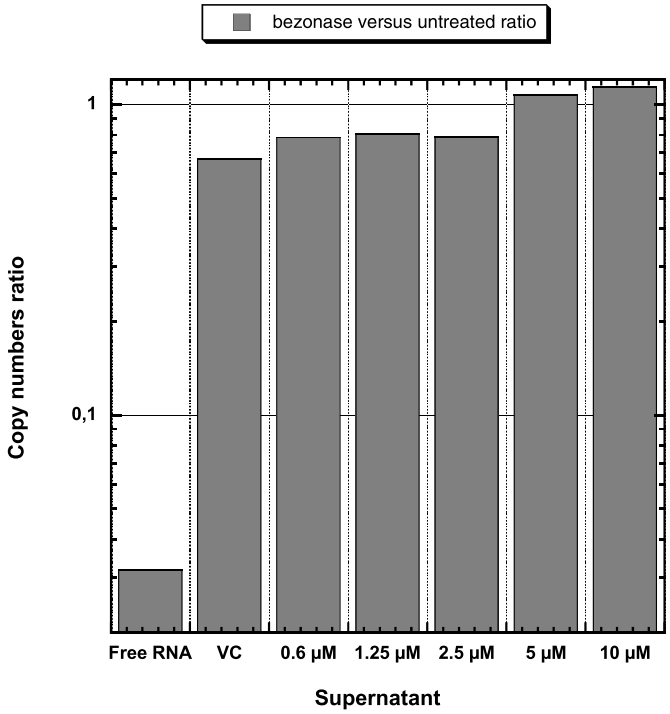


Fig. 5. Benzonase digestion of cell supernatant: ratio of viral RNA copy numbers in benzonase digested samples versus untreated ones are plotted.

released from cells that have been treated with higher doses of **16** did not lead to any detectable secondary infection by our qRT-PCR system suggesting that viruses produced in presence of large amount of **16** were completely ineffective. Accordingly, viral replication level measured by IC RNA at later time point (24 h p.i.) decreased in a dose-response mode when incoming viruses were derived from cells treated with increasing amount of **16**. These results show that the exposition to **16** during the first cycle of replication led to the release of defective viruses, unable to complete a second round of infection even in absence of the inhibitor.

3.6. Integrity of released virions

In order to check whether the reduced infectivity of the viral particles was related to virion-packaging defects, we treated the viral samples with benzonase, a genetically engineered RNA and DNA endonuclease (homodimer of 60 kDa) able to digest free nucleic acids. In conditions where free RNA was highly sensible, viral RNA was protected from nuclease digestion, showing that treatment with **16** (up to 10 μM) did not appear to induce macroscopic defects in virions packaging (Fig. 5). Although the experiment with benzonase is not conclusive to exclude structural defects of viral particles, to date we are unable to safely run electron microscopic studies of virion budding and maturation in our institutions.

3.7. Genomic defects

We hypothesized that the defective virions could result from the encapsidation of an abnormal genome after a single round of replication in presence of compound **16**. Thus, we looked at genome defects by analyzing a representative 2051 bp genomic segment of the YFV genome to search for any excess mutations. One per 1000 variations of an average of 15,000 reads were registered for control and for viruses treated with 2.5 or 10 μM of compound **16**. No significant excess mutations were observed either in total number or in frequency of specific nucleotide transitions (Table S2).

To further explore if **16** was able to affect the polymerase reliability,

we evaluated the level of incorporation of nucleotide inhibitors (NIs) by measuring the EC_{50} of NITD-008 and ribavirin in absence/presence of **16** (5 μM). We observed no significant modification of the EC_{50} of these NIs, excluding an easier incorporation by RdRp promoted by compound **16** (Table S3). These results demonstrated that compound **16** did not affect RdRp fidelity during elongation.

4. Conclusions

We have reported the design, synthesis and biological evaluation of functionalized PBTZs derived from the previously described hit-compound **1** (Tarantino et al., 2016). Some compounds described in this study and especially **16** resulted potent and selective inhibitors of a wide panel of relevant flaviviruses. The activity of **16** was specific for flavivirus with respect to other viral families (*i.e.* alphaviruses and phleboviruses). In order to have insights about the mode of action, we investigated the effect of **16** on RNA synthesis, virion integrity, release and infectivity using YFV as a model. We showed that **16** does not affect the IC and SPN RNA levels, ruling out any effect on viral RNA synthesis or virions release. However the recovered viruses were unable to complete a second round of replication, likely due to the production of an abortive viral phenotype. The experiments with benzonase showed that the new virions, synthetized in presence of **16,** lacked macroscopic structural defects but still they showed highly reduced infectivity. To our knowledge, such PBTZ-based compounds are the first-in-class anti-flavivirus agents that exert their activity by causing the release of viruses unable to complete a second round of infection. Thanks to this peculiar mode of action, this class of molecules can represent a valid starting point to develop new pan-antiflavivirus agents. Future studies will be focused on the identification of the mechanism of action for this compound class and results will be published on due course.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.03.004.

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