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Letter

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Synthesis and evaluation of bifunctional aminothiazoles as antiretrovirals targeting the HIV-1 nucleocapsid protein.

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KEYWORDS: nucleocapsid protein, HIV, NC inhibitors, aminothiazole, drug resistance, antiretroviral

ABSTRACT: Small molecule inhibitors of the HIV-1 nucleocapsid protein (NC) are considered as promising agents in the treatment of HIV/AIDS. In an effort to exploit the privileged 2-amino-4-phenylthiazole moiety in NC inhibition, here we conceived, synthesized, and tested *in vitro* eighteen NC inhibitors (NCIs) bearing a double functionalization. In these NCIs, one part of the molecule is deputed to interact non-covalently with the NC hydrophobic pocket, while the second portion is designed to interact with the N-terminal domain of NC. This binding hypothesis was verified by molecular dynamics simulations, while the linkage between these two pharmacophores was found to enhance antiretroviral activity both on the wild-type virus and on HIV-1 strains with resistance to currently licensed drugs. The two most interesting compounds **6** and **13** showed no cytotoxicity, thus becoming valuable leads for further investigations.

The HIV-1 nucleocapsid protein (NC) is a zinc-finger protein acting as a nucleic acid chaperone that is involved in multiple steps of the HIV-1 replication cycle, thus becoming a desirable target for antiretroviral therapy.[1] To date, pharmacological modulation of the NC has been achieved by means of three different strategies: *i)* small molecules able to promote the ejection of zinc from the NC and to induce protein unfolding, which are considered rather toxic and poorly specific although some of them have been recently profiled in preclinical studies;[2] *ii)* non-covalent NC binders that inhibit the interaction between NC and nucleic acids, which are potentially more specific and less toxic than *i)*;[1,3-5] *iii)* non-covalent binders to nucleic acids that compete with the NC, which are currently more suited as tool compounds than lead candidates.[6-7]

Our research has long focused on strategy *ii)* with the aim to develop different chemotypes of effective NC inhibitors (NCIs) endowed with antiretroviral activity against wild-type and drug-resistant HIV-1 strains.[8-10] These molecules are designed to bind to a hydrophobic pocket located in the C-terminal zinc finger of NC, which accommodates the critically-recognized guanosine of nucleic acid targets through interaction with Gly35, Trp37, Gln45 and Met46, as shown by NMR studies (Figure 1a).[11-12] Most of these residues belong also to the more extended hydrophobic platform that is instrumental for the specific recognition and destabilization of nucleic acids.[12-15]

In a previous work, NC inhibition by the 2-amino-4-phenylthiazole derivative AN3 (Figure 1b) was characterized by a multidisciplinary approach, and AN3 was shown to anchor the NC by interacting within the hydrophobic pocket described above.[8,12,15] In an effort to obtain rough structure-activity relationships (SAR) of AN3, the derivative AN1 (Figure 1b) was designed,[8] but unfortunately it proved less effective than AN3 in NC inhibition. Moreover, AN1 was shown to bind preferentially to residues located near the N-terminal region of NC, i.e. Met1–Lys14 (N-ter) (Figure 1a), which are crucial for nucleic acids recognition and annealing.[16-21] By using NMR spectroscopy, Goudreau et al. have recently disclosed an NCI able to form a 2:1 complex with the NC; one molecule binds the hydrophobic pocket, while the second molecule binds the N-ter as well as the first molecule in a head-to-tail stacking conformation (Supporting Information, Figure S1).[22]

Based on these evidences, here we designed and synthesized eighteen bifunctional NCIs by linking pharmacophores and chemical moieties from AN3, namely the hydrophobic pocket binder (HPB), to those from AN1, namely the N-ter binder (NTB) (Figure 1b and 1c) in individual molecular entities endowed with improved antiretroviral activity.

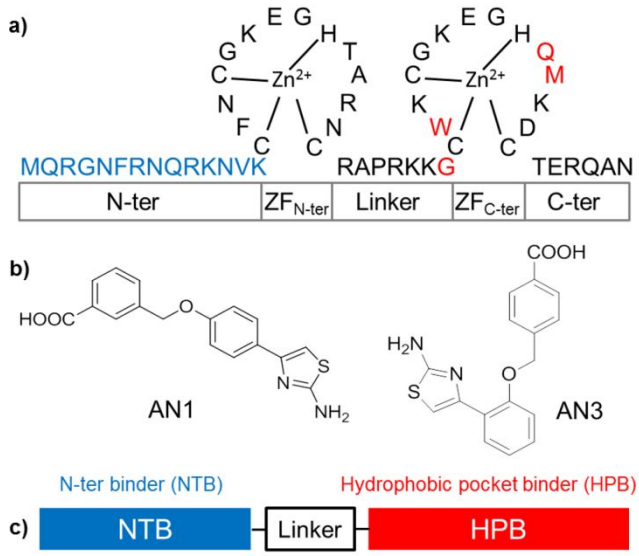

Figure 1. Design strategy adopted in this work. a) Sequence of the HIV-1 nucleocapsid protein from the LAV strain; the N-ter is colored blue, while residues of the hydrophobic pocket targeted also by AN3 are colored red. The various regions of the protein are labeled in the bottom bar (ZF means zinc finger). b) Chemical structures of AN1 and AN3. c) Schematic representation of the bifunctional aminothiazoles studied in this work, in which hydrophobic pocket binder (HPB) and N-ter binder (NTB) moieties are connected by a linker.

Structural information on AN3/NC complex[8] identified the carboxylic acid group as the preferred site for the introduction of the linker, as it is solvent exposed and not involved in binding to the NC. In a first attempt, AN1 and AN3 were coupled through four linkers with different length and flexibility including piperazine, to understand which distance is required to interact with both the hydrophobic pocket and the N-ter of the NC (compounds **1-4**, Figure 2a). Evaluation of the antiviral activity of **1-4** showed that the molecules exhibit enhanced efficacy (Table 1) compared to parent compound AN3, this latter having IC_{50} = 95 μM as determined previously.[8] Particularly, compound **1** bearing a two-carbon linker proved to be the most effective NCI of this preliminary series, which guided the design of additional derivatives. Thus, modifications were first introduced within the HPB portion by means of a fluorine (**5**) or a chlorine (**6**) substituent in position 4 of the (benzyloxy)benzene ring. Moreover, both aminothiazoles were replaced by aminoxazole rings (**7**) or a single aminothiazole was replaced by a benzothiazole (**8**) (Figure 2b). In addition, derivatives **9-18** bearing modifications to the NTB were designed and synthesized by removing and/or replacing AN1 pharmacophores. Progressive shortening of the NTB portion was planned to optimize the interaction with the N-ter as well as to improve the drug-likeness of the compounds (Figure 2c). Details of chemistry are reported in Supporting Information.

The antiviral activity of compounds **1-18** was tested against the HIV-1 wild type NL4-3 strain (sequence alignment between NC from LAV and NL4-3 strains is described in Supporting Information, Figure S2) in a TZM-bl cell line-based phenotypic assay (Table 1).[23]

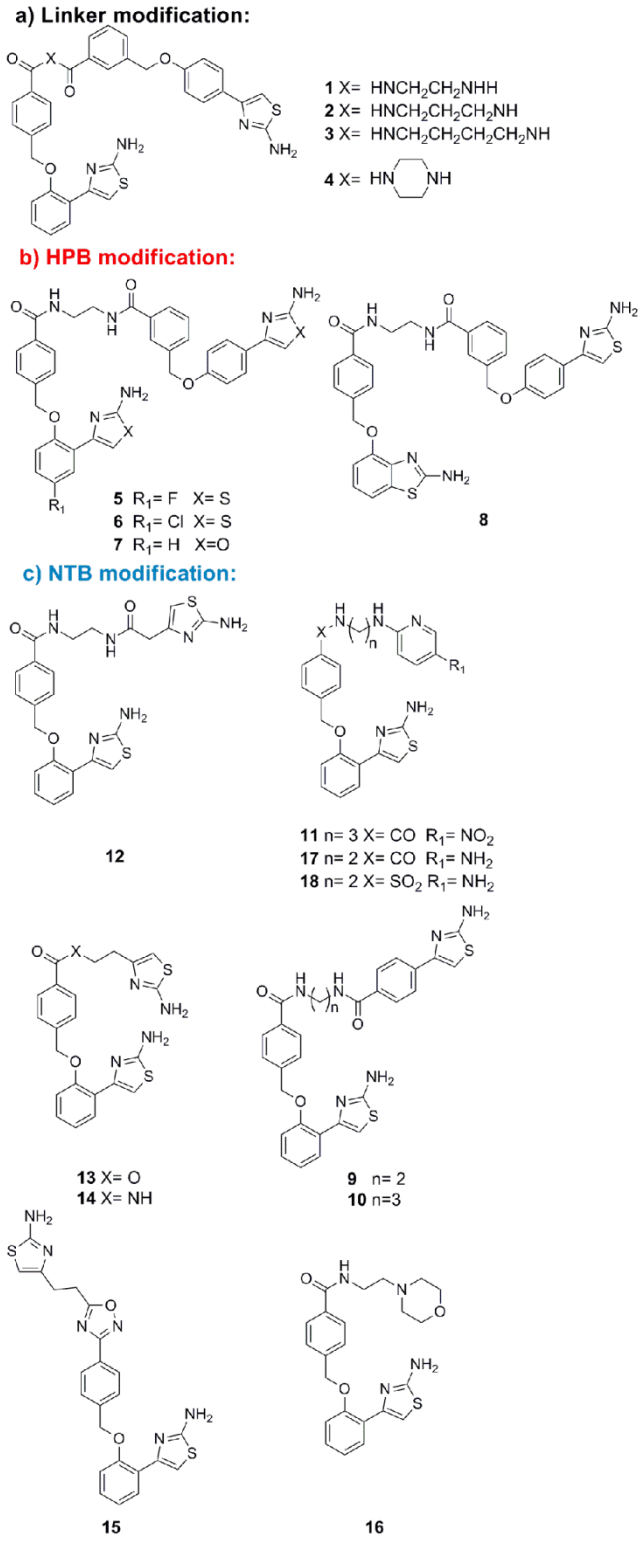

Figure 2. Chemical structures of bifunctional aminothiazoles. a) derivatives bearing different linkers; b) derivatives bearing modifications on the HPB portion; c) derivatives bearing modifications on the NTB portion.

The reporter cell line TZM-bl allows the detection of HIV-1 replication through the activation of luciferase and β–galactosidase genes integrated in the cell genome under the control of HIV-1 long terminal repeat (LTR) promoter. After infection, the expression of the reporter genes is activated by

the viral Tat protein, which is mainly produced in the late phase of HIV-1 life cycle. In particular, two approaches were adopted to evaluate the effect of candidate NCIs in the early phase of viral replication (MonoCycle assay) and on the whole replication cycle (BiCycle assay).[23] While the Monocycle assay consists in a single cycle of infection in TZM-bl cells in the presence of dilutions of compounds, the BiCycle assay includes a first infection in MT-2 cells with dilutions of compounds, then the viral particles produced in this step are subsequently measured by infecting TZM-bl cells. Most compounds showed a good antiviral activity with IC_{50} ranging from 0.3 to 37 μM with the only exception of **12**, **16** and **18** that were inactive up to 100 μM concentration. Most compounds showed an IC_{50} generally lower in the BiCycle assay than the MonoCycle assay, suggesting a multi-step inhibition of viral replication that is consistent with the multiple roles of NC in the HIV replication cycle.[24-25] Notably, compared to the parent AN3, antiretroviral activity was increased around 100 folds.

Table 1. Antiretroviral activity (two different assays), NC inhibition, and cytotoxicity of the tested molecules.

Cmpd	BiCycle IC_{50} ± SD (μM)	MonoCycle IC_{50} ± SD (μM)	% NC inhibition at 10 μM	MTS[a] TD_{50} (μM)	SI[b]
1	1.3 ± 0.8	5 ± 1	24 ± 6	30	23
2	12 ± 10	>50	16 ± 2	-	-
3	5 ± 2	16 ± 3	19 ±3	>100	>20
4	28 ± 5	>50	15 ± 2	-	-
5	1.1 ± 1	>50	16 ± 1	>50	>45
6	0.8 ± 0.3	>50	20 ± 6	>50	>63
7	6.2 ± 0.5	>50	21 ± 7	-	-
8	37 ± 6	>50	17 ± 2	-	-
9	15 ± 3	12 ± 5	15 ± 1	-	-
10	5 ± 4	30 ± 3	not active	-	-
11	12 ± 10	28 ± 5	21 ± 6	-	-
12	>100	>100	-	-	-
13	0.3 ± 0.1	1.2 ± 1	18 ± 3	16	53
14	36 ± 9	>50	16 ± 2	-	-
15	6 ± 4	35 ± 6	not active	-	-
16	>100	>100	-	-	-
17	3.8 ± 2.1	>50	15 ± 2	45	12
18	>100	>100	-	-	-
AN3	95[c]	-	6 ± 1	-	-

[a] MTS assay was performed only on compounds showing an IC_{50} (BiCycle) ≤ 5μM, and being positive in the NC inhibition assay.

[b] Selectivity Index SI = TD_{50} (MTS)/IC_{50} (BiCycle). SD: standard deviation.

[c] From reference[8], first cycle of infection in HeLa P4.R5MAGI cells.

- means not performed

To monitor whether the target of the antiretroviral activity *in vitro* could be the NC, compounds showing efficacy in the BiCycle assay were then tested for their ability to inhibit the NC chaperone activity towards nucleic acids. To this aim, a well-established fluorescence assay that monitors the destabilization of cTAR DNA labeled with the Alexa488 dye and the Dabcyl quencher at its 5′ and 3′ ends, respectively, was used.[26-27] A threshold of 15% of NC inhibition at compound concentration of 10 μM was arbitrarily set to classify NCIs. Such threshold was based on NC inhibition by the parent compound AN3 at 10 μM (6%), and on the limited water solubility of some compounds at higher concentrations. Results are summarized in Table 1 and show that all tested compounds except **10** and **15** can target the NC and inhibit NC-induced cTAR destabilization with efficacy above the threshold.

Coupling this information with the antiretroviral activity (BiCycle assay) led to define some preliminary SAR. Introduction of a fluorine or a chlorine (compounds **5** and **6**) in the HPB portion of the scaffold did not significantly affect the antiviral activity, but induced a slight decrease in IC_{50} values in comparison to the dehalogenated compound **1**. Replacement of the aminothiazole with the benzothiazole ring (compound **8**) in the HPB portion led to a significant drop of antiviral activity compared to **1**, suggesting that the phenylaminothiazole is a privileged moiety to bind within the hydrophobic pocket. The NTB portion proved more suitable to substitutions and modifications than the HPB, particularly with respect to molecular shortening. Compound **13** showed the best antiviral activity, while its amidic analog **14** displayed a lower efficacy. Whether water solubility of most bifunctional aminothiazoles was not an issue in antiretroviral assays, one may note that it could impair further development. In this respect, replacement of the aminothiazole ring in the NTB portion with an aminopyridine (compound **17**) led to a good antiviral activity in the low micromolar range, as well as an improved water solubility (i.e. up to 100 μM compared to an approximate value of 10 μM for **6** and **13**, as determined by monitoring absorbance spectra – data not shown). Compound **17** might thus represent the starting point for the development of a second generation of bifunctional NCIs endowed with lead-like features.

The possible binding mode of the most potent antiretroviral hits **6** and **13** towards the full length NC was investigated by means of molecular dynamics (MD) simulations. Details of the computational methods are reported in the Supporting Information. After energy minimization, heating and density equilibration, unrestrained MD trajectories were produced for 300 ns in explicit water solvent. A cluster analysis on the ligand/NC complexes highlighted the most abundant cluster of binding conformations, which was used to discuss the structural details of the interaction between NC and **6** or **13**.

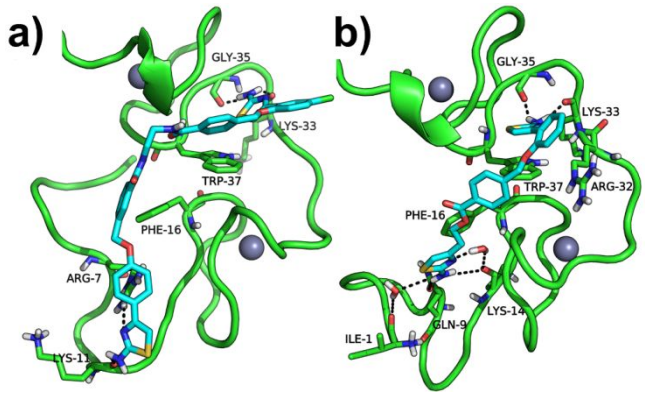

Figure 3. Binding mode of hits **6** (a) and **13** (b) to the NC. The protein is colored green and is show as cartoon. Residues contacted by the ligands are showed as sticks. NCIs are showed as cyan sticks, H-bond interactions are highlighted by black dashed lines. Zn(II) ions are showed as grey spheres.

Both compounds can bind to the hydrophobic pocket of NC and interact with the side chain of the key residue Trp37 (Figure 3). Additional interactions of the HPB portion are established with the backbone of Lys33 and Gly35 residues, with the aromatic ring of Phe16, and with Arg32 (only **13**), in good agreement with the binding mode of the parent AN3 and other non-covalent NCIs.[3-4,8,10] Consistent with our design, the NTB portion of the molecules interacts within the N-ter region of NC. In detail, **6** establishes a cation-pi and a H-bond interaction with the side chain of Arg7, and an H-bond with Lys11 (Figure 3a). Compound **13** establishes a network of direct and water-bridged H-bonds with residues of the N-ter, such as Ile1, Gln9, and Lys14 (Figure 3b). Overall, the slightly higher efficacy of **13** as compared to **6** could be due to its stronger interaction with the NC, as highlighted by the network of contacts shown in Figure 3b, and by the binding energy calculated through the MM-GBSA approach (delta energy of binding of **6** and **13** to the NC is -24.7± 1.2, and -30.6 ± 0.4 kcal/mol, respectively).[28] It is worth mentioning that MD simulations were carried out using the only available structure of NC in complex with a small molecule (Supporting Information, Figure S1),[22] whose sequence corresponds to that of the NL4-3 strain. Among the few differences with the LAV strain (Supporting Information, Figure S2), which are conservative and do not affect the interactions observed by MD, this structure has the residue Ile1 instead of Met1. Since **13** was shown to bind the backbone of Ile1 and not its side-chain, it is reasonably expected that this interaction observed by MD simulations could be exploited also in the presence of Met1 in different HIV-1 strains.

In order to assess the potentiality of this class of NCIs to become lead candidates, the cytotoxicity of active hits showing an $IC_{50} \leq 5$ μM in the BiCycle assay, and being positive for NC inhibition, was evaluated in Peripheral Blood Mononuclear Cells (PMBC) through the MTS assay (Table 1). The selectivity index (SI) was then calculated as the ratio between TD_{50} and IC_{50} measured in the respective assays. Overall, active hits showed poor cytotoxicity, and thus, high SI values.

Consistent with the role of NC in HIV-1 replication,[1,24-25] it is expected that NCIs are active against wild-type and drug-resistant HIV-1 strains. To prove this hypothesis, the antiretroviral activity of the most interesting hits **1**, **5**, **6**, **13**, and **17** was tested against a panel of HIV-1 strains harboring mutations conferring high levels of resistance to drugs currently used for the treatment of HIV-1 infection. IC_{50} values on drug-resistant strains were obtained by the BiCycle assay. Fold change values were calculated by comparison with IC_{50} values determined with the wild-type strain NL4-3 (Table 2). Results clearly indicated that all resistant viruses are as susceptible as the wild-type strain to the tested NCIs, which is consistent with inhibition of the NC and the lack of interference with other validated anti-HIV targets. By coupling these data with the results of Table 1, and in particular the SI values, it is further suggested that the NCI hits identified in this work are safe and could be truly considered for further development.

The impact of NCIs in HIV-1 life cycle was evaluated through the quantification of viral nucleic acid intermediates produced in the course of viral replication. To this end, MT-2 cells infected with NL4-3 wild-type strain at multiplicity of infection of 0.1 were incubated with **6** or **13**, the two most potent NCI hits, or the reference integrase inhibitor (INI) raltegravir (RAL), the protease inhibitor (PI) darunavir (DRV), or the non-nucleoside reverse transcriptase inhibitor (NNRTI) rilpivirine (RPV).

According to the amount of the total and integrated HIV-1 DNA measured at 16 and 30 hours post infection (p.i.), respectively, and the intra/extracellular HIV-1 genomic RNA detected at 30 hours p.i., our data suggested that compound **13** impaired reverse transcription but with a lower efficacy than RPV, while compound **6** did not affect this process at all (Figure 4a). In the presence of **13**, the integrated HIV-1 DNA levels reflected the amount of HIV-1 DNA produced during reverse transcription, suggesting that the integrase activity was not affected by **13** (Figure 4b). Similarly, compound **6** did not decrease the level of integrated HIV-1 DNA, in sharp contrast to the integrase inhibitor RAL.

Table 2. Determination of susceptibility to NCIs of viral strains harboring resistance to drugs currently used in clinical practice.

		IC_{50} ± SD μM (fold change)[a]						
Cmpd	NL4-3 IC_{50} μM	11808[b] (PI)	12465[b] (PI)	7400[b] (NRTI)	7401[b] (NRTI)	12231[b] (NNRTI)	12229[b] (NNRTI)	11845[b] (INI)
1	1.3	2.1± 1 (1.3)	1.2± 0.5 (0.9)	0.8± 0.5 (0.6)	1.2± 0.8 (0.9)	0.8± 0.8 (0.6)	2.2± 1 (1.7)	1±1 (0.8)
5	1.1	0.8± 0.4 (0.7)	2.0± 0.7 (1.8)	1.1± 1.0 (1.0)	0.6± 0.2 (0.5)	4±1 (3.6)	0.9± 0.4 (0.8)	3±1 (2.7)
6	0.8	0.6± 0.4 (0.7)	1.6± 1 (2.0)	0.7± 0.8 (0.9)	1.4± 1 (1.8)	6±2 (7.5)	1±1 (1.2)	2.2± 1 (2.7)
13	0.3	1.1± 0.4 (3.6)	0.4± 0.2 (1.2)	0.1± 0.1 (0.4)	0.5± 0.3 (1.5)	0.7± 0.5 (2.2)	0.3± 0.2 (1.0)	0.7± 1.0 (2.3)
17	3.8	3±1 (0.8)	6±3 (1.6)	1.5± 0.7 (0.4)	2.7± 0.4 (0.7)	5±1 (1.3)	12± 4 (3.1)	2.3± 1 (0.6)

[a] Fold change values indicate the ratio between IC_{50} values from drug-resistant and NL4-3 wild type reference strains. [b] NIH AIDS Reagent Program catalogue number of resistant strains (www.aidsreagent.org)

SD: standard deviation; PI: resistance to Protease Inhibitor; NRTI: resistance to Nucleoside Reverse Transcriptase Inhibitor; NNRTI: resistance to Non-Nucleoside Reverse Transcriptase Inhibitor; INI: resistance to Integrase Inhibitor

Viral transcripts measured approximately after one infection cycle (30 hours p.i.) indicated that compound **6** had a minimal impact on the amount of intracellular RNA, but caused a decrease in extracellular RNA similar to DRV, supporting antiviral activity in the late phases of viral replication (Figure 4c). Differently, viral transcripts measured in presence of compound **13** were lower than with compound **6**, in agreement with the impairment of earlier steps such as reverse transcription. Taken together, these data are consistent with the results of antiretroviral assays showed in Table 1, where compound 6 was found active only in the BiCycle assay, thus indicating a possible effect during maturation and/or on the infectivity of viral particles, while compound **13** interfered with early steps of viral replication as observed in both Mono- and BiCycle assay.

In conclusion, to follow-up a prior work and optimize a validated scaffold of NCIs, bifunctional aminothiazoles were designed, synthesized and tested as anti-HIV agents that target the NC. The design strategy was aimed to target simultaneously the hydrophobic pocket and N-ter domain of the NC, in order to increase the antiretroviral efficacy with respect to parent compounds that target only the hydrophobic pocket or the N-ter. Most synthesized compounds proved effective in inhibiting HIV-1 replication at low- to sub-micromolar concentrations, while cytotoxicity in PMBC was not detected for compounds able to inhibit the replication of HIV-1 in infected cells. Notably, the NC inhibition assay *in vitro* confirmed that almost all antiretroviral compounds target the NC, as further suggested by MD simulations. Similar findings were also obtained by further characterization of the mechanism of action in infected MT-2 cells, which was carried out by monitoring HIV-1 nucleic acids levels at different time-points in the presence of the most potent hits (namely, **6** and **13**). All the results point to the relevance of these molecules for NC inhibition, particularly in the context of resistance acquired to currently licensed drugs. Future experiments will be carried out to test whether candidate NCIs select mutations in the target NC, as a proof of concept of NC inhibition in HIV-1 infected cells. Of note, this approach will also allow to estimate the genetic barrier to resistance of NCIs and the frequency of emerging mutations among naturally circulating HIV-1 strains. These NCI hits represent therefore a valuable starting point for further optimization.

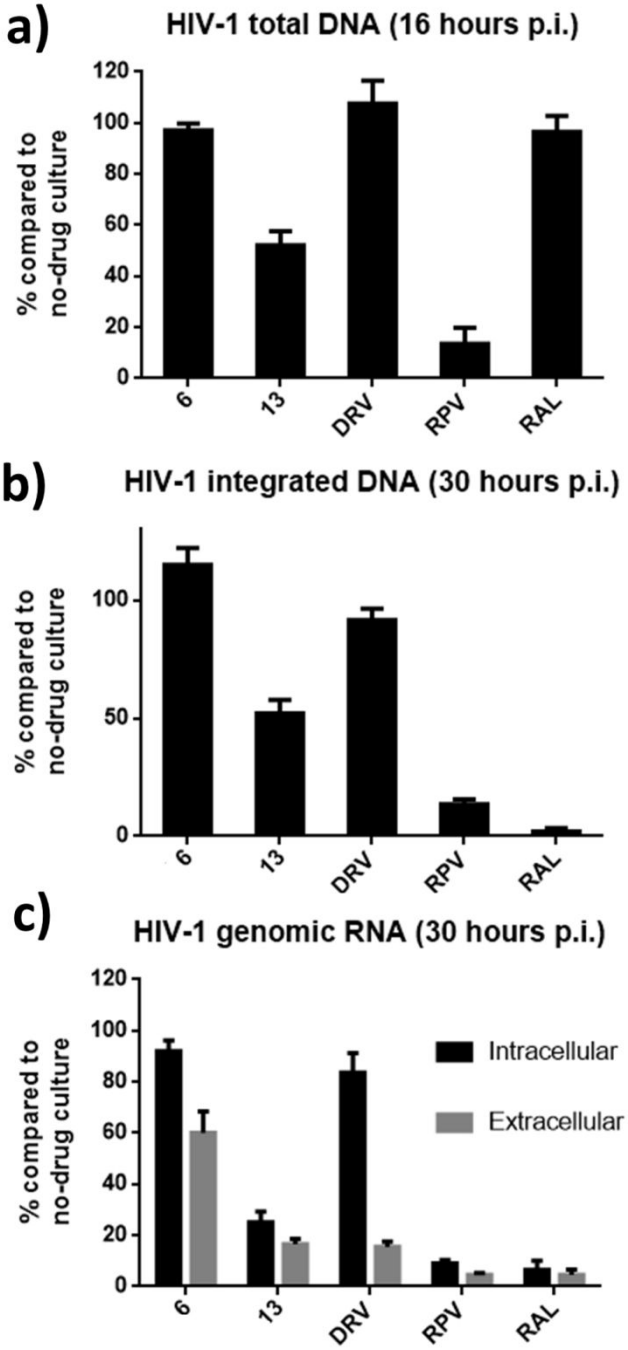

Figure 4. Real-time PCR quantification of HIV-1 nucleic acid species produced in the presence of compound **6** (20 μM), compound **13** (5 μM) or 1 μM reference compounds darunavir (DRV), rilpivirine (RPV), and raltegravir (RAL). The percentage of viral nucleic acid species was calculated with respect to the no drug control culture. The different graphs show the effects on total HIV-1 DNA at 16 hours post infection (p.i.; a), integrated HIV-1 DNA at 30 hours post infection (b), and both intracellular and extracellular genomic HIV-1 RNA at 30 hours post infection (c).

ASSOCIATED CONTENT

Supporting Information

Supporting Information Figures, Chemistry, characterization of chemical compounds, and experimental details on molecular dynamics, NC inhibition assay, antiretroviral assay, cytotoxicity evaluation, and virology are described. The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

All authors have given approval to the final version of the manuscript.

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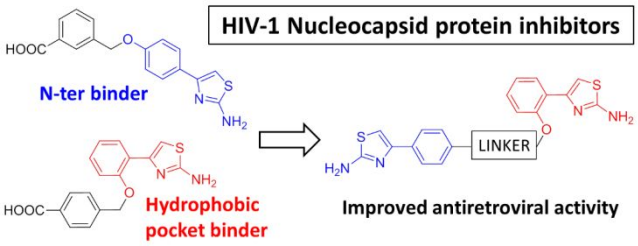

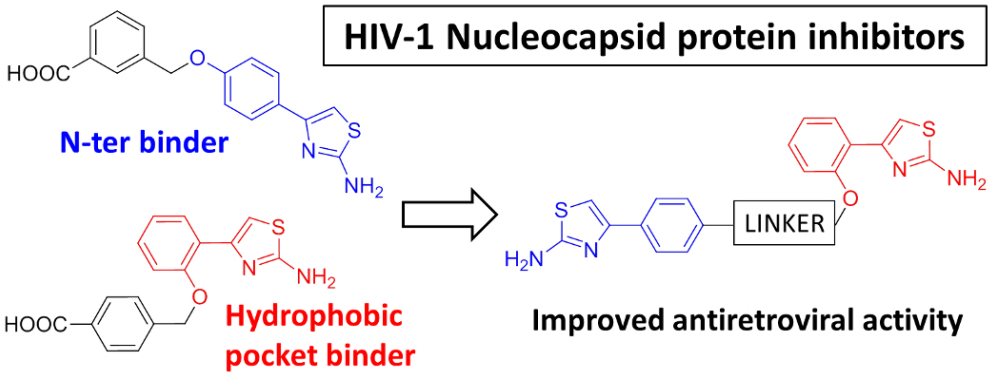

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