Letter

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5,6-Dihydroxypyrimidine Scaffold to Target HIV-1 Nucleocapsid Protein

Savina Malancona[1*°], Mattia Mori[2°], Paola Fezzardi[1], Marisabella Santoriello[1], Andreina Basta[1,†], Martina Nibbio[1], Lesia Kovalenko[3], Roberto Speziale[1], Maria Rosaria Battista[1], Antonella Cellucci[1], Nadia Gennari[1], Edith Monteagudo[1], Annalise Di Marco[1], Alessia Giannini[4], Rajhans Sharma[3], Manuel Pires[3], Eleonore Real[3], Maurizio Zazzi[4], Maria Chiara Dasso Lang[2], Davide De Forni[5], Francesco Saladini[4], Yves Mely[3], Vincenzo Summa[1,††], Steven Harper[1,#] and Maurizio Botta[2#]

[1]IRBM S.p.A., Via Pontina Km 30.600, 00071 Pomezia (RM), Italy

[2]Department of Biotechnology, Chemistry and Pharmacy, University of Siena, via Aldo Moro 2, 53100 Siena, Italy

[3]Laboratoire de Bioimagerie et Pathologies, UMR 7021 CNRS, Faculté de Pharmacie, Université de Strasbourg, 74 Route du Rhin, 67401 Illkirch, France

[4]Department of Medical Biotechnologies, University of Siena, Viale Mario Bracci, 16, 50100 Siena, Italy

[5]ViroStatics S.r.l, Viale Umberto I 46, 07100 Sassari, Italy

[#]This work is dedicated to the beloved memory of Prof. Maurizio Botta (August 2, 2019) and Steven Harper (June 30, 2019)

KEYWORDS: Nucleocapsid protein, HIV, NC inhibitors, Dihydroxypyrimidine, Drug Resistance, Antiretroviral.

ABSTRACT: The HIV-1 Nucleocapsid Protein (NC) is a small basic DNA and RNA binding protein which is absolutely necessary for viral replication and thus represent a target of great interest to develop new anti-HIV agents. Moreover, the highly conserved sequence offers the opportunity to escape the drug resistance (DR) emerged following the highly active antiretroviral therapy (HAART) treatment. Based on our previous research nordihydroguaiaretic acid **1** acts as NC inhibitor showing moderate antiviral activity and sub-optimal drug-like properties due to the presence of the catechol moieties. A bio-isosteric catechol replacement approach led us to identify the 5-dihydroxypyrimidine-6-carboxamide substructure as privileged scaffold of a new class of HIV-1 NC inhibitors. Hit validation efforts led to the identification of optimised analogs, as represented by compound **28**, showing improved NC inhibition and antiviral activity as well as good ADME and PK properties.

Nowadays AIDS (Acquired Immune Deficiency Syndrome) is still a major global public health issue. According to the Joint United Nation Program on HIV/AIDS (UNAIDS) nearly 38 million people were living with HIV in 2018.[1] The current pharmacological strategy relays on the combination of drugs (HAART, highly active antiretroviral therapy) targeting various steps of the viral replication: entry, fusion, reverse transcription, integration, protein maturation. Despite successful in mortality and morbidity reduction, HAART long-term treatment is associated with the insurgence of drug resistance as results of the ability of HIV-1 to mutate.[2] In this context there is an urgent need to develop new drugs to treat HIV infection based on novel mechanism of action or targets.[3] One emerging approach to overcome drug resistance is to target HIV-1 proteins that are highly conserved among phylogenetically distant viral strains and currently not targeted by available therapies. The Nucleocapsid Protein (NC) of the Human Immunodeficiency Virus type-1 (HIV-1) is a small and basic protein with two zinc fingers that is highly conserved among the retroviruses. This nucleic acids (NA) binding protein is involved in both early and late steps of the HIV-1 replication cycle, through its ability to chaperone NAs towards their most stable conformation.[4,5,6,7] Thus, NC appears as an ideal target for the development of new drugs to prevent HIV-1 replication and complement the highly active anti-retroviral therapy.[8] Since NC is highly conserved, anti-NC drugs are expected to provide a sustained replication inhibition of the wild type and HIV-1 drug-resistant strains. Three classes of NC inhibitors have been reported based on the mechanism of action: (i) zinc ejectors,[9,10] (ii) noncovalent NCIs binding to nucleic acid partners of NC,[11,12] and (iii) noncovalent NCIs binding to NC.[13,14] The development of NCIs directly binding to NC is currently our main research area, as it is expected to overcome the selectivity and toxicity issues intrinsically related to NCIs belonging to the classes (i) and (ii). In this work, we describe the most recent advances achieved, leading to the identification of a new class of NCIs showing both good antiviral activity as well as promising *in-vitro* and *in-vivo* profiles.

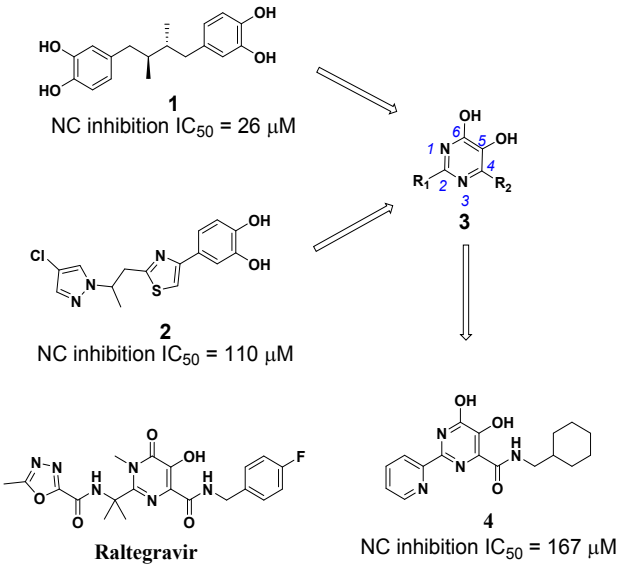


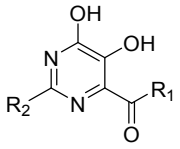
Figure 1. NC inhibitors based on catechol template **1** and **2**. 5,6-dihydroxypyrimidine **3** as catechol replacement and hit compound **4** active in the NC-inhibition assay. Raltegravir, first in class HIV integrase inhibitor.

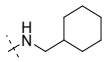
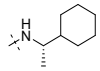
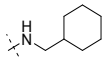
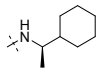
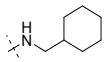
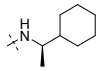
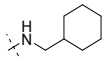
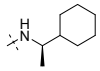
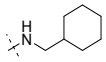
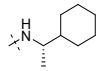
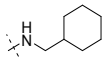
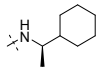
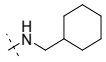
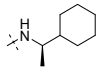
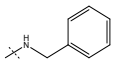
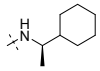
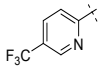
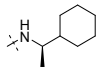
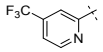
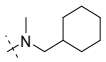
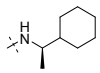
Recently, we have developed non-covalent NC inhibitors,[15] by applying a computational structure-based screening of commercial compounds collection followed by *in-vitro* assessment of the NC inhibitory activity. This led to the identification of the natural product nordihydroguaiaretic acid **1** and compound **2** as NC inhibitors sharing a catechol moiety (Figure 1). Based on our pharmacophore, the catechol moiety is a common structural feature that plays a critical role in binding NC in a guanidine-like manner by means of the two hydroxyl groups.[16,17,18] Biophysical and computational studies of compound **1** interacting with the protein confirmed the binding postulated by modeling.[15] While valuable as a tool compound, the low metabolic profile associated with the catechol group limits the further development of both compounds **1** and **2**. The replacement of undesirable functional groups with bioisosters is a common strategy applied in drug discovery to modulate the activity profile, reduce potential toxicities and circumvent metabolic liabilities.[19] In this perspective, using the protocol previously described,[16] a virtual screening campaign on the ZINC catalog of commercial products was conducted to identify catechol-like compounds as putative candidate inhibitors of NC. Following virtual screening and clusterization of the top-ranking compounds,[15] the dihydroxypyrimidine core scaffold **3** (Figure 1) emerged as a potential replacement for the catechol moiety and thus offering the possibility to develop a new class of NCIs.

Along this line further scaffold expansion was achieved through the design of a focused virtual library of analogs with the scope of selecting a pool of compounds to be evaluated *in-vitro* for their ability to inhibit the NC chaperone activity. To this aim, a well-established fluorescence assay[20] (NC-inhibition assay) was used; it allows to monitor the destabilization of cTAR DNA, the complementary sequence to the transactivation response element of the HIV-1 genome labeled with the Alexa488 dye and Dabcyl quencher.[21,22] The protein concentration used in the inhibition assay is 1 μM and thus 0.5 μM is the lowest IC_{50}s values measurable. This effort conducted to the identification of compound **4** as a positive hit (Figure 1), showing an IC_{50} below 200 μM (Table 1). Despite its modest potency, compound **4** represented an optimal starting point from the development perspective due to the favorable drug-like properties of the dihydroxypyrimidine carboxamide class. This class of compounds is known to have led to the discovery of Raltegravir[23] (Figure 1), the first in class HIV integrase inhibitor. Furthermore, in the antiviral field, the dihydroxypyrimidine core has been investigated to target other NA processing enzymes, such as HIV-1 RT[24] or HCV NS5B RNA-dependent RNA polymerase.[25] Due to the close structural similarity between compound **4** and Raltegravir, a possible polypharmacology profile could be expected in the development of this class of compounds as NC inhibitors. Nonetheless, first rough structural comparison and lack of activity of Raltegravir in the NC inhibition assay (data not shown), suggested that a degree of selectivity was already present in the hit compound **4**. The structure activity relationship (SAR) of the dihydroxypyrimidine carboxamide as inhibitors of the HIV-integrase enzyme has been fully elucidated and well documented in literature.[26,27,28,29] Optimized HIV-integrase inhibitors show a marked preference for the N-methylpyrimidone core, benzylic carboxamide and neutral aryl or alkyl substituent at the 2-position of the pyrimidine core. In contrast, the catechol-like dihydroxypyrimidine core of the hit compound **4**, is decorated with an alkyl amide that in the HIV integrase inhibitor abolish completely the activity against this target, and a basic aromatic substituent in the 2-position of the pyrimidine.[28] A hit validation and optimization process was thus conducted on **4** with the aim of improving the potency as NC inhibitor. The compounds were also tested for cell-based antiretroviral activity. The *in-vitro* and *in-vivo* ADME properties were finally evaluated for the most interesting analogs.

While retaining the central dihydroxypyrimidine core, structural modifications were introduced first in the aromatic region at the 2-position (R_2, Table 1). The 2-(pyridin-2-yl) group emerged as the privileged substitution for the activity: the replacement with a simple phenyl **5** or replacement with the 2-(pyridin-3-yl) or 2-(pyridin-4-yl) isomers, respectively compounds **6** and **7,** resulted in complete loss of the activity. Neither a 2-phenethyl, 2-(1-phenylethyl) or bulky 2-(*tert*-butyl) substituents were tolerated, compounds **8**, **9** and **10** respectively. The amide moiety (R_1, Table 1) of compound **4** is believed from modelling to be oriented towards the hydrophobic binding site of the protein. The SAR in the amide region showed that the replacement of the *N*-(cyclohexylmethyl) group with *N*-benzyl **11**, *N*-ethyl **12** and the tertiary amide **13** are not tolerated. The exploration of the linker length pointed out that anchoring the cyclohexyl directly to the amide nitrogen as for **14** produces a 2-fold loss of activity in the NC inhibition assay with respect to the hit compound **4**, whereas the elongation of the linker by one carbon atom **15** is beneficial for the activity and accounts for a 4-fold improved activity. Similar gain of potency is achieved with the branched analogs *(R)-N*-(1-cyclohexylethyl) **16** and *(S)-N*-(1-cyclohexylethyl) **17**, showing the same activity regardless of chirality. The results obtained are in line with the proposed binding mode that suggests the amide occupying the hydrophobic pocket and that the lipophilic substituents are thus well tolerated, chirality is not influencing conformation and binding of the amide moiety.

Table1. *In-vitro* NC inhibition for the analogs exploring the amide and aromatic substituent regions



ID	R_1	R_2	NC inhibition IC_{50} (μM)[a]	ID	R_1	R_2	NC inhibition IC_{50} (μM)[a]
4			167 ± 7	17			47 ± 5
5			N.A.[b]	18			20 ± 1
6			N.A.[b]	19			20[c]
7			N.A.[b]	20			8 ± 0.7
8			N.A.[b]	21			41 ± 10
9			N.A.[b]	22			21 ± 15
10			N.A.[b]	23			20[d]
11			N.A.[b]	24			23 ± 6
12			N.A.[b]	25			12 ± 6
13			N.A.[b]	26			20[d]
14			300 ± 100	27			22 ± 7
15			38 ± 10	28			2 ± 1
16			42 ± 10	29			4 ± 1

[a] Data are average ± SD of a least two independent experiments. [b]N.A. = not active. [c]IC_{40}. [d]IC_{35}.

Preliminary SAR at the 2-position of the 5,6-dihydroxypyrimidine core has shown that 2-(pyridin-2-yl) substituent is required for the activity. Further optimization was thus conducted introducing substituents on the pyridine ring. First modifications introduced were the methyl (**18**-**22**) and trifluoromethyl (**23**-**26**) groups which were selected considering the differences in the electron donating and withdrawing ability, as well as lipophilicity. Regardless of the electronic nature, the introduction of a substituent in position 4- and 5 is beneficial for the activity, affording compounds **20** and **25** showing IC_{50}s around 10 μM. In the other positions of the pyridine ring, two-fold activity gain was achieved with respect to the hit **4**. A variety of other substituents were evaluated, in particular, the most relevant results were obtained with the introduction of 5-methoxy and 5-chlorine groups on the pyridine ring (respectively, compounds **27** IC_{50} =20 μM and **28** IC_{50} = 2 μM). To complete the exploration at the 2- position of the pyrimidine core, a series of heterocycles retaining the 2-nitrogen atom present in the 2-pyridine substituent were explored. Best results were achieved with fused bicyclic groups, such as the quinoline analogs **29** showing more than 40-fold improvement in the NC inhibition with respect to the original compound **4**. Smaller 5-member ring heterocycles such as thiophene or thiazole were not tolerated (data not shown).

Modification at the dihydroxypyrimidine core showed that both hydroxyl groups are essential for the activity. Indeed, compounds **30** and **31** lacking respectively the 4- and 5-hydroxyl substituent, were found inactive in the NC inhibition assay (Figure 2).

The 5,6-dihydroxypyrimidine core exists in two tautomeric forms, namely dihydroxypyrimidine and pyrimidone. A different hydrogen bond donator/acceptor asset, potentially able to influence the binding to the nucleocapsid protein, is associated to each form. The N-methyl pyrimidone analog **32** resulted to be completely inactive in the NC inhibition assay, indicating the catechol form as decisive for NC inhibition, in contrast to the preference for the pyrimidinone form in the HIV-1 integrase area. The SAR conducted on the hit compound **4** has shown that it is possible to modulate NC activity and improve 80-fold its potency (cmpd **28**), thus obtaining a number of compounds in the low micromolar range of activity.

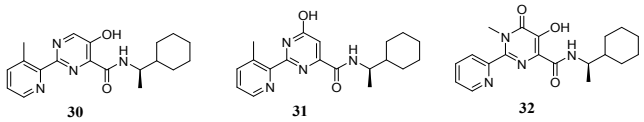


Figure 2. Dihydroxypyrimidine core modifications leading to lack of NC inhibition.

The binding mode of most potent NC inhibitors identified was investigated by molecular docking simulations and reported in Figure 3 for compound **28** and Figure S1 for compounds **4**, **20** and **25**. To this aim, we took advantage of the computational protocol already refined by our research group for the NC protein, and based on molecular docking with the FRED program (OpenEye) against the NMR structure of the NC in complex with a small molecule inhibitor (PDB: 2M3Z), see also the Supporting Information.[15,17,16] Overall, the molecules shared a highly similar binding mode, and proved to fit the hydrophobic pocket of the NC. Besides a π-stacking interaction with the side chain of Trp37, the core dihydroxypyrimidine established H-bonds with the backbone of Gly35, and Met46, and with the side-chain of Gln45. In addition, the amide moiety established hydrophobic interactions with the side chain of residues from the NC hydrophobic platform[17] such as Phe16, Ala25, Trp37, and Met46 (Figure 3). Overall, this binding mode is highly consistent with the binding mode of NA nucleotides[30] and of nordihydroguaiaretic acid **1**[15]. Most notably, the predicted binding for these dihydroxypyrimidines also overlaps with the binding mode of the NC inhibitor characterized by NMR spectroscopy in complex with the NC, whose structure was used as a receptor in molecular docking simulation[31] (Supporting Information, Figure S2). A good correlation between the score of compounds **4**, **20**, **25**, and **28** and their $-\log IC_{50}$ was observed (R^2 = 0.939) (Supporting Information, Figure S3). Finally, the binding mode described above and shown in Figure 3 and Figure S1 clearly accounts for the lack of NC inhibition by derivatives deprived of the dihydroxyl moiety, such as **30**, and **31**, or bearing the N-methyl pyrimidone substructure such as **32**.

The antiviral activity on the whole replication cycle of HIV-1 was evaluated for selected compounds by means of the cell-based assay named BiCycle (Table 2). The assay consists of a first infection round of the T-cell line MT-2 using HIV-1 wild-type reference strain NL4-3, followed by a second infection round of the reporter cell line TZM-bl.[32] Hit compound **4** shows activity in the low micromolar range. A similar activity is found for the analog **15**, which is more active in the biochemical assay as result of the amide elongation.

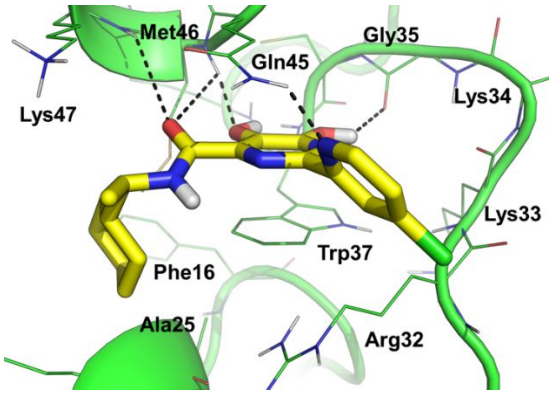


Figure 3. Predicted binding mode of **28** within the hydrophobic pocket of the NC. The protein is shown as green cartoon. Residues within 5 Å from the ligands are shown as lines, and are labeled. Compound **28** is shown as yellow sticks. H-bond interactions are highlighted by black dashed lines.

A beneficial effect on the antiviral activity is seen when the branched amide is present, with both **16** and **17** enantiomers active in the sub-micromolar range. The difference between the *R* and *S* enantiomers in term of antiviral activity is very modest (3-fold), confirming the behavior observed in the NC inhibition assay. Analogs substituted in the aromatic region such as **20**, **25**, **27**, **28** and **29** exhibit nanomolar antiviral activity with EC_{50}s ranging from 40 to 400 nM. In parallel, cytotoxicity was evaluated in Hela and peripheral blood mononuclear cells (PMBC) and the selectivity indexes (SI) were calculated as the ratio between the CC_{50} and EC_{50} values measured in their respective assays (see footnote of Table 2). Despite the narrow difference between the activity and cytotoxicity found for **4** and close analogues (SI range 2-5-fold), much higher SI values were

found for compounds **20**, **27**, **28**, **29** as a result of their improved activity in cells (SI in the range 13-75). Overall, these data clearly show that the potency and selectivity can be modulated and improved in future investigations of the series. The inhibition of NC is expected to overcome drug resistance. To prove this hypothesis most potent compounds were tested against a representative panel of drug-resistant HIV-1 strain (Table 2). Notably, only a 1-2-fold IC_{50} shift was measured compared to wild type IC_{50}, consistent with the hypothesis that NC inhibitors retain their full potency against resistant strains.

In order to further characterize the new class of NC inhibitors (NCIs), a series of *in-vitro* and *in-vivo* tests were performed on a small set of selected compounds. The metabolic stability was evaluated *in-vitro* in rat and human species, plasma and hepatocytes matrices (Table 3). Data show that compounds **25**, **27**, **28** and **29** were stable up to one hour or more in both plasma and hepatocytes matrices. The same compounds were not inhibitors of major human cytochrome P450 isoforms (CYP1A2, CYP2D6, CYP3A4) and the hERG ion channels. Based on the potency profile in the different assays and the *in-vitro* DMPK data, compounds **27**, **28** and **29** were selected for *in-vivo* PK studies in C57BL/6 mice (Table 3). Compounds were administrated IV and PO at 2 and 5 mg/kg, respectively. Eight time points were taken up to 24h to obtain a plasma PK profile (Table 4).

Table 4: PK parameters for compounds **27**, **28** and **29** following i.v. and p.o administration in C57BL/6 mice at 2 mg/kg and 5 mg/kg respectively

	in-vivo profiling			
ID	**$t_{1/2}$ (h)**	**Vdss (L/kg)**	**Cl (ml/min/kg)**	**%F**
25				
27	1.4	0.8	33.1	25
28	3.1	1.2	37.1	40
29	1.1	0.8	30.2	26

Compounds **27** and **29** showed similar PK parameters: medium-low plasma clearance (33 and 30 mL/min/kg, respectively) and medium volume of distribution (Vdss 0.8 L/kg for both) with a corresponding half-life of 1.4 and 1.1 hours respectively for **27** and **29**. The oral bioavailability was good (25% and 26%, respectively). Compared to **27** and **29**, compound **28** showed a better profile presenting higher oral bioavailability (40 %), prolonged half-life of 3.1 hours and volume of distribution of 1.2 L/kg.

To assess a preliminary polypharmacology profile compounds **4**, **27** and **29** were tested for their ability to inhibit the HIV Integrase enzyme by means of the well-established LEDGF-dependent IN activity assay[33] resulting not active up to 10 uM, Raltegravir used as positive control showed $IC_{50} = 0.027 \pm 0.01 \mu M$.

In summary, based on our previous studies the catechol moiety is a privileged structure to achieve NC inhibition as demonstrated by compound **1**. In the prospective of drug development, the intrinsic metabolic liability of the catechol group is an issue to be considered. To overcome this issue, we decided to search for catechol-like scaffold with improved physicochemical properties able to bind NC protein. The process allowed the identification of the 5,6-dihydroxypyrimidine-4-carboxamide analog **4** as representative of a new class of HIV-1 nucleocapsid inhibitors. Initial SAR studies were conducted in order to prove the bona fide nature of the hit and identify the key structural features needed to achieve and modulate NC inhibition. Optimized analogs such as **27**, **28**, **29** not only showed nanomolar antiviral activity and improved selective index, but were active against a panel of HIV-1 resistant strains in agreement with the proposed mechanism of action. Furthermore, the compounds showed a very clean profile against the major CYP-P450 isoforms and the hERG channel. The pharmacokinetic profile in mice was good for all three compounds but in particular for compound **28,** which shows 40% oral bioavailability, good half-life and moderate clearance. In conclusion, we identified an unprecedented structural class of NC inhibitors with good drug like properties. In particular compound **28** represents a novel NC inhibitor lead with low nanomolar potency in cell-based assay, low cytotoxicity, and good PK profile. It represents a good starting point for further characterization and optimization.

Table 2: Antiviral and cytotoxicity profile. Susceptibility to viral strains harbouring resistance to drug used in clinical practice

	BiCycle Wild type strain NL4-3	Hela		PBMC MTS		Strain 11808 (PI)[e]	Strain 7401 (NRTI)[e]	Strain 12231 (NNRTI)[e]	Strain 11845 (INI)[e]
ID	**EC_{50} (µM)[a]**	**CC_{50} (µM)[a]**	**SI-Hela[b]**	**CC_{50} (µM)[a]**	**SI-PBMC[c]**	**EC_{50} (µM)[a]**	**EC_{50} (µM)[a]**	**EC_{50} (µM)[a]**	**EC_{50} (µM)[a]**
4	1.2 ± 0.7	3.6 ± 0.7	3	2.6 ± 1.7	2				
15	2.3 ± 2	4.2 ± 3.4	2	11 ± 6.0	5				
16	0.7 ± 0.1	3.2 ± 0.9	5	3.0 ± 1.4	4				
17	0.2 ± 0.1	0.6 ± 0.04	3	2.4 ± 1	12				
20	0.2 ± 0.1	2.7 ± 0.4	13	3.1 ± 0.8	16				
25	0.4 ± 0.3	6.0 ± 0.2	15	1.1 ± 0.1	3				
27	0.04 ± 0.03	3.0 ± 0.1	75	2.7 ± 0.1	75	0.056 ± 0.02 (1.4)[d]	0.031 ± 0.02 (0.8)[d]	0.11 ± 0.04 (2.8)[d]	0.09 ± 0.04 (2.3)[d]
28	0.1 ± 0.03	3.0 ± 0.5	30	2.5 ± 0.2	25	0.02 ± 0.01 (0.2)[d]	0.01 ± 0.005 (0.1)[d]	0.08 ± 0.06 (0.8)[d]	0.07 ± 0.08 (0.7)[d]
29	0.1 ± 0.05	2.1 ± 0.3	21	5 ± 2	50	0.16 ± 0.1 (1.6)[d]	0.13 ± 0.08 (1.3)[d]	0.21 ± 0.07 (2.0)[d]	0.09 ± 0.02 (0.9)[d]

[a]Data are average ± SD for a least two independent experiments; [b]SI-Hela: HELA CC_{50} / BiCycle EC_{50}; [c]SI-PBMC: PBMC CC_{50} / BiCycle EC_{50}; [d]Fold change values indicate the ratio between IC_{50} values from drug-resistant and NL4−3 wild type reference strains. [e]NIH AIDS Reagent Program catalogue number of resistant strains (www.aidsreagent.org). 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.

Table 3: In-vitro profiling for compounds 25, 27, 28 and 29; PK parameters for compounds 27, 28 and 29 following i.v. and p.o administration in C57BL/6 mice at 2 mg/kg and 5 mg/kg respectively

	in-vitro profiling							
ID	**Mouse plasma $t_{1/2}$ (h)**	**Human plasma $t_{1/2}$ (h)**	**Mouse hepatocytes $t_{1/2}$ (h)**	**Human hepatocytes $t_{1/2}$ (h)**	**HERG IC_{50} (µM)**	**CYP1A2 IC_{50} (µM)**	**CYP2D6 IC_{50} (µM)**	**CYP3A4 IC_{50} (µM)**
25	>1	>1	2.4	>4	>30	>30	>30	>30
27	>24	>6	1.9	>4	>30	>30	>30	>30
28	>6	>6	1.7	>4	>30	>30	>30	>30
29	3.7	ND	1.7	2.6	>30	>30	>30	>30

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Synthetic experimental details and characterization data, description of biological assay protocols, modeling (PDF).

AUTHOR INFORMATION

Corresponding Author

* Email: s.malancona@irbm.com

ORCID

Savina Malancona: 0000-0002-5388-6533

Present Addresses

[†]Tubilux Pharma spa, via Costarica 20, 00071 Pomezia (Rome)
[††]Department of Pharmacy, University of Naples "Federico II", Via D. Montesano 49, 80131 Naples, Italy

Author Contributions

°These authors equally contributed to this work. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

AIDS, acquired immune deficiency syndrome; HAART, highly active antiretroviral therapy; NC, nucleocapsid protein; HIV-1, human immunodeficiency virus type-1; NCIs, nucleocapsid protein inhibitors; HIV-IN, human immunodeficiency virus integrase; SI, selectivity index; SAR, structure-activity relationship; NMR, nuclear magnetic resonance; PK, pharmacokinetics; hERG, human Ether-à-go-go-Related Gene.

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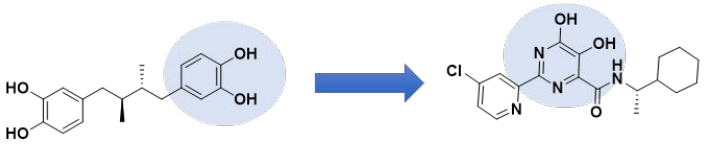

1

- HIV- 1 NC inhibition IC_{50} = 26 μM
- Antiviral activity EC_{50} = 4.3 μM
- Low *in-vitro* metabolic stability

28

- NC inhibition IC_{50} = 2 μM
- Antiviral activity EC_{50} = 0.1 μM
- Good *in-vitro* metabolic stability
- F% 40