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Hindered nucleoside analogs as antiflaviviridae agents*

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Abstract: Flaviviridae are an important family of viruses, responsible for widely spread diseases such as dengue and West Nile fever and hepatitis C. Despite the severity of the related diseases, no effective antiviral treatments for infection are available. Following our discovery of adenosine-hindered analogs as potent antiflaviviridae agents, we have continued our investigation on guanosine and inosine derivatives, which were evaluated for activity against BVDV, YFV, DENV, and WNV viruses in cell-based assays. The present study allowed us to identify some newer features that led to improve the antiviral potency (down to the μM range) and to selectively inhibit BVDV and YFV viruses. The molecular modeling results were consistent with the hypothesis that test analogs act as RNA-dependent RNA polymerase (RdRp) inhibitors by interacting with a surface allosteric binding pocket.

INTRODUCTION

Flaviviridae are positive, single-stranded RNA viruses. This virus family contains three genera: hepacivirus [hepatis C virus (HCV)], flavivirus [e.g., yellow fever virus (YFV), dengue fever virus (DENV), West Nile virus (WNV)], and pestivirus [bovine viral diarrhea virus (BVDV), border disease virus (BDV)], which include numerous important human and animal pathogens [1–4]. Notwithstanding, flaviviridae belonging to different genera show great similarity in viral morphology, genome organization, and replication strategy [5]. A number of these viruses are responsible for important worldwide human deadly pathologies.

Chronic or early-diagnosed acute hepatis C, the flaviviridae infection endowed with the major social impact, are normally treated with interferon, alone or in combination with ribavirin, which possesses a significant activity against some RNA viruses [6]. However, the efficacy of these drugs is very limited, leading to suboptimal responses in only less than 30 % of the patients infected with the most

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frequent HCV genotypes in Europe (Ia and Ib) [7]. Similarly, no specific treatments are available for dengue or West Nile virus infections, although they would be particularly desirable in view of the recently observed spread of these viral agents [8–10]. As already well established in the case of HIV infections/AIDS, viral pathologies need a continuous evolution of specific and highly effective treatments. In fact, an inadequate control of viral replication may lead to the rapid emergence of drug-resistant mutants and, consequently, to treatment failure.

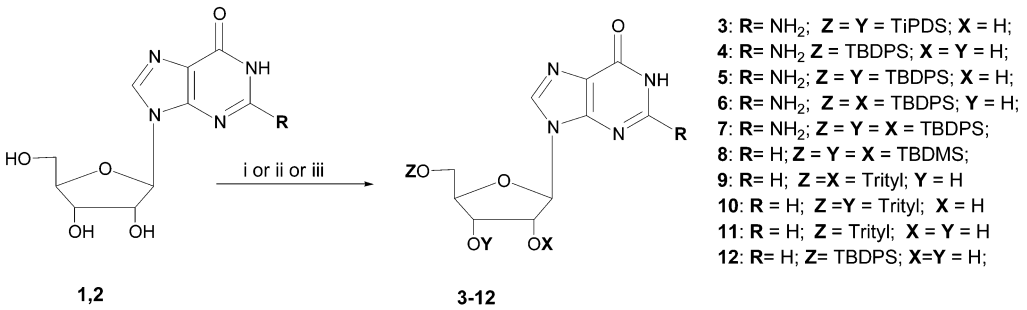
One of the most relevant aspects in the research of new antiviral agents is the availability of in vitro models able to predict their potential biological activity. In the case of flaviviridae, we used an in vitro methodology involving viruses, that belong to the flavivirus and pestivirus genera. Among them, BVDV (bovine viral diarrhea virus) has been also widely used as an HCV surrogate. Among a first generation of compounds, the two adenine derivatives FEVB28 [11] and FEG118 turned out to be selectively active, exhibiting a potency far superior to that of ribavirin, used as reference compound. With the aim of improving their activity, we then performed a structure–activity relationship study that led us to envision modifications at both the base and sugar portions. In particular, adenosine and 8-substituted-adenosine derivatives, as well as 2′-deoxy- and arabino-furanosyl derivatives of adenine, differently substituted at the sugar portion with hindered groups, were prepared and tested in cell-based assays [12]

Some derivatives, and in particular *tert*-butyl-diphenylsilyl-adenosines (TDSAs), resulted in being endowed with potent and selective activity against BVDV (EC_{50} 5–50 μM). Taking this into account, and considering that the presence of hindered groups at sugar positions appeared as a relevant feature for these compounds, we decided to extend the same modification to other nucleosides, in particular to guanosine and inosine. An extensive structure–activity relationship study was then conducted taking into account compounds carrying sugar modifications at the 2′ position (ribo- and arabino-derivatives) as well as different hindered groups. Moreover, being FEG118, the first lead compound, an 8-substituted nucleoside, this feature was also explored by introducing simple modifications in the guanine and hypoxantine bases.

Molecular modeling studies were also conducted in order to corroborate indications coming from biological activity evaluations and to assist and direct synthetic studies.

CHEMISTRY

Guanosine and inosine TBDPS and TBDMS derivatives (Scheme 1) and corresponding arabino-furanosyl compounds (Scheme 2), were prepared according to the procedure described by Ogilvie et al. [13].

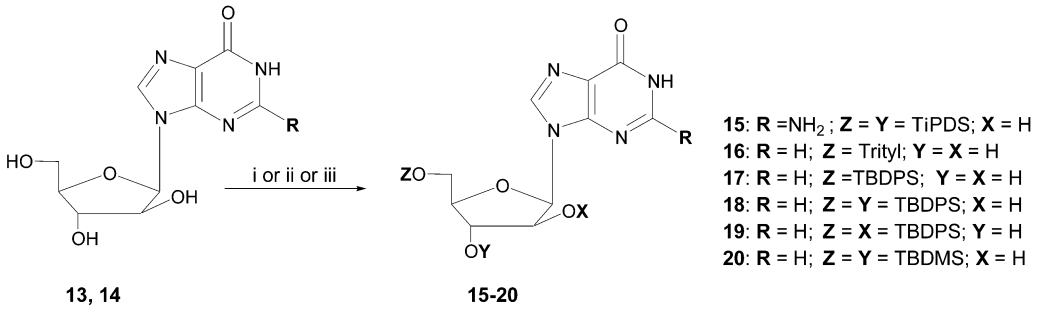


1: **R**: NH_2; **2**: **R**: H

i: DMF, TBDMSCl or TBDPSCl, imidazole, r.t.; ii: Pyr, $TiPDSCl_2$, Rfx, iii: Pyr, Trityl-Br, r.t.-60°C.

Scheme 1

Trityl derivatives were prepared as described in the literature starting from compounds **2** and **14** [14]. The synthesis of the 5′,3′-TIPDS derivative of guanosine was carried out following and adapting the procedure reported by Robins et al. [15]. Modifications of reaction conditions were required to obtain the different positional isomers (see Schemes 1 and 2).

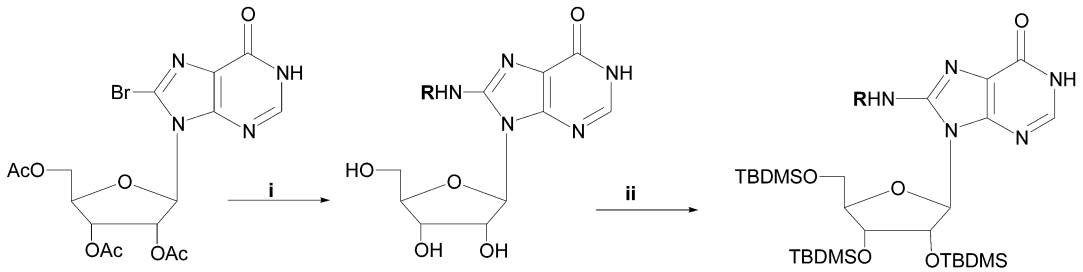


13: **R** = NH_2; **14**: **R** = H

i: DMF, TBDMSCl or TBDPSCl, imidazole, r.t.; ii: Pyr, $TiPDSCl_2$, Rfx, iii: Pyr, Trityl-Br, r.t..

Scheme 2

8-Substituted inosine derivatives were prepared by heating at reflux conditions compound **21** [16] in anhydrous dioxane in the presence of an excess of the corresponding amine [17]. The next introduction of the TBDMS groups, carried out as reported above (Scheme 1), gave the compounds **32**–**41** in good yields (Scheme 3).

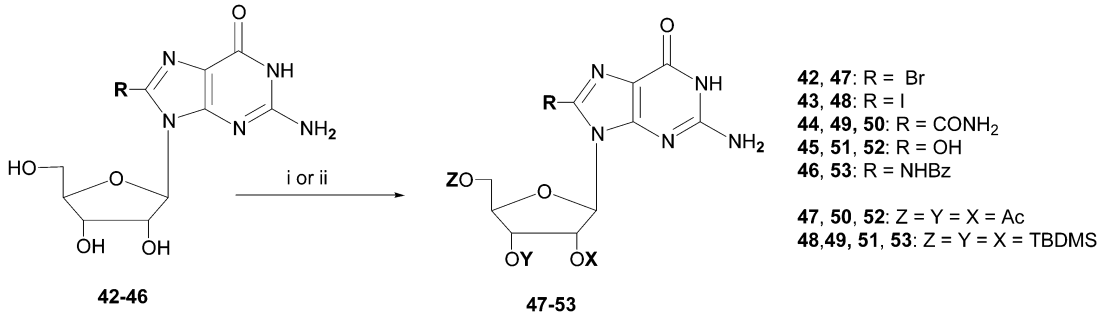


22, 32: R= nBut-
23, 33: R= Bn-
24, 34: R= cyclopentyl-
25, 35: R= $Ph(CH_2)_3$-
26, 36: R= *p*F-Bn-
27, 37: R = 3,4-dimethoxy-Bn-
28, 38: R = *p*Me-Bn-
29, 39: R= cycloexylmethyl-
30, 40:R= 3,4,5-trimethoxy-Bn-
31, 41:R = cycloexyl-

i: Dioxane, amine, 80°C; **ii:** DMF,TBDMSCl, imidazole, rt

Scheme 3

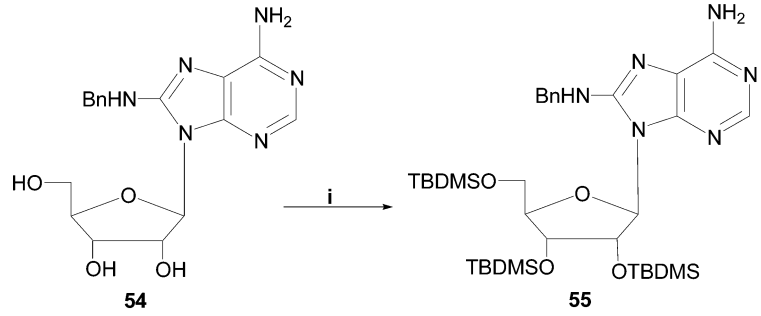
8-Substituted guanosine derivatives **42–45** (Scheme 4) were prepared mainly according to synthetic strategies described in the literature [16,18,19,20]. Compound **46** was synthesized from compound **42** with benzylamine in methanol at reflux conditions. The further synthetic step was performed with acetic anhydride in pyridine (**47**, **55**, **52**) or with TBDMSCl and imidazole in anhydrous DMF (**48**, **49**, **51**, **53**).



i: DMF,TBDMSCl, imidazole, rt; **ii:** Pyr., Ac_2O, rt.

Scheme 4

The only 8-substituted adenosine analog reported here (**55**) was prepared starting from compound **54** [21], by persilylation of the sugar ring as previously described [13] (Scheme 5).



i: DMF,TBDMSCl, imidazole, rt

Scheme 5

BIOLOGY

Test compounds were dissolved in DMSO at 100 mM and then diluted in culture medium.

Cytotoxicity

Exponentially growing human $CD4^+$ lymphocytes (MT-4), baby hamster kidney (BHK-21), and Madin Darby bovine kidney (MDBK) cells were resuspended in growth medium containing serial dilutions of the drugs. Cell viability was determined after 96 h at 37 °C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method.

MTT assay

Activity against YFV, DENV-2, and WNV was based on inhibition of virus-induced cytopathogenicity in acutely infected BHK-21 cells. Activity against BVDV was based on inhibition of virus-induced cytopathogenicity in acutely infected MDBK cells. Cells were seeded overnight at a rate of 5×10^4/well into 96-well plates in growth medium at 37 °C, in a humidified CO_2 (5 %) atmosphere. Cell monolayers were infected with 50 µl of a proper virus diluition to give an m.o.i = 0.01. Then, serial dilutions of test

compounds in Dulbecco's modified Eagle's medium, supplemented with 2 % inactivated fetal calf serum, were added. After a 3-day incubation at 37 °C, cell viability was determined by the MTT method.

MOLECULAR MODELING

All simulations were run on a cluster of Silicon Graphics Octane and performed by using the program packages AutoDock (v. 3.0) [22], AMBER 6.0 [23,24], Materials Studio (v. 2.2) [25], Discover [26], and in-house developed codes (stand-alone and add-on to the commercial software). The starting 3D model of the RNA-dependent RNA-polymerase (RdRp) was based on its X-ray crystallographic structure [27] (chain B, PBD Code: 1CSJ). The all-atom force field (FF) parameters by Cornell et al. [28] (in *parm94.dat* file of the AMBER 6.0 code) was applied for protein relaxation. The GB/SA continuum solvation model [29,30] was used to mimic a water environment.

The model structures of all nucleoside analogs considered were generated using the 3D sketcher tool of Materials Studio. The conformational search was carried out using a combined molecular mechanics/molecular dynamics simulated annealing (MDSA) protocol [31]. The electrostatic charges for the geometrically optimized nucleoside analogs were obtained by quantum mechanical calculations. [32].

For the docking of each inhibitor to the protein, Monte Carlo/simulated annealing (MC/SA) runs were performed with Autodock [33]. Each best drug/RdRp complex resulting from the automated docking procedure was further refined in the AMBER suite using the quenched molecular dynamics method (QMD) [34]. Extended molecular dynamics simulations at 298 K were further conducted to both qualify and quantify the interaction between the enzyme and the nucleoside analogs [35].

RESULTS AND DISCUSSION

The results of a preliminary investigation conducted on adenine derivatives [12] pointed out two main requisites for the activity against flaviviridae: (i) the specific structure of the glycosidic moiety (ribo- and arabino-furanosyl) and (ii) the presence of bulky groups at the hydroxyl functions. In particular, modifications at the glycosidic portion by the introduction of hindered groups led to compounds endowed with increased inhibitory activity against test viruses (i.e., 2′,5′-bis-*O*-TBDPS-araA, $CC_{50} > 100$ μM, EC_{50} 11 μM, against BVDV in MDBK cell lines) in the respect of the lead FEVB-28. However, in addition to a greater potency, adenine derivatives showed increased cytotoxicity in MT4 cells (CC_{50} 19 μM), that prompted us to continue the study considering different heterocyclic bases

In this regard, the biological data here reported (Table 1) can be considered of great interest. The introduction of different silyl or non-silyl hindered groups at the glycosyl moiety of guanosine and inosine derivatives led to compounds maintaining and improving antiflaviviridae activity (**5**, **6**, **9**, **10**, **19**). Moreover, an important outcome of the present study concerned the discovery of the contribution to the biological activity of a substituent at position 8 of the purine base. In particular, introduction of benzylamine at position 8 on 2′,3′,5′-tri-*O*-TBDMS inosine, led to derivatives showing interesting antiviral activity and also a good selectivity index (**33**, SI > 10). The corresponding guanosine and adenosine derivatives, **53** and **55**, did not display similar behavior. When benzylamine was substituted, at para position of the phenyl ring, with small groups like fluorine and methyl, an increase in potency and SI was observed (**36** SI > 20, **38** SI > 50). A similar increase in potency was obtained, introducing on the aromatic ring two or three methoxy groups (compounds **37** and **40**), but with concomitant increase in cytoxicity. Furthermore, basic features for the biological activity appear to be the maintenance of an aromatic ring and the length of the spacer between nitrogen and the aromatic ring. In fact, elongation of the spacer and saturation of the aromatic ring led to less active and cytotoxic compounds (**35**, **39**).

Table 1 Biological activity of study compounds.

Compd.	MT4	YFV		BVDV		DEN-2	WNV
	CC_{50}[a]	CC_{50}[b]	EC_{50}[c]	CC_{50}[d]	EC_{50}[e]	EC_{50}[c]	EC_{50}[c]
3	49	>10	>100	>100	100	>100	>100
4	≥100	>100	>100	>100	>100	>100	>100
5/6	16	16	>16	48	14	>16	>16
7	>100	>100	>100	>100	>100	ND	ND
8	>100	>100	>100	>100	>100	>100	>100
9	16	13	>13	>100	3.8	ND	ND
10	17	6	>6	15	3	ND	ND
11	53	44	17	57	>57	ND	ND
12	18	24	11	46	24	ND	ND
15	17	≥100	>100	>100	>100	ND	ND
16	47	43	>43	59	>59	ND	ND
17	39	24	>24	33	>33	ND	ND
18	20	>100	>100	19	>19	ND	ND
19	12	5	>5	14	6.7	ND	ND
20	20	16	>16	8.4	>8.4	ND	ND
22	>100	>100	>100	>100	>100	>100	>100
23	>100	>100	>100	>100	>100	>100	>100
25	>100	>100	>100	100	>100	ND	ND
26	>100	>100	>100	>100	>100	ND	ND
27	>100	>100	>100	>100	>100	ND	ND
32	50	25	>25	63	11	>25	>25
33	16	29	>29	≥100	8.8	>29	>29
34	56	40	>40	>100	>100	ND	ND
35	58	68	>68	>100	21	ND	ND
36	25	24	>24	≥100	4	ND	ND
37	19	6.4	>6.4	11	0.5	ND	ND
38	41	16	>16	≥100	2	ND	ND
39	50	51	>51	≥100	32	ND	ND
40	20	6.4	>6.4	7	3	ND	ND
41	54	54	>54	>100	>100	ND	ND
45	>100	>100	>100	>100	>100	>100	>100
46	>100	>100	>100	>100	>100	>100	>100
47	>100	>100	>100	>100	>100	>100	>100
48	30	19	>19	>100	9	>19	>19
49	6	7	>7	16	11	>7	>7
50	>100	>100	>100	>100	>100	>100	>100
51	10	5	>5	19	5	>5	>5
52	>100	>100	>100	>100	>100	>100	>100
53	51	35	>35	>100	47	>35	>35
55	>100	>100	>100	>100	>100	>100	>100

[a]Compound concentration (μM) required to reduce the viability of mock-infected MT4 cells by 50 %, as determined by the MTT method.
[b]Compound concentration (μM) required to reduce the viability of mock-infected BHK cells by 50 %, as determined by the MTT method.
[c]Compound concentration (μM) required to achieve 50 % protection of BHK cells from the YFV-induced cytopathogenicity, as determined by the MTT method.
[d]Compound concentration (μM) required to reduce the viability of mock-infected MDBK cells by 50 %, as determined by the MTT method.
[e]Compound concentration (μM) required to achieve 50 % protection of MDBK cells from the BVDV-induced cytopathogenicity, as determined by the MTT method.
ND: not determined.

Finally, very preliminary results, coming from ongoing biological studies (competition studies using known inhibitors, data not shown) seems to indicate in the RNA-dependent RNA polymerase (RdRp) a possible target of the activity.

However, in order to support the synthetic studies and since the exact molecular target of the observed activity has not been yet confirmed, a preliminary investigation, by molecular modeling techniques, has been conducted in order to explore the possible interaction with the putative target. As an example of the evidences obtained by the application of the modeling techniques, we report here in detail the results obtained with one of the most active compounds, **33**. As seen in Fig. 1, the compound is partially buried within a long cleft ($30 \times 10 \times 10$ Å), that extends nearly the entire length of the thumb's surface and eventually connects with the finger domain. The bottom of the cleft is formed by the side chains of Trp 528, Met 423, Leu 419, Tyr 477, and the main chain atoms of His 475 and Leu 474. The molecule occupies the central portion of the extended cleft, and the detailed interactions between **33** and the enzyme are illustrated in Fig. 2. The interface consists primarily of nonpolar surfaces, along with

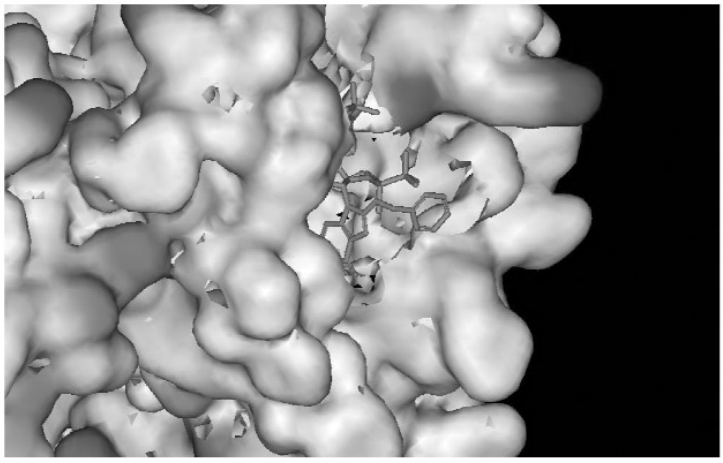


Fig. 1 Binding of nucleoside analog **33** to a surface allosteric site of RdRp.

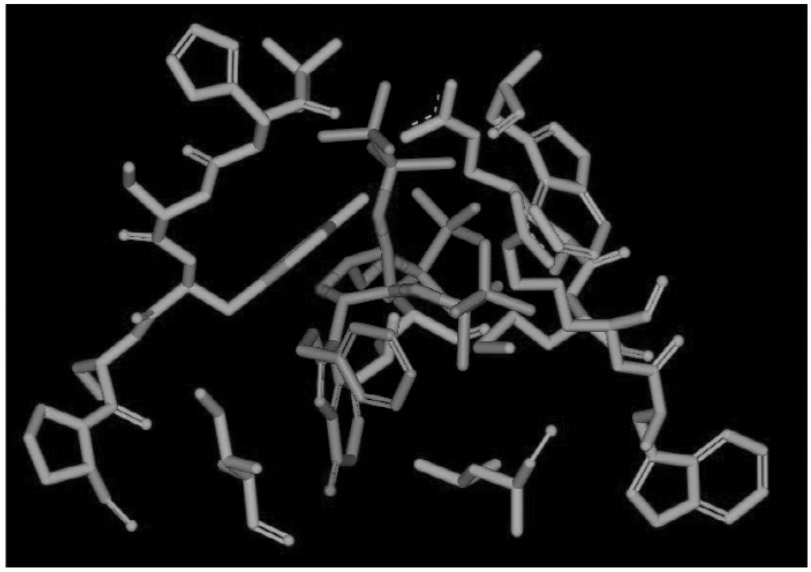


Fig. 2 Detailed interaction of nucleoside analog **33** and the amino acid of the enzyme allosteric binding site.

some hydrogen bonds. Extensive van der Waals and hydrophobic interactions involve both the phenyl substituent on the base, and the *tert*-butyl bulky substituents on the sugar ring. The first is lined by the side chain of Arg 501 (alkyl portion of the side chain), and is further stabilized by the presence of the *tert*-butyl group linked to *O*-2′. This, in turn, positively interacts with the side chains of Met 423 and Trp 528. The *O*-3′ linked *tert*-butyl moiety fits into a hydrophobic region, lined by the alkyl portion of the side chain of Arg 422, and the main chain atoms of Leu 474 and His 475. The remaining bulky substituent on *O*-5′ of **33** rests on side chains of Arg 422 and Leu 419, while the sugar ring is aptly lined by the polar residues Ser 476 and Tyr 477, and by the amide bonds of the main chain. In particular, two persistent, alternative H-bond stabilizing interactions are realized between the 5′-oxygen atom of the sugar and the hydroxyl group of Tyr 477 (characterized by an average dynamic length, ADL, of 2.55 Å), and with the charged amino group of Arg 422 (ADL = 2.97 Å). The above description of NS5B-inhibitor interactions applies, with minor modifications, to the other study compounds.

It is interesting to note that: (1) interaction of these compounds with HCV NS5B results only in minimal changes in overall protein architecture and (2) the compound binding site may retain a high degree of 3D similarity across NS5B enzymes derived from known HCV genotypes. Accordingly, NS5B inhibitors that interact with this binding site have the potential to function as selective, broad-spectrum anti-HCV agents. In order to obtain information useful to the confirmation of the molecular target, and to address computational and synthetic in vitro studies on the specific enzymes involved with the viral RNA replication are currently being conducted.

In conclusion, the current investigations confirm the results previously obtained and consent to characterize these compounds as a new class of antiflaviviridae agents featured by highly hindered nucleosidic structures which we termed: hindered nucleoside analogs (HNAs). Further studies are currently ongoing in order to obtain newer compounds deriving from the suggestions of the biological and modelistic studies.

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