



**HAL**  
open science

# **(Thia)calixarenephosphonic Acids as Potent Inhibitors of the Nucleic Acid Chaperone Activity of the HIV-1 Nucleocapsid Protein with a New Binding Mode and Multitarget Antiviral Activity**

Nicolas Humbert, Lesia Kovalenko, Francesco Saladini, Alessia Giannini, Manuel Pires, Thomas Botzanowski, Sergiy Cherenok, Christian Boudier, Kamal Sharma, Eleonore Real, et al.

## ► To cite this version:

Nicolas Humbert, Lesia Kovalenko, Francesco Saladini, Alessia Giannini, Manuel Pires, et al.. (Thia)calixarenephosphonic Acids as Potent Inhibitors of the Nucleic Acid Chaperone Activity of the HIV-1 Nucleocapsid Protein with a New Binding Mode and Multitarget Antiviral Activity. ACS Infectious Diseases, 2020, 10.1021/acsinfecdis.9b00290 . hal-02493003

**HAL Id: hal-02493003**

**<https://hal.science/hal-02493003>**

Submitted on 16 Dec 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# (Thia)calixarenephosphonic acids as potent inhibitors of the nucleic acid chaperone activity of the HIV-1 nucleocapsid protein with a new binding mode and multi-target antiviral activity

Nicolas Humbert<sup>1§</sup>, Lesia Kovalenko<sup>1,2§</sup>, Francesco Saladini<sup>3</sup>, Alessia Giannini<sup>3</sup>, Manuel Pires<sup>1</sup>, Thomas Botzanowski<sup>4</sup>, Sergiy Cherenok<sup>5</sup>, Christian Boudier<sup>1</sup>, Kamal K. Sharma<sup>1</sup>, Eleonore Real<sup>1</sup>, Olga A. Zaporozhets<sup>2</sup>, Sarah Cianféran<sup>4</sup>, Carole Seguin-Devaux<sup>6</sup>, Federica Poggialini<sup>7</sup>, Maurizio Botta<sup>7</sup>, Maurizio Zazzi<sup>3</sup>, Vitaly I. Kalchenko<sup>5</sup>, Mattia Mori<sup>7</sup>, Yves Mély<sup>1\*</sup>

<sup>1</sup>Laboratoire de Bioimagerie et Pathologies, UMR 7021 CNRS, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France

<sup>2</sup>Department of Chemistry, Taras Shevchenko National University of Kyiv, 01601 Kyiv, Ukraine

<sup>3</sup>Department of Medical Biotechnologies, University of Siena, viale Mario Bracci n.16, 53100 Siena, Italy

<sup>4</sup>Laboratoire de Spectrométrie de Masse BioOrganique, IPHC UMR 7178 CNRS, Université de Strasbourg, 67000 Strasbourg, France

<sup>5</sup>Institute of Organic Chemistry, National Academy of Science of Ukraine, Murmanska str., 5, Kyiv 02660, Ukraine

<sup>6</sup>Department of Infection and Immunity, Luxembourg Institute of Health, 29 rue Henri Koch, L-4354 Esch-sur-Alzette, Luxembourg

<sup>7</sup>Department of Biotechnology, Chemistry and Pharmacy, Department of Excellence 2018-2022 Università degli Studi di Siena, via Aldo Moro 2, I-53019 Siena, Italy

§ NH and LK equally contributed to this work

\* To whom correspondence should be addressed. Tel: +33 (0)3 68 85 42 63, Fax: +33 (0)3 68 85 43 13. E-mail address: yves.mely@unistra.fr

## ABSTRACT

The nucleocapsid protein (NC) is a highly conserved protein that plays key roles in HIV-1 replication through its nucleic acid chaperone properties mediated by its two zinc fingers and basic residues. NC is a promising target for antiviral therapy, particularly to control viral strains resistant to currently available drugs. Since calixarenes with antiviral properties have been described, we explored the ability of calixarene hydroxymethylphosphonic or sulfonic acids to inhibit NC chaperone properties and exhibit antiviral activity. By using fluorescence-based assays, we selected four calixarenes inhibiting NC chaperone activity with sub-micromolar  $IC_{50}$  values. These compounds were further shown by mass spectrometry, isothermal titration calorimetry and fluorescence anisotropy to bind NC with no zinc ejection, and to compete with nucleic acids for the binding to NC. Molecular dynamic simulations further indicated that these

compounds interact via their phosphonate or sulfonate groups with the basic surface of NC, but not with the hydrophobic plateau at the top of the folded fingers. Cellular studies showed that the most soluble compound CIP201 inhibited the infectivity of wild-type and drug-resistant HIV-1 strains at low micromolar concentration, primarily targeting the early steps of HIV-1 replication, and notably the entry step. Moreover, CIP201 was also found to inhibit the flipping and polymerization activity of reverse transcriptase. Calixarenes form thus a class of non-covalent NC inhibitors, endowed with a new binding mode and multi-target antiviral activity.

Keywords: Nucleocapsid protein, HIV-1, Fluorescence, calixarenes, NC inhibitors

57

## INTRODUCTION

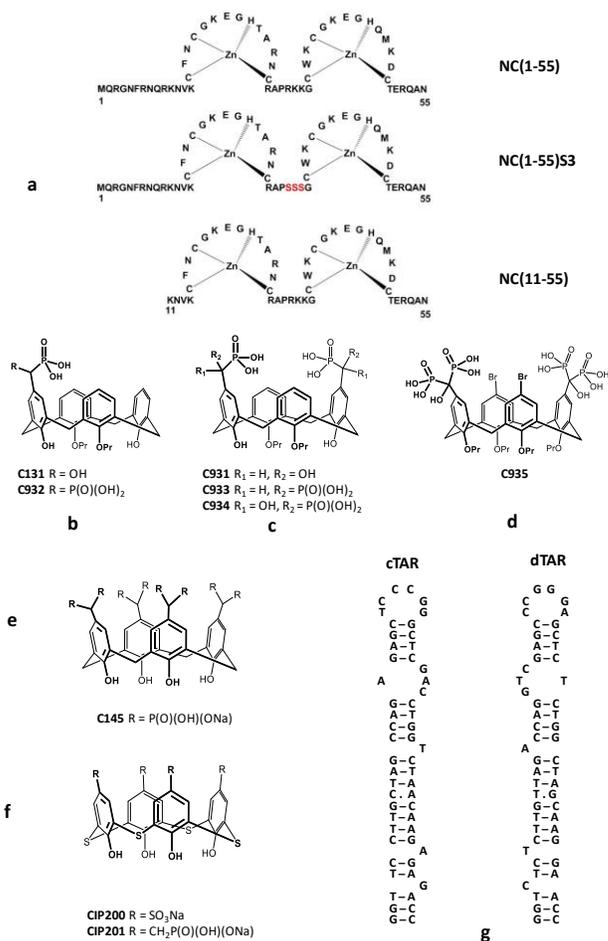
1 During the last two decades, the number of drugs acting as  
 2 inhibitors of entry/fusion, reverse transcription, integration,  
 3 and protease cleavage developed to fight HIV disease has  
 4 gradually increased. Though being endowed with efficient  
 5 antiviral activity, all these compounds select for resistant  
 6 strains, as a result of the ability of HIV-1 to rapidly mutate<sup>1</sup>.  
 7 Even in the classical treatment scheme, namely the Highly  
 8 Active Anti-Retroviral Therapy (HAART), which combines  
 9 anti-retroviral drugs of different classes, treatment failures  
 10 due to drug resistance are still observed.

11 Thus, new compounds targeting novel and highly conserved  
 12 viral targets such as the nucleocapsid protein (NC) could be  
 13 helpful to limit or prevent the emergence of drug resistance.  
 14 NC exhibits a chaperone activity which enables the structural  
 15 rearrangement of nucleic acids (NAs) into their most stable  
 16 conformation. This activity relies on the ability of NC to bind  
 17 and dissociate rapidly from its NA targets, to destabilize  
 18 moderately stable NA secondary and tertiary structures and to  
 19 promote the annealing of complementary NA sequences<sup>2-5</sup>.  
 20 This NC activity is thought to be critical in the reverse  
 21 transcription process, where NC likely chaperones the  
 22 annealing of the primer tRNA to the primer binding site (PBS)  
 23 and the two strand transfers that are required for the synthesis  
 24 of the proviral DNA by reverse transcriptase<sup>6,7</sup>. NC is a small  
 25 basic protein that binds with high affinity ( $10^{13} - 10^{14} \text{ M}^{-1}$ )<sup>8</sup>  
 26 two zinc ions on two strictly conserved finger motifs (CCHC)  
 27 through their cysteine and histidine residues (Figure 1a). Upon  
 28 zinc binding, the NC protein adopts its functional folding  
 29 leading to the formation at the top of the folded fingers of a  
 30 hydrophobic plateau that involves the amino acids Val13,  
 31 Phe16, Thr24, Ala25, Trp37, Gln45 and Met46<sup>9-14</sup>. This  
 32 plateau is critical for interaction with target NAs, notably  
 33 through a stacking of the Trp37 residue with the  
 34 nucleobases<sup>15-17</sup>. This key role of the plateau is further  
 35 illustrated by the fact that single mutation of any of its residues  
 36 results in non-infectious viral particles<sup>18</sup>.

37 Due to its key functions in HIV-1 replication and its high  
 38 conservation, NC appears as an ideal target for antiretroviral  
 39 therapy. Several strategies, including zinc ejectors, non-  
 40 covalent NC binders and noncovalent NA binders of different  
 41 chemical classes (see Discussion section for more details)  
 42 have been developed, but were not fully successful, due to low  
 43 specificity or to limited antiviral activity or therapeutic  
 44 index<sup>19-21</sup>. In order to develop new compounds directed  
 45 against NC, we have focused our interest on calixarenes, a  
 46 large family of macrocyclic aromatic compounds bearing  
 47 phenolic groups, widely used in supramolecular  
 48 chemistry<sup>22,23</sup>. Calixarenes are rigid vase-like macrocycles  
 49 with a well-established chemistry. These molecules can be  
 50 obtained through accessible and relatively cheap organic  
 51 synthesis routes, which also allow the versatile  
 52 functionalization of their macrocyclic core<sup>24-26</sup>. Calixarenes  
 53 are also receptors in supramolecular recognition studies<sup>27,28</sup>  
 54 that can bind and/or host small molecules through their  
 55 cavity<sup>29,30</sup> making them suitable for drug delivery purposes.<sup>31-  
 56 33</sup>. Calixarenes have been investigated among others as  
 57 biologically-active molecules in a number of applications, and  
 58 are recently emerging as candidate therapeutics against  
 59 multiple diseases<sup>34-36</sup>. However, their mechanism of action is  
 60 generally poorly elucidated, so that calixarenes are mostly  
 61 used as tool compounds in chemical biology studies.

62 We have also considered this family for two reasons. First,  
 63 calixarenes endowed with antiviral activity were first  
 64 described in the patent of Hwang *et al.*<sup>37</sup>, in which the para-  
 65 sulfonatocalix[4]arene and its analogs were characterized as  
 66 anti-HIV agents. Thereafter, synthetic strategies were  
 67 developed by inserting carboxylate substituents on the  
 68 calixarene backbone leading to antiviral molecules that have  
 69 been tested on humans<sup>38-41</sup>. Additional calixarene derivatives  
 70 with anti-HIV activities were further developed<sup>42-44</sup> and  
 71 recently reviewed.<sup>45</sup> Their mechanism of action revealed that  
 72 they may act as inhibitors of HIV fusion, integration and viral  
 73 protease cleavage<sup>25,46-48</sup>. Second, calixarene  
 74 hydroxymethylphosphonic acids<sup>49-51</sup> have been shown to form  
 75 host-guest complexes with free amino acids<sup>52,53</sup> that are  
 76 stabilized by electrostatic interactions between the positively  
 77 charged terminal  $-\text{NH}_3^+$  group of the amino acids and the  
 78 negatively charged phosphonic acid moiety of calixarenes.  
 79 Similarly, since the NC protein is highly basic with a large  
 80 number of solvent-exposed positively charged lysine and  
 81 arginine residues<sup>16,54,55</sup>, we hypothesized that this class  
 82 of calixarenes may inhibit NC and thus, HIV infection by  
 83 competing with nucleic acids for the binding to NC.

84



85

86 **Figure 1.** NC peptides (a), calixarene compounds (b to f), and  
 87 cTAR and dTAR oligonucleotides used in this study (g).

88 In this manuscript, we tested the ability of a series of  
 89 calixarene hydroxymethylphosphonic or sulfonic acids  
 90 (Figure 1b-f) to interact with NC and inhibit the binding of

1 this protein to its target nucleic acids, preventing the  
 2 promotion of the destabilization and annealing of the  
 3 complementary sequences. Molecular modelling and  
 4 mutational studies indicated that calixarenes exhibit a new  
 5 binding mode where their phosphonate or sulfonate groups  
 6 interact with the basic surface of NC. The antiviral activity  
 7 and cellular cytotoxicity of the most efficient compound were  
 8 tested using HIV pseudoviruses, as well as replication-  
 9 competent wild-type (WT) and drug-resistant strains. This  
 10 compound was also tested in humanized mice infected with  
 11 HIV-1. Finally, the mechanism of action of this calixarene  
 12 was investigated through biochemical and cell-based assays.

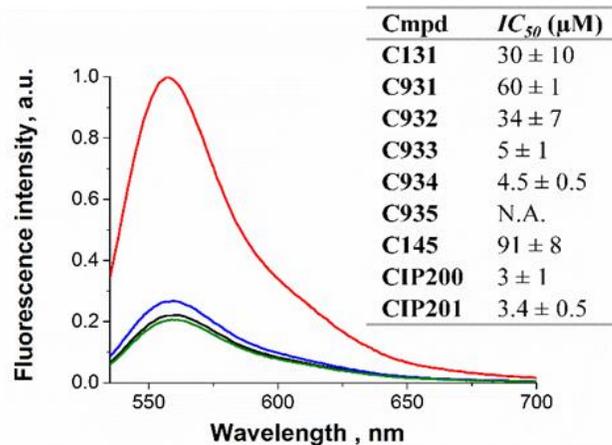
## 13 RESULTS

### 14 Inhibition of NC chaperone properties: 15 selection of the most active compounds

16 A series of calixarenes was synthesized and characterized as  
 17 described in supplementary information (Figures S1 and S2).  
 18 To test their ability to inhibit NC chaperone activity, we used  
 19 a fluorescence assay based on the NC-mediated  
 20 destabilization of the stem-loop structure of cTAR DNA  
 21 (Figure 1g), the complementary sequence of the  
 22 transactivation response element, involved in minus strand  
 23 DNA transfer during reverse transcription<sup>56</sup>. This assay as  
 24 well as all fluorescence-based assays in this study were  
 25 performed with the truncated NC(11-55) peptide (Figure 1a)  
 26 that contains the zinc finger domain responsible of the specific  
 27 nucleic acid binding and destabilization activities<sup>10,57</sup>, but is  
 28 deleted of the basic N-terminal domain that is responsible of  
 29 the strong NA aggregation activity of NC and thus, of strong  
 30 light scattering<sup>58</sup>. The assay is based on the use of cTAR  
 31 labelled at its 5' and 3' ends by a Rh6G fluorophore and a  
 32 Dabcyl fluorescence quencher, respectively<sup>59</sup>. In the absence  
 33 of NC(11-55), the proximity of cTAR ends induces a strong  
 34 fluorescence quenching of Rh6G by the Dabcyl group (Figure  
 35 2, black curve). Addition of a 10-fold molar excess of NC(11-  
 36 55) to cTAR induces the melting of the bottom of the cTAR  
 37 stem, leading to an increase in the distance between the two  
 38 fluorophores which restores the Rh6G fluorescence (Figure 2,  
 39 red curve). As a consequence, a positive hit will be detected  
 40 by its ability to inhibit partially or totally the NC(11-55)-  
 41 induced increase of Rh6G fluorescence.

42 Calixarenes were first added to the NC(11-55)/cTAR complex  
 43 at a concentration of 10  $\mu$ M, which corresponds to a molar  
 44 ratio of 10:1 in respect to NC(11-55). Interestingly, C933,  
 45 C934, CIP200 and CIP201 induced a 75 to 97% reversion of  
 46 the NC(11-55)-promoted increase in Rh6G emission,  
 47 suggesting that these four compounds can efficiently inhibit  
 48 the NC destabilization activity. In contrast, the five other  
 49 compounds induced only a marginal change in the same  
 50 conditions. After further increase of the calixarene  
 51 concentration to 100  $\mu$ M, a full reversion of the NC(11-55)-  
 52 induced increase in Rh6G emission was observed with C933,  
 53 C934, CIP200, CIP201, C131 and C932, while a 53 and 78 %  
 54 reversion was observed for C145 and C931, respectively.

55



56

57 **Figure 2.** Inhibition of the NC(11-55)-induced cTAR  
 58 destabilization by the calixarenes. Emission spectra of Rh6G-5'-  
 59 cTAR-3'-Dabcyl (0.1  $\mu$ M) were recorded in the absence (black)  
 60 and in the presence of 1  $\mu$ M NC(11-55) before (red) and after  
 61 addition of 10  $\mu$ M (blue) or 100  $\mu$ M (green) CIP201. Buffer: 25  
 62 mM Tris (pH = 7.5), 30 mM NaCl and 0.2 mM MgCl<sub>2</sub>.  
 63 Temperature: 20°C. Excitation wavelength was 520 nm. The  
 64 DMSO concentration was less than 2% v/v for all experiments.  
 65 The  $IC_{50}$  values for the inhibition of NC(11-55)-induced cTAR  
 66 destabilization by the tested calixarenes are shown in the table.  
 67 N.A. = Not Active

68

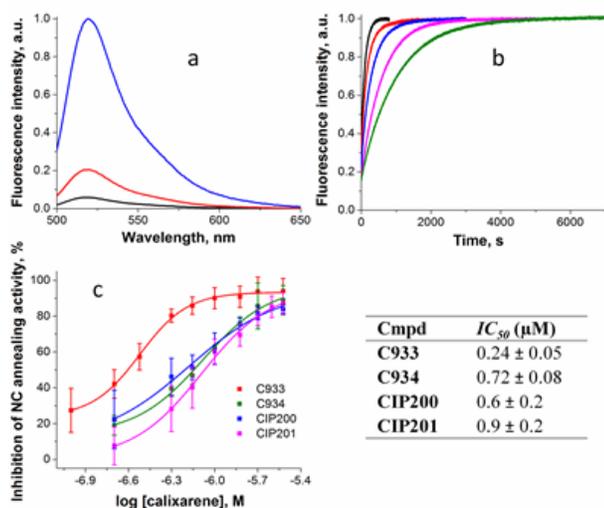
69 In contrast, no fluorescence change was induced by C935,  
 70 indicating that it is unable to inhibit the NC(11-55)-induced  
 71 destabilization of cTAR. Notably, this compound differs from  
 72 the other tested calixarenes by the fact that it possesses four  
 73 propyl chains at the lower rim and two bromine substituents  
 74 at the upper rim of the calixarene core. Assuming that the OH  
 75 groups may be solvent exposed when calixarenes are bound  
 76 to NC<sup>60</sup>, the O-propyl chains become then unfavorably  
 77 solvent-exposed. Moreover, the two bromine substituents  
 78 may sterically hinder the inclusion of NC residues in the  
 79 calixarene central core cavity or form a steric obstacle for the  
 80 binding of C935 to the NC. Determination of the  $IC_{50}$  values  
 81 revealed that the other tested calixarenes could be divided into  
 82 two classes (Figure 2, table). The first class comprising the  
 83 four most active compounds (C933, C934, CIP200 and  
 84 CIP201) is characterized by  $IC_{50}$  values in the range 3-5  $\mu$ M,  
 85 close to the concentration of the NC peptide used in the assay.  
 86 The second class (C131, C932, C145 and C931) is  
 87 characterized by  $IC_{50}$  values in the range 40-90  $\mu$ M and is thus  
 88 far less efficient. Based on the difference in activities of the  
 89 two classes, we focused our next efforts only on the  
 90 compounds of the first class.

91 In order to confirm the ability of the four selected compounds  
 92 to inhibit the NC chaperone properties, we checked their  
 93 effect on the NC(11-55)-promoted cTAR/dTAR annealing  
 94 reaction (Figure 3), which relies on both the nucleic acid  
 95 destabilizing and aggregating activities of NC<sup>61,62</sup>. We used  
 96 in this test a cTAR sequence labelled with Alexa488  
 97 (fluorophore) and Dabcyl (quencher) at its 5' and 3' ends,  
 98 respectively. In the absence of NC, the fluorescence signal is  
 99 low (Figure 3a) due to the close proximity of both cTAR ends  
 100 and the strong fluorescence quenching of the Alexa488  
 101 emission by the Dabcyl group. Of note, Alexa 488 was  
 102 preferred to Rh6G, due to its lower tendency to bind to

1 calixarenes at the high concentrations used in this assay (data  
 2 not shown). After addition of NC(11-55) at 8 eq, we observed  
 3 an increase of the fluorescence signal as a result of the  
 4 destabilization of cTAR by NC(11-55) (Figure 3a)<sup>56</sup>. A further  
 5 increase in the fluorescence signal was observed when both  
 6 NC-coated sequences were mixed together in order to allow  
 7 the annealing reaction to proceed (Figure 3a and b). Real-time  
 8 annealing kinetics were monitored in pseudo-first order  
 9 conditions by following the increase of the Alexa488  
 10 fluorescence at 519 nm. The same plateau was reached in the  
 11 absence and in the presence of calixarenes, indicating that the  
 12 same final product (extended duplex) was formed. However,  
 13 the reaction was slowed down in the presence of all four  
 14 selected calixarenes (Figure 3b), clearly indicating that they  
 15 efficiently counteract the NC-chaperoned annealing reaction.  
 16 The kinetic traces were fitted using<sup>61,63</sup>:

$$17 \quad I(t) = I_F - (I_F - I_0)e^{-k_{obs}(t-t_0)} \quad (1)$$

19 where  $t_0$  is the dead time,  $k_{obs}$  is the observed kinetic rate  
 20 constant, and  $I_0$  and  $I_F$  are the fluorescence intensities before  
 21 dTAR addition and at completion of the reaction, respectively.  
 22 NC annealing activity was calculated as the ratio  $k_{obs}/k_{obs0}$ ,  
 23 where  $k_{obs}$  and  $k_{obs0}$  are the kinetic rate constants in the  
 24 presence and in the absence of calixarenes, respectively.  $IC_{50}$   
 25 values were obtained by fitting to equation (4) the variation of  
 26 the percentages of inhibition (% inh = 100 - % of NC  
 27 annealing activity) as a function of the concentration of  
 28 calixarene (Figure 3c). The  $IC_{50}$  values were in the  
 29 submicromolar range, up to 10 times lower than those  
 30 obtained for the destabilizing activity (Figure 2, table). The  
 31 lowest value was observed for C933, which was about two to  
 32 three times more active than the other three compounds  
 33 (Figure 3, table).



35 **Figure 3.** Inhibition of NC(11-55)-promoted cTAR/dTAR  
 36 annealing reaction by selected calixarenes. (a) Fluorescence  
 37 spectra of 10 nM Alexa488-5'-cTAR-3'-Dabcyl in the absence  
 38 (black line) and in the presence of 80 nM of NC(11-55) (red line)  
 39 and after annealing with 100 nM of dTAR in the presence of 800  
 40 nM of NC(11-55) (blue line). Excitation was at 480 nm. Buffer:  
 41 25 mM Tris (pH = 7.5), 30 mM NaCl and 0.2 mM MgCl<sub>2</sub>.  
 42 Temperature: 20 °C. (b) Effect of CIP201 on the NC-promoted  
 43 cTAR/dTAR annealing reaction. Kinetic traces of the annealing

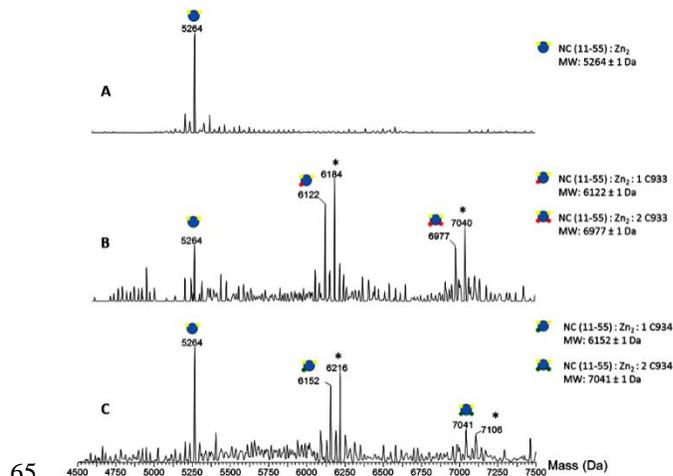
44 reaction in the absence of CIP201 (black) and in the presence of  
 45 0.5  $\mu\text{M}$  (red), 1  $\mu\text{M}$  (blue), 2  $\mu\text{M}$  (magenta) and 2.5  $\mu\text{M}$  (green)  
 46 of CIP201. The kinetic traces were normalized. Excitation was at  
 47 480 nm. Emission was recorded at 519 nm. (c) Inhibition of the  
 48 NC(11-55)-promoted cTAR/dTAR annealing reaction as a  
 49 function of the concentration of C933 (red), C934 (green),  
 50 CIP200 (blue), and CIP201 (magenta). The curves were fitted to  
 51 equation (4). The concentration of DMSO was less than 2% v/v.  
 52 Table:  $IC_{50}$  values for the inhibition of the NC(11-55)-promoted  
 53 cTAR/dTAR annealing reaction by the tested calixarenes

54

55 Thus, the selected calixarenes inhibit efficiently both the  
 56 nucleic acid destabilization and aggregation components of  
 57 NC(11-55) chaperone activity.

## 58 Binding of calixarenes to NC

59 To determine whether the inhibitory activity observed in  
 60 Figures 2 and 3 resulted from the direct binding of the  
 61 calixarenes to NC(11-55) and not from zinc ejection, we used  
 62 non-denaturing mass spectrometry for NC(11-55) in the  
 63 absence or presence of a 10-fold molar excess of C933 or  
 64 C934 (Figure 4).



66 **Figure 4.** Binding of C933 and C934 to NC(11-55) monitored by  
 67 non-denaturing mass spectrometry. Deconvoluted mass spectra  
 68 in non-denaturing conditions of 20  $\mu\text{M}$  NC(11-55) in 50 mM  
 69 AcONH<sub>4</sub> at pH 7.0 in the absence (A) or in the presence of 200  
 70  $\mu\text{M}$  of C933 (B) or C934 (C) in 1% DMSO. \* [M + CH<sub>3</sub>COO]  
 71 adduct. Experimental mass of each observed complex is given at  
 72 the right part of the spectrum.

73

74 As previously described<sup>56,64-66</sup>, NC(11-55) is detected in its  
 75 zinc-bound form with a measured mass of  $5264 \pm 1$  Da (Figure  
 76 4A). In the presence of C933 (Figure 4B), signals  
 77 corresponding to free NC(11-55) (with its 2 Zn<sup>2+</sup> ions) as well  
 78 as to 1:1 and 1:2 NC(11-55):C933 complexes are detected. No  
 79 peak corresponding to zinc-free NC(11-55) or zinc-free  
 80 NC(11-55):C933 species is detected, indicating that C933  
 81 binding occurs without zinc ejection. Similar results were  
 82 obtained with C934, for which 1:1 and 1:2 NC(11-55):C934  
 83 complexes were detected along with free NC(11-55) (Figure  
 84 4C). Again no zinc-free peptide species were detected,  
 85 demonstrating the absence of zinc ejection upon C934 binding  
 86 to NC(11-55). Similar experiments were performed with  
 87 CIP200 or CIP201, but led to weak MS signals not suitable

1 for interpretation (data not shown), due either to lower  
2 ionization efficiencies of CIP200 and CIP201 in electrospray  
3 or neutralization of the charges of the resulting complexes.  
4 Altogether non-denaturing MS analysis clearly indicates that  
5 C933 or C934 binds to NC(11-55) but does not induce zinc  
6 ejection.

7 The absence of zinc ejection with CIP200 and CIP201 was  
8 checked by monitoring the time-dependence of their  
9 inhibition of the NC(11-55)-induced cTAR destabilization. A  
10 fast inhibition was observed with both compounds (Figure S3)  
11 in sharp contrast with the slow time-dependent inhibition  
12 observed with zinc ejectors (see WDO-217, Figure S3), as a  
13 consequence of the time requested to chemically react with  
14 the zinc fingers and eject zinc<sup>64</sup>. This fast inhibition further  
15 suggests that CIP200 and CIP201 do not eject zinc, but rather  
16 compete with nucleic acids for binding to NC(11-55).

17 To further characterize the binding of the calixarenes to  
18 NC(11-55), we next determined their binding parameters by  
19 ITC (Figure S4). The titration curves were adequately fitted  
20 to equation (5), giving  $K_d$  values ranging from 0.7 to 2  $\mu$ M  
21 (Table 1), close to the  $IC_{50}$  values determined for the  
22 inhibition of NC chaperone activity (Figures 2 and 3). We  
23 found that the reaction of NC(11-55) was accompanied by a  
24 favorable enthalpy contribution, ranging from -2.8 to -5.7  
25 kcal/mol that represents roughly half of the total binding  
26 energy (Table 1). The positive  $\Delta S$  values found for all  
27 compounds indicated favorable entropic contributions,  
28 suggesting that the formation of the complexes is partly driven  
29 by a release of water molecules and/or ions.

30

31 **Table 1.** Thermodynamic parameters for the binding of  
32 calixarenes to NC peptides.

Cmpd	NC	$K_d$ ( $\mu$ M)	$\Delta H$ (kcal.mol <sup>-1</sup> )	$\Delta S$ (cal.mol <sup>-1</sup> .K <sup>-1</sup> )
C933	11-55	0.7 $\pm$ 0.2	-3.8 $\pm$ 0.5	15 $\pm$ 2
	1-55	0.15 $\pm$ 0.1	-4.4 $\pm$ 0.3	16 $\pm$ 1
C934	11-55	1.8 $\pm$ 0.6	-2.8 $\pm$ 0.3	17 $\pm$ 2
	1-55	0.12 $\pm$ 0.1	-2.5 $\pm$ 0.2	23 $\pm$ 2
CIP200	11-55	2 $\pm$ 1	-5 $\pm$ 2	8 $\pm$ 3
	1-55	0.7 $\pm$ 0.1	-3.1 $\pm$ 0.1	17.7 $\pm$ 0.5
CIP201	11-55	1.1 $\pm$ 0.5	-5.7 $\pm$ 0.9	8 $\pm$ 2
	1-55	0.2 $\pm$ 0.1	-7.3 $\pm$ 0.4	5.5 $\pm$ 0.3
	(1-55)S3	1.7 $\pm$ 0.2	-4.7 $\pm$ 0.9	10 $\pm$ 3

33 Buffer: 25 mM Tris (pH = 7.5), 30 mM NaCl and 0.2 mM  
34 MgCl<sub>2</sub>, T = 20°C. The thermodynamic parameters are  
35 reported as means  $\pm$  standard error of the mean for at least  
36 three independent measurements.

37

38 To investigate the contribution of the strong basic N-terminal  
39 domain of the peptide on calixarene binding, we performed  
40 ITC titrations with the full length NC. We found that  $K_d$  values  
41 are 3 to 15 times lower (Table 1) than those determined with  
42 the NC(11-55) mutant, suggesting that the basic N-terminal  
43 domain of NC significantly contributes to calixarene binding.  
44 Moreover, the basic residues between the two zinc fingers

45 play an important role too in the binding of CIP201 to NC, as  
46 indicated by the about one order of magnitude change in the  
47  $K_d$  value when these residues were replaced by Ser residues in  
48 the NC(1-55)S3 mutant (Table 1).

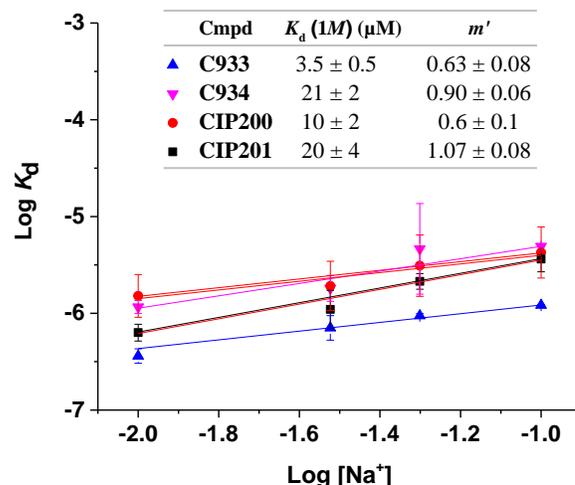
49 To provide further information on the binding process, the  
50 electrostatic and non-electrostatic binding contributions were  
51 determined from the variation of  $K_d$  with the concentration of  
52 Na<sup>+</sup> ions<sup>67</sup>. The number  $m'$  of ion pairs and the non-  
53 electrostatic binding constant  $K_d(1M)$  between NC(11-55) and  
54 the calixarenes were derived from:

55

$$56 \log K_d = \log K_d(1M) + \psi_{Na^+} m' \log[Na^+] \quad (2)$$

57

58 The value of the fraction of Na<sup>+</sup> ions thermodynamically  
59 bound per negatively charged moiety,  $\psi_{Na^+}$ , was assumed to  
60 be 0.71 as for single-stranded DNA<sup>67,68</sup>. The plots of  $\log K_d$   
61 versus  $\log [NaCl]$  were found to be linear in the 10-100 mM  
62 salt concentration range (Figure 5). Interestingly, the  
63 contribution of the non-electrostatic interactions deduced  
64 from the  $K_d(1M)$  values was found to be dominant,  
65 representing 80 % to 90 % of the binding energy at 30 mM  
66 NaCl. Moreover, the number  $m'$  of ion pairs between NC(11-  
67 55) and the calixarenes was about 1 in all the complexes. Of  
68 note, a similar distribution of electrostatic and non-  
69 electrostatic contributions was observed for the binding of  
70 NC(12-53) to a small d(AACGCC) oligonucleotide<sup>13,69</sup>.



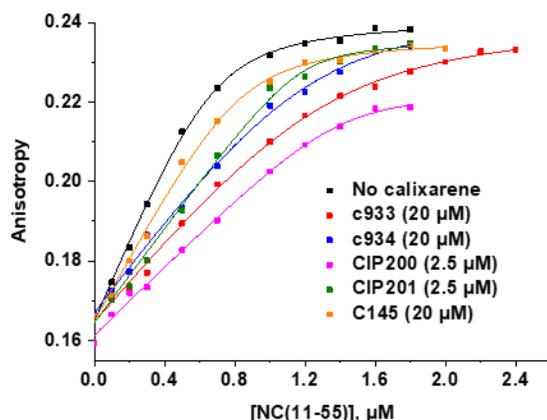
71

72 **Figure 5.** Dependence on Na<sup>+</sup> ion concentration of the  
73 equilibrium dissociation constant  $K_d$  for the binding of  
74 calixarenes C933, C934, CIP200 and CIP201 to NC(11-55), as  
75 determined by ITC at 20°C. The graph depicts the dependence of  
76  $\log K_d$  on  $\log [Na^+]$ . Lines are least-squares fits of equation (2) to  
77 the experimental points. The table provides the equilibrium  
78 dissociation constant at 1 M Na<sup>+</sup> ( $K_d(1M)$ ) and the numbers of  
79 ion pairs ( $m'$ ) between NC(11-55) and the calixarenes. Buffer: 25  
80 mM Tris (pH = 7.5), 0.2 mM MgCl<sub>2</sub>.

81

82 Finally, we performed fluorescence anisotropy titrations to  
83 determine whether the calixarenes can compete with NAs for  
84 the binding to NC(11-55). To this end, we titrated cTAR  
85 labeled by tetramethylrhodamine, TMR-5'-cTAR, by NC(11-  
86 55) in the absence and in the presence of calixarenes (Figure

6). As expected, NC(11-55) was found to markedly increase the fluorescence anisotropy of TMR-5'-cTAR, as a result of the formation of the NC(11-55)/cTAR complex. All four selected calixarenes were observed to shift the titration curves toward higher NC(11-55) concentrations as compared to the curve in the absence of calixarenes (Figure 6, black curve). Thus, all four calixarenes compete with cTAR for the binding to NC(11-55), which explains their ability to inhibit the NA chaperone properties of the peptide. It should be noted that the C145 used as negative control has, as expected, a marginal impact on the binding of NC (11-55) with cTAR (Figure 6, compare orange and black curves). Moreover, a control experiment was performed to check both by ITC and fluorescence titrations that calixarenes do not bind to cTAR (Figure S5A and B). Thus, the calixarenes hinder the binding of NC(11-55) to cTAR, only by their binding to the peptide.

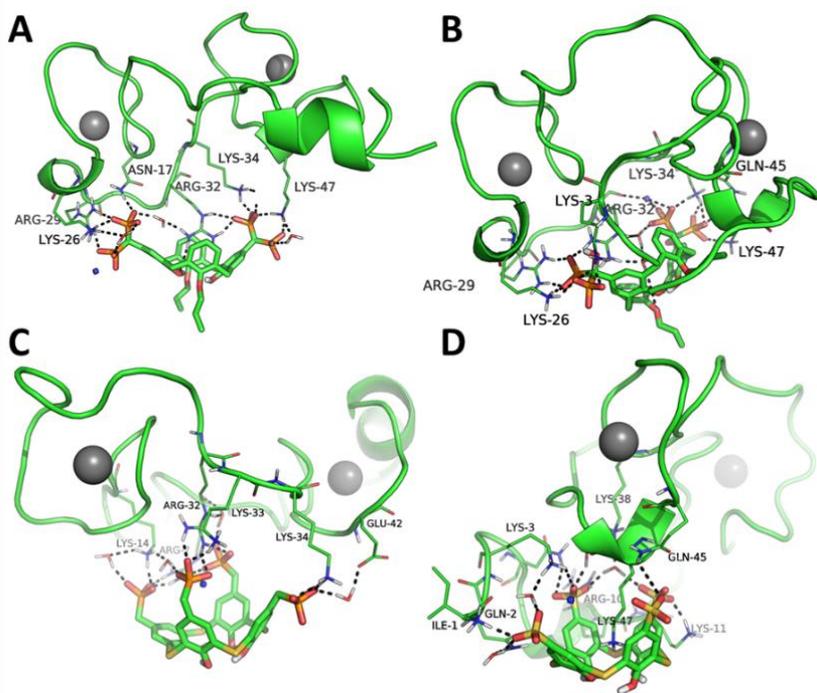


**Figure 6.** Binding of NC(11-55) to TMR-5'-cTAR in the presence of calixarenes. The interaction between NC(11-55) and 0.1 μM TMR-5'-cTAR was monitored through the fluorescence anisotropy changes of TMR. Titrations were performed at 20°C either in the absence (black) or in the presence of 20 μM C933 (red), 20 μM C934 (blue), 2.5 μM CIP200 (magenta) or 2.5 μM CIP201 (green) or 20 μM C145 (orange). Buffer: 25 mM Tris pH

7.5, 30 mM NaCl and 0.2 mM MgCl<sub>2</sub>. Excitation wavelength was 550 nm.

## Molecular dynamics simulations of NC/calixarene complexes

To further rationalize our experimental data, and to characterize the binding mode of selected calixarenes to NC, we performed recognition experiments *in silico* through molecular dynamics (MD) simulations. This method was selected because of the complexity of calixarenes and the flexibility of NC, which render other approaches (such as molecular docking) ineffective towards this study. Each calixarene was placed at a non-binding distance from the NC surface (> 40 Å) in a box of explicit water molecules, and then unrestrained MD trajectories were produced for 0.5 μs in the NPT ensemble. Using this approach, the four selected calixarenes were found to recognize and bind NC, by interacting with the basic surface at the top of the zinc fingers. Similar to the previously reported complexes of calixarenes with isolated amino acids<sup>70,71</sup> or proteins<sup>60</sup>, the recognition between calixarenes and the NC *in silico* is mostly driven by electrostatic forces between the calixarene phosphonate or sulfonate groups and basic NC residues (Figure 7 and Figure S6 in the supplementary data). In contrast, OH or O-propyl groups at the lower rim are exposed to the solvent and do not seem to contribute to the binding of the protein. Similarly, no interaction was observed with the specific hydrophobic pocket of NC or through inclusion of the side chain of the key residue Trp37 into the calixarene core. The formation of NC/calixarene complexes is rather fast in MD simulations, as observed by monitoring the distance between the target residue and the mass center of the carbons bearing the phosphonate or sulfonate groups (supplementary data, Figure S7). Moreover, the NC/calixarene complexes were found to be stable with time, since by analogy with other NC inhibitors<sup>66</sup>, we did not observe any dissociation of the complexes during MD simulation.



65

66 **Figure 7.** Binding mode of calixarenes to NC predicted by a recognition experiment performed along 0.5  $\mu$ s of unrestrained MD  
 67 simulations. The panels show the conformations of the most abundant clusters of MD frames for the complexes between NC and  
 68 C933 (A), C934 (B), CIP201 (C) and CIP200 (D). The protein is shown as a green cartoon. Residues establishing polar contacts with  
 69 calixarenes are shown as lines and are labeled. Bridging water molecules are shown as lines, calixarenes as sticks,  $Zn^{2+}$  ions as grey  
 70 spheres, and  $Na^+$  ions from the solvent as blue small spheres. Polar contacts between calixarenes and NC are shown as black dashed  
 71 lines. The complexes were oriented to have the calixarene in the bottom part, with the upper rim projected towards the top of the  
 72 panel.

73 As highlighted by the salt-dependence data of Figure 5, the  
 74 Coulomb electrostatic contribution in the final complexes  
 75 may be rather weak, due to the strong dielectric constant of  
 76 the solvent that considerably lowers electrostatic forces. In  
 77 fact, the NC/calixarene complexes are stabilized by cation- $\pi$   
 78 stacking<sup>72,73</sup> and a number of H-bonds established directly, or  
 79 bridged by water molecules (Figure 7). In particular,  
 80 phosphonate groups at the upper rim of calixarenes C933,  
 81 C934, and CIP201 bind preferentially the side chain of Arg32  
 82 by both H-bond interactions and cation- $\pi$  stacking. The Arg32  
 83 residue is well included in the calixarene core of C933 (Figure  
 84 7A), and partially included in the core of C934 (Figure 7B).  
 85 In CIP201, the Arg32 residue is located at the upper rim of the  
 86 calixarene molecule because the inner core is occupied by a  
 87  $Na^+$  ion (Figure 7C), whereas CIP200 targets the side chain of  
 88 Lys47, which is well included in the calixarene core (Figure  
 89 7D).

90 C933 and C934 proved to bind the NC in a highly comparable  
 91 manner by interacting with Arg32, as well as with Lys26,  
 92 Arg29, Lys34, and Lys47. C933 further interacts with Asn17  
 93 (Figure 7A), whereas C934 interacts with Lys3 and Gln45  
 94 (Figure 7B), this latter being also involved in the binding of  
 95 other NC inhibitors<sup>65,66,74</sup>. Besides Arg32, CIP201 establishes  
 96 H-bonds with Arg7, Lys14, Lys33, Lys34, and Glu42 (Figure  
 97 7C). In the case of CIP200, in addition to Lys47, the  
 98 compound interacts with Ile1, Gln2, Lys3, Arg10, Lys11,  
 99 Lys38, and Gln45 (Figure 7D).

100 Finally, to provide a structural explanation to the noticeable  
 101 entropic effect observed by ITC (Table 1), the change of NC  
 102 hydration associated to the binding of CIP200 was evaluated

103 along MD trajectories. The comparison of the solvation of NC  
 104 target residues in the free protein (Figure S8A) with that in the  
 105 complex (Figure S8B) clearly shows that CIP200 displaces a  
 106 large number of water molecules from solvent-exposed NC  
 107 residues such as for example Lys47, in full line with the  
 108 measured increase in entropy.

109 Overall, MD simulations suggest that the phosphonate and  
 110 sulfonate groups of calixarenes are responsible for NC  
 111 recognition through long-range electrostatic interactions and  
 112 for the stabilization of the final NC/calixarene complexes,  
 113 mainly through H-bond and cation- $\pi$  interactions with NC  
 114 basic residues. As a result, these molecules may inhibit NC by  
 115 competing with NAs for the binding to the protein and  
 116 occluding the accessibility to its hydrophobic pocket from the  
 117 solvent area.

118 To validity of the MD simulations is substantiated by the ITC  
 119 data with the NC(1-55)S3 mutant where the three positively  
 120 charged basic residues (<sup>32</sup>RKK<sup>34</sup>) of the linker acting as a  
 121 binding site for CIP201 are replaced by non-charged Ser  
 122 residues. ITC titration of NC(1-55)S3 by CIP201 revealed an  
 123 about one order of magnitude decrease in affinity as compared  
 124 to NC, with a significant change in the balance between the  
 125 enthalpy and entropy contributions (Table 1). Indeed, we  
 126 observed a two-fold less favourable  $\Delta H$  value together with a  
 127 two-fold increase in the  $\Delta S$  value. These changes in  $\Delta H$  and  
 128  $\Delta S$  values strongly suggest that the binding of CIP201 is less  
 129 specific with the NC mutant as compared to NC<sup>75</sup>. Thus, the  
 130 binding data with NC(1-55)S3 are fully in line with the MD  
 131 simulations and confirm the key role played by the <sup>32</sup>RKK<sup>34</sup>  
 132 sequence in the binding of CIP201.

1

## 2 Antiviral activity of CIP201 against wild-type 3 and drug-resistant HIV-1 strains

4 Next, our objective was to determine the antiviral activity of  
5 the water soluble calixarene CIP201 acting as NC inhibitor.  
6 Indeed, we selected this compound, as it remains highly  
7 soluble in water (up to 100 mM) while the others calixarenes  
8 were low soluble in aqueous solutions in the absence of  
9 DMSO (solubility limits of C933, C934 and CIP200 were  
10 measured at 400  $\mu$ M, 6.5 mM and 150  $\mu$ M respectively). To  
11 determine the antiviral activity of CIP201, we used two  
12 cellular assays. The first one being based on Hela cells  
13 infection with non-replicative pseudo-particles (anti-  
14 pseudovirus assay in Table 2) monitors only the inhibition of  
15 the early steps of infection. The second one being based on  
16 cell infection by a wild-type HIV virus (NL4-3) is sensitive to  
17 inhibition in either the early phase of HIV infection  
18 (MonoCycle) or both the early and late phases of HIV  
19 infection (BiCycle). In both the anti-pseudovirus and  
20 MonoCycle assays, CIP201 was found to inhibit HIV  
21 infection with  $IC_{50}$  values of 3.3 - 21  $\mu$ M with comparable  
22 values in both MonoCycle and BiCycle assay, indicating that  
23 this compound inhibited mainly, if not only, the early phases  
24 of HIV replication. These  $IC_{50}$  values were found to be close  
25 to the  $IC_{50}$  values of the NC chaperone assays (Figures 2 and  
26 3) and the  $K_d$  values (Table 1). These similar values  
27 determined by different independent experiments strongly  
28 suggest that the antiviral activity of CIP201 may be related to  
29 its ability to target NC. The cytotoxicity of CIP201 was very  
30 low ( $CC_{50} > 5$  mM), giving an excellent selectivity index for  
31 this compound. However, it cannot be excluded at this step  
32 that the low toxicity might be related to a limited cell  
33 permeability of the compound.

34 **Table 2.** Antiviral activity and cytotoxicity of CIP201.

Anti pseudovirus assay <sup>a</sup>			Antiviral assays <sup>d</sup>	
			MonoCycle	BiCycle
			assay	assay
$IC_{50}$ <sup>a</sup>	$CC_{50}$ <sup>b</sup>	SI <sup>c</sup>	$IC_{50}$ <sup>d</sup>	$IC_{50}$ <sup>d</sup>
( $\mu$ M)	( $\mu$ M)		( $\mu$ M)	( $\mu$ M)
21 $\pm$ 3	>5000	>230	3.3 $\pm$ 2	1.7 $\pm$ 0.2

35 <sup>a</sup> Inhibition activity of CIP201 on the infection of Hela cells  
36 by viral pseudoparticles.  $IC_{50}$  corresponds to the concentration  
37 at which the infection is decreased by 50%. <sup>b</sup> $CC_{50}$ :  
38 concentration required to cause 50% of cell death. <sup>c</sup> Selectivity  
39 index  $SI = CC_{50}/IC_{50}$ . <sup>d</sup> Inhibitory activities tested on HIV-1  
40 wild-type reference strain NL4-3.

41

42 The compound CIP201 was then tested on a series of mutant  
43 strains resistant to reference protease inhibitors (PI, strain  
44 11808), nucleoside reverse transcriptase inhibitors (NRTI,  
45 strain 7406), non-nucleoside reverse transcriptase inhibitors  
46 (NNRTI, strain 12231) and integrase inhibitors (INI, strain  
47 11845) (Table 3). CIP201 showed an antiviral activity against

48 these representative resistant HIV-1 viral strains in the low  
49 micromolar range, similar to that against the wild-type virus  
50 (strain 114 i.e. NL4-3, Table 3). These data indicate that the  
51 resistance mutations to drugs currently employed in clinical  
52 practice do not affect the susceptibility to CIP201, reinforcing  
53 the conclusion that NC could be the main target of the  
54 calixarenes. Due to its favourable  $IC_{50}$  values and low toxicity,  
55 CIP201 appears as a good starting point for the development  
56 of new anti-HIV agents.

57 **Table 3.** Antiviral activity of CIP201 against drug-resistant  
58 strains.

<sup>a</sup> Strain	$IC_{50}$ ( $\mu$ M)	<sup>b</sup> Fold Change
<b>114</b> (NL4-3)	1.7 $\pm$ 0.2	1.0
<b>11845</b> (INI)	2.3 $\pm$ 0.4	1.3
<b>11808</b> (PI)	1.8 $\pm$ 0.1	1.1
<b>12231</b> (NNRTI)	1.0 $\pm$ 0.5	0.6
<b>7406</b> (NRTI)	1.5 $\pm$ 0.3	0.9

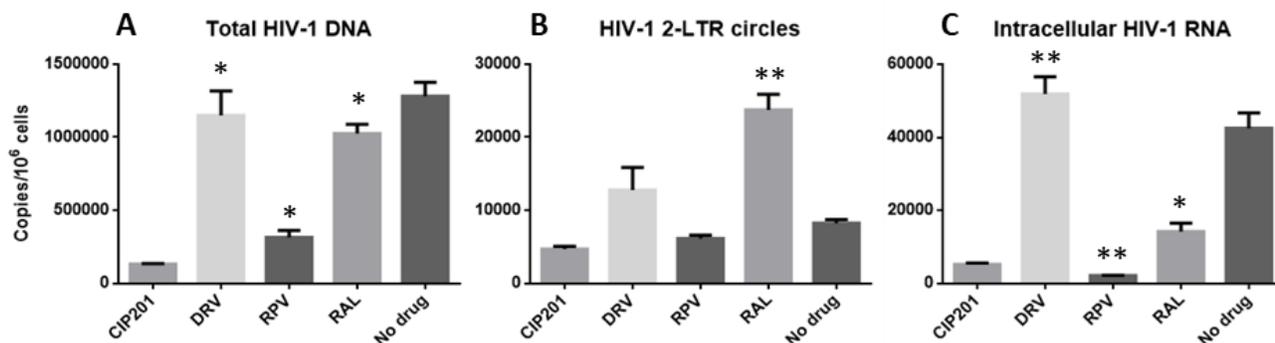
59 <sup>a</sup> Reference number of the drug resistant strains obtained  
60 through the NIH AIDS Reagent Program  
61 (ARP, www.aidsreagent.org). <sup>b</sup>Ratio of the resistant virus  
62  $IC_{50}$  to wild-type reference virus  $IC_{50}$ .

## 63 Mechanism of action of calixarenes

64 As CIP201 was found to be active in the early steps of HIV  
65 replication, we performed complementary investigation to  
66 further characterize its mode of action. As a first step, we  
67 investigated the effects of CIP201 on the production of viral  
68 nucleic acid intermediates. The data in Figure 8 suggests that  
69 inhibition of HIV replication occurred mainly during pre-  
70 integration events. In particular, the amount of total HIV-1  
71 DNA (Figure 8A) produced after reverse transcription  
72 (approximately 16 h post infection) was significantly  
73 decreased in presence of CIP201. This decrease was similar  
74 to that measured in the presence of rilpivirine (RPV),  
75 indicating an impairment of reverse transcription or earlier  
76 events. In contrast, CIP201 did not affect the levels of 2-LTR  
77 circles (Figure 8B). This absence of effect is in contrast with  
78 the approximately 3 times higher amount of 2-LTR circles  
79 observed with the integrase inhibitor raltegravir (RAL) as  
80 compared to “no drug” control culture. This suggests that  
81 CIP201 does not impair viral DNA integration. In addition,  
82 the levels of intracellular HIV-1 RNA were comparable to  
83 those measured in presence of RPV, reinforcing the  
84 hypothesis of an early inhibitory activity for CIP201 (Figure  
85 8C).

86

87



88

89 **Figure 8.** Quantification of HIV-1 nucleic acid intermediates produced in the presence of CIP201 (100  $\mu$ M), or reference compounds  
 90 darunavir (DRV), rilpivirine (RPV), raltegravir (RAL) (2  $\mu$ M each) or no drug. (A) Total HIV-1 DNA measured at 16 hours post infection.  
 91 (B) HIV-1 2-LTR DNA circles and (C) Intracellular HIV-1 RNA measured at 30 hours post infection. Values are shown as means with  
 92 standard deviation bars derived from two measurements. Pairwise comparisons between CIP201 and each reference drug were made by  
 93 Student t test. \*:  $p < 0.05$ ; \*\*:  $p < 0.005$ .

94 In a second step, since compound CIP201 was observed to  
 95 possibly impair reverse transcription, we tested whether this  
 96 compound could directly inhibit RT activity. Using a FRET-  
 97 based assay previously developed in the laboratory<sup>76</sup>, CIP201  
 98 (Figure S9a) was found to strongly alter RT flipping and  
 99 polymerisation activity, indicating it may directly target RT.  
 100 Its effect was similar to that of Nevirapine, a non-nucleoside  
 101 RT inhibitor (NNRTI) that binds non-competitively to RT  
 102 allosteric sites<sup>77,78</sup>. By fitting the dependence of RT inhibition  
 103 as a function of the concentration of CIP201 with equation (4),  
 104 an  $IC_{50}$  value of  $0.9 (\pm 0.1) \mu$ M was determined for CIP201  
 105 (Figure S9b), as compared to  $49 (\pm 4) \text{ nM}$  for Nevirapine in

106 the same assay<sup>76</sup>. Altogether our data suggest that CIP201  
 107 may have multiple molecular targets in HIV-1. However, as  
 108 CIP201 is also active on RT resistant strains, it is believed that  
 109 RT is not a major target of CIP201 in cellular assays and/or  
 110 that CIP201 inhibits RT via a novel binding mode that is  
 111 different from that of the established inhibitors.

112 To further decipher the mode of action of CIP201, we used a  
 113 Multi-Dosing Time protocol to test its effect comparatively to  
 114 reference inhibitors of entry (maraviroc), RT (lamivudine and  
 115 didanosine) and integrase (raltegravir) (Table 4).

116 **Table 4.**  $IC_{50}$  values obtained in the Multi-Dosing Time protocol using the pseudotype virus assay.

CIP201 incubation	Compounds $IC_{50}$				
	CIP201	Maraviroc	Lamivudine	Didanosine	Raltegravir
Pre-incubation with the cells before infection	Inactive	$4.5 \pm 0.8 \text{ nM}$	Inactive	Inactive	Inactive
Incubation for 2 h during HIV-1 infection.	$19 \pm 2 \mu\text{M}$	$5 \pm 2 \text{ nM}$	$1.3 \pm 1 \text{ nM}$	$99 \pm 6 \text{ nM}$	$2 \pm 3 \mu\text{M}$
Standard protocol for 48 h	$20 \pm 3 \mu\text{M}$	$7 \pm 3 \text{ nM}$	$1.3 \pm 0.6 \text{ nM}$	$6.4 \pm 0.9 \text{ nM}$	$4 \pm 1 \text{ nM}$
incubation for 2 h after viral entry	Residual activity	$3 \pm 1 \mu\text{M}$	$0.6 \pm 0.4 \text{ nM}$	$9 \pm 2 \text{ nM}$	$1.2 \pm 1 \text{ nM}$
Incubation for 48 h after viral entry	Residual activity	$2.5 \pm 2 \mu\text{M}$	$1.3 \pm 0.7 \text{ nM}$	$120 \pm 40 \text{ nM}$	$4 \pm 3 \text{ nM}$

117  $IC_{50}$  values are expressed as a mean  $\pm$  SD. Two independent experiments were performed in triplicate.

118 In the standard protocol, CIP201 was incubated with the cells for 2 h during spinoculation and then, again for 48 h.

119

120

121 When CIP-201 was pre-incubated with the cells for 2 hours  
 122 and then washed out before HIV-1 incubation, no effect was  
 123 observed on virus infection, suggesting that CIP201 likely  
 124 does not bind with high affinity to the plasma membrane in  
 125 order to protect the cells against HIV-1 infection. In contrast,  
 126 CIP201 protected U373-CD4-CCR5 cells against virus  
 127 infection when incubated together with the pseudotyped  
 128 viruses for 2 h ( $IC_{50} = 19 \pm 2 \mu$ M) or in the standard protocol  
 129 where CIP201 was added both during spinoculation (for 2 h)  
 130 and at 2 h post-infection (for 48 h) ( $IC_{50} = 20 \pm 3 \mu$ M).  
 131 Moreover, CIP201 showed only residual activity when  
 132 incubated with cells 2 h post-infection (Figure S10 and Table

133 4). Thus, CIP201 resembles to the entry inhibitor maraviroc,  
 134 but differs from RT inhibitors that remain fully active when  
 135 provided for 2 or 48 h at 2 h post-infection. Therefore, our  
 136 data suggest that CIP201 mainly targets the HIV-1 entry step  
 137 during the first two hours of infection. The low activity of  
 138 CIP201 when provided 2 h after infection, in spite of its high  
 139 activity on NCp7 and RT in molecular assays, suggests that  
 140 CIP201 might not enter efficiently into cells. The lack of  
 141 activity on HIV-1 infection after cell pre-treatment with  
 142 CIP201 and subsequent wash, confirms that CIP201 does not  
 143 enter efficiently into cells, and may primarily act by binding  
 144 to viral particles. Finally, its residual activity when added 2 h  
 145 after infection (Figure S10) may tentatively be attributed to

1 CIP201 molecules that have been shuttled into cells through  
2 viral particles docked on the cell surface, in order to inhibit  
3 HIV-1 replication possibly by acting on RT and NCp7.

#### 5 CIP201 activity in HIV-infected humanized NSG 6 mice

7 Finally, CIP201 was tested in humanized NSG mice<sup>79</sup> and  
8 compared with the triple regimen Triumeq (abacavir,  
9 dolutegravir, lamivudine) on viral replication and CD4  
10 depletion after 7 weeks of treatment. After 5 weeks of HIV  
11 infection, although the mice have drunk the same volume of  
12 sucralose solution among the three groups, we did not find any  
13 effect of CIP201 on plasma viral load (Figure S10A) and on  
14 the CD4/CD8 ratio (Figure S10B) after 6 weeks of treatment.  
15 In contrast, the mice treated with Triumeq reached  
16 undetectable viral load and restored CD4 cell count. The  
17 concentrations of CIP201 was further measured in the plasma,  
18 spleen, liver and kidneys but was found to be below the LOD  
19 (i.e. 10 µg/mL). These results indicate that the lack of efficacy  
20 of CP201 in infected humanized mice was probably due to  
21 poor absorption, distribution, metabolism, and excretion  
22 (ADME) and/or pharmacokinetic properties. Thus, CIP201  
23 needs to be optimized for further development in therapy.

#### 25 DISCUSSION

26 Following our strategy to develop antiretroviral drug  
27 candidates targeting the HIV-1 NC protein<sup>19</sup>, we evaluated the  
28 NC inhibitory properties and antiretroviral activity of a  
29 number of functionalized calixarenes by means of an  
30 integrated multidisciplinary approach. Using fluorescence-  
31 based methods, calixarenes C933, C934, CIP200 and CIP201  
32 proved to inhibit efficiently the NC(11–55)-induced  
33 destabilization of cTAR and subsequent annealing with  
34 dTAR. Different from the largest class of NC inhibitors  
35 available to date, no zinc ejection was observed for these  
36 molecules, making them valuable candidates for antiviral  
37 therapy. Moreover, NC inhibition in the low to sub-  
38 micromolar range places these calixarenes within the most  
39 potent non-zinc-ejecting NC inhibitors reported to date<sup>80,81</sup>.

40 By using ITC, the calixarenes were found to bind to NC with  
41 affinity in the low to sub-micromolar range in agreement with  
42 the NC inhibition data. Moreover, the binding was found to  
43 rely on balanced entropy and enthalpy contributions. This was  
44 further corroborated by MD simulation for each molecule,  
45 showing that the recognition between NC and calixarenes is  
46 driven by long-range electrostatic forces, and that calixarenes  
47 bind basic residues at NC surface by means of H-bond and  
48 cation- $\pi$  interactions, in line with the strong observed enthalpy  
49 energy changes. Moreover, according to the binding mode  
50 observed in the most populated cluster of MD frames, a  
51 number of water molecules are released from NC residues  
52 upon binding of calixarenes, consistent with the favourable  
53 entropy changes deduced from ITC. This release of water may  
54 compensate for the loss of overall NC flexibility induced by  
55 calixarene binding. From the ionic strength dependence of the

56 affinity of calixarenes for NC, we further showed that the  
57 binding of calixarenes C933, C934, CIP200 and CIP201 to  
58 NC(11–55) involved about 1 ion pair and that non-  
59 electrostatic interactions represented 80% to 89% of the  
60 binding energy at 30 mM NaCl. This suggests that long-range  
61 electrostatic interactions are likely required for recognition  
62 between the two interacting species, but as suggested by MD  
63 simulations, the complex is mainly stabilized by H-bonds,  
64 cation- $\pi$  interactions and water release. Calixarenes C933,  
65 C934, and CIP201 that bear phosphonate groups at the upper  
66 rim show a similar interaction pattern. They recognize and  
67 bind basic residues of the NC linker (and notably Arg32) by  
68 H-bond and cation- $\pi$  interactions, and establish a number of  
69 H-bond contacts with basic residues at the top of zinc-fingers,  
70 a region of NC that is crucial for its biological functions.<sup>82,83</sup>  
71 The key role of the basic residues of the NC linker in binding  
72 to CIP201 was clearly confirmed using a NC mutant where  
73 these residues were replaced by Ser residues (NC(1-55)S3,  
74 Figure 1a). Different from the other active calixarenes,  
75 CIP200 is functionalized with sulfonate groups at the upper  
76 rim, and targets the Lys47 by means of cation- $\pi$  interactions  
77 through inclusion of the residue's side chain in its aromatic  
78 core. CIP200 also interacts with a cluster of residues within  
79 the N-terminal domain of NC, suggesting that the binding site  
80 of calixarenes on NC may be modulated by specific  
81 functionalization at the upper rim of the aromatic core.

82 Importantly, the binding mode predicted for calixarenes was  
83 not observed before for any type of NC inhibitors. The main  
84 hypothesis that emerged by coupling computational with  
85 biophysical and biochemical data is that this new binding  
86 mode efficiently prevents NC to bind to its nucleic acid  
87 targets. Moreover, the water soluble compound CIP201 was  
88 found to inhibit replication of wild-type and drug-resistant  
89 HIV-1 strains in a cellular model at low micromolar  
90 concentration, by targeting the early steps of HIV-1  
91 replication. Time of addition assays further revealed that  
92 CIP201 has low cell permeability, likely due to its high  
93 amount of negative charges, and mainly targets the viral entry  
94 step. In addition, the residual activity observed after the HIV-  
95 1 entry step suggested that CIP201 molecules could have  
96 entered cells when bound to viral particles and exerted their  
97 antiviral activity on RT and NCp7 molecules. Both modes of  
98 action are fully consistent with the low levels of HIV-1 DNA  
99 and intracellular HIV-1 RNA measured in cells infected by  
100 HIV-1 in the presence of CIP201.

101 Finally, CIP201 showed low cytotoxicity that can be at least  
102 partly ascribed to the low cell permeability of the compound.  
103 The low toxicity of CIP201 was further confirmed on infected  
104 humanized mice, which showed no evidence of acute toxicity  
105 during the treatment. Therefore, CIP201 stands as a very  
106 interesting lead, since its efficacy in inhibiting HIV-1  
107 replication is highly comparable to other NC inhibitors (Mori  
108 et al, Sancineto et al, Iraci et al). Unfortunately, CIP201 was  
109 found to be inactive in this animal model. This lack of efficacy  
110 *in vivo* was related to the low compound's concentration in  
111 blood and different organs, suggesting a high clearance and  
112 poor tissue distribution likely due to its highly polar nature<sup>99</sup>.

113  
114  
115 In this study, we identified the CIP201 calixarene as an  
116 antiviral compound active against both HIV-1 reference virus  
117 and resistant viral strains, mainly by inhibiting the HIV-1

1 entry step. CIP201 was also found to inhibit the nucleic acid  
2 chaperone activity of the HIV-1 NC as well as the flipping  
3 and polymerisation activity of RT in molecular assays. The  
4 inhibition of NC was predicted to result from the interaction  
5 of CIP201 via its phosphonate or sulfonate groups with the  
6 basic surface of NC, which blocks the binding of NC to its  
7 nucleic acid targets. This binding mode differs from that of  
8 most other noncovalent NC inhibitors that target the  
9 hydrophobic platform at the surface of the NC zinc fingers  
10 (Mori et al, 2015). Importantly, due to its low permeability  
11 the antiviral activity of this multi-target compound is certainly  
12 underestimated. Thus, CIP201 appears as a very promising  
13 candidate for the development of anti-HIV drug candidates  
14 targeting the NC.

15 However, efforts are still needed to further increase its  
16 efficacy and improve its ADME and pharmacokinetic  
17 properties. To this aim, hit-to-lead optimization of CIP201  
18 could be driven by the necessity to tune the hydrophilic-  
19 hydrophobic balance in order to ensure a better  
20 pharmacokinetics profile and to enhance *in vivo* activity. This  
21 could be accomplished by chemical modifications at the lower  
22 (narrow) rim or the upper (wide) rim of the macrocycle.  
23 Interestingly, thiacalixarenes offer almost unlimited  
24 possibilities for chemical modifications, including the  
25 derivatization of the hydroxyl groups at the lower rim, or the  
26 four reactive para-positions of benzene rings at the upper rim  
27 by the inclusion of different functional groups with specific  
28 chemical or physicochemical features. In the specific case of  
29 CIP201, the phosphonate groups could be replaced by  
30 bioisosters or other negatively charged groups such as for  
31 instance the carboxylic acid, sulfone, triazole, and tetrazole  
32 rings. In addition, sulfur atoms can be oxidized to convert the  
33 thiacalixarenes into calixarene sulfoxides or calixarenes  
34 sulfone with four or six valent sulfur atoms respectively.  
35 Besides chemical modifications, conformational changes to  
36 the macrocyclic core can be designed in order to achieve  
37 optimal interaction with NC. Thanks to the accessible  
38 chemistry of calixarenes, and the reliable computational  
39 protocol developed in this work, a large number of  
40 thiacalixarene derivatives could be preliminarily screened and  
41 prioritized *in silico* for their interaction with NC, although this  
42 will be the subject of future works.

## 43 EXPERIMENTAL SECTION

### 44 Materials

45 All solvents for spectroscopic measurements were from  
46 Sigma-Aldrich (Darmstadt, Germany). Acetonitrile was  
47 purchased from Sigma-Aldrich (Darmstadt, Germany) with  
48 high degree of purity for the HPLC analysis.

49 The synthesis and the characterization of the calixarenes used  
50 in this study are described in the supplementary information.  
51 Calixarenes C145 and CIP201 were directly solubilized in  
52 water while the other calixarenes were first dissolved in  
53 DMSO. Unless mentioned otherwise, experiments were  
54 carried out in Tris buffer (25 mM Tris-HCl pH 7.5, 30 mM  
55 NaCl, and 0.2 mM MgCl<sub>2</sub>)<sup>59</sup>. The percentage of DMSO was

56 between 1.3 and 2 % v/v in the final reaction mixtures. Fresh  
57 calixarene solutions were prepared before all experiments.

### 58 Proteins

59 In order to reduce the NA-aggregating properties<sup>58,101,102</sup> of the  
60 full-length NC which causes substantial light-scattering, we  
61 performed the majority of experiments with the NC(11-55)  
62 peptide (Figure 1a). This truncated peptide possesses the zinc  
63 finger domain and aggregates NAs only at high  
64 concentrations<sup>58,103</sup>. NC, NC(1-55)S3 and NC(11-55) peptides  
65 were synthesized by solid-phase peptide synthesis on a 433A  
66 synthesizer (ABI, Foster City, CA), as previously  
67 described<sup>56,104</sup>. Peptides were stored lyophilized. The zinc-  
68 bound form of the peptides was prepared by dissolving them  
69 in water, adding a 2.5-fold molar excess of zinc sulphate, and  
70 raising the pH to its final value by adding buffer. The pH was  
71 increased to its final value only after addition of zinc to avoid  
72 the oxidation of the zinc-free peptide. The peptide  
73 concentration was determined by using an extinction  
74 coefficient of  $5.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at 280 nm for NC and NC(11-  
75 55).

### 76 Oligonucleotides

77 All unlabelled and labelled oligodeoxynucleotides (ODNs)  
78 were synthesised and purified by IBA GmbH Nucleic Acids  
79 Product Supply (Göttingen, Germany). Their concentrations  
80 were determined by UV absorbance at 260 nm. For the doubly  
81 labelled cTAR, the 5' terminus was labelled with 6-  
82 carboxyrhodamine (Rh6G) or with Alexa Fluor 488 via an  
83 amino linker with a six carbon spacer arm. The 3' terminus of  
84 cTAR was labelled with 4-(4'-  
85 dimethylaminophenylazo)benzoic acid (Dabcyl) using a  
86 special solid support with the dye already attached. The singly  
87 labelled cTAR was labelled with  
88 carboxytetramethylrhodamine (TMR) at its 5' terminus. The  
89 ODNs were purified by reverse-phase high performance  
90 liquid chromatography (HPLC) and polyacrylamide gel  
91 electrophoresis. Extinction coefficients at 260 nm of  $5.15 \times$   
92  $10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $5.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $5.775 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ , and  $5.73$   
93  $\times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  were used to determine the concentration of  
94 dTAR, TMR-5'-cTAR, Rh6G-5'-cTAR-3'-Dabcyl, and  
95 Alexa488-5'-cTAR-3'-Dabcyl, respectively.

### 96 Absorption and fluorescence spectroscopy

97 Absorption spectra were recorded with a Cary 400 or a Cary  
98 4000 spectrophotometer (Agilent). Fluorescence spectra and  
99 anisotropy measurements were recorded at 20°C with a  
100 Fluorolog or a Fluoromax 3 spectrofluorometer (Horiba  
101 Jobin-Yvon) equipped with a thermostated cell compartment.  
102 Excitation wavelengths were 520 nm for Rh6G-5'-cTAR-3'-  
103 Dabcyl, 550 nm for TMR-5'-cTAR, and 480 nm for  
104 Alexa488-5'-cTAR-3'-Dabcyl. The spectra were corrected  
105 for dilution, buffer fluorescence and wavelength-dependent  
106 response of the optics and detectors.

107 To screen calixarenes (Cal), we used an assay in which we  
108 tested their ability to inhibit the NC-induced destabilization of  
109 cTAR<sup>59</sup>. The percentage of inhibition (% inh) obtained for  
110 each calixarene concentration was calculated with equation  
111 (3):

$$112 \quad \%inh = \frac{I_{(cTAR+NC)} - I_{(cTAR+NC+Cal)}}{I_{(cTAR+NC)} - I_{(cTAR)}} \times 100$$

(3)

where  $I_{(cTAR)}$ ,  $I_{(cTAR+ NC)}$ , and  $I_{(cTAR+ NC+ Cal)}$  correspond to the fluorescence intensity of cTAR alone, cTAR in the presence of the NC(11-55)peptide, and cTAR in the presence of both NC(11-55) and calixarene, respectively. To determine the  $IC_{50}$  values, we plotted the percentage of inhibition against the calixarene concentration ( $C$ ) and fitted it with a modified version of the dose-response equation<sup>105</sup> :

$$\% inh = A_1 + \frac{(A_2 - A_1)}{1 + 10^{(\log(IC_{50}) - \log(C)) \times p}}$$

(4)

where,  $A_1$  and  $A_2$  represent the percentage of inhibition in the absence and with saturating concentrations of calixarene, respectively.  $IC_{50}$  represents the half maximal inhibitory concentration, and  $p$  denotes the Hill coefficient.

Annealing kinetics were measured in pseudo-first order conditions by adding a 10-fold excess of unlabelled dTAR to the doubly labelled complementary sequence Alexa488-5'-cTAR-3'-Dabcyl. Excitation and emission wavelengths were set at 480 nm and 519 nm, respectively to monitor the Alexa 488 fluorescence. NC(11-55) was added at a 8:1 molar ratio to each reactant separately, and then the reaction was initiated by mixing the peptide-coated ODNs together. Calixarenes (C933, C934, CIP200 and CIP201) were added at different concentrations to monitor their ability to perturb the NC-promoted cTAR/dTAR annealing rate constants  $k_{obs}$ . All fitting procedures were carried out with Origin 8.6 software using nonlinear least-squares methods and the Levenberg-Marquardt algorithm.

## Mass spectrometry in non-denaturing conditions

Electrospray ionization mass spectrometry (ESI-MS) analysis was performed in the positive ion mode on an electrospray time-of-flight mass spectrometer (LCT, Waters, Manchester, UK) equipped with an automated nanoESI source (TriversaNanomate, Advion, Ithaca, NY). Calibration of the instrument was performed using a 2  $\mu$ M horse heart myoglobin solution prepared in a mixture of water:acetonitrile (1:1) acidified with 1% of formic acid. MS data were acquired and analysed using the MassLynx 4.1 software (Waters, Manchester, UK). For mass spectrometry experiments in non-denaturing conditions, NC(11-55) was prepared in water with 2.2 molar equivalents of  $ZnSO_4$  and the concentration was measured using an extinction coefficient of 5700  $M^{-1} cm^{-1}$  at 280 nm. Before use, the protein was buffered with 50 mM ammonium acetate ( $AcONH_4$ ) at pH 7.0. NC(11-55) protein (20  $\mu$ M) was incubated with 200  $\mu$ M of calixarenes (10 fold excess, 1% of DMSO v/v in the final volume) at 20 °C for 15 min, before recording the mass spectra. In order to balance the weak ionization due to the presence of DMSO, the analyses under non-denaturing conditions were carried out with an accelerating voltage (Vc) of 100 V, and the pressure in the first pumping stage (backing pressure) of the instrument was fixed to 6 mbar.

## Isothermal Titration Calorimetry (ITC)

The binding of NC, NC(1-55)S3 and NC(11-55) to calixarenes was investigated by Isothermal Titration Calorimetry (ITC), using a Nano ITC microcalorimeter (TA

Instruments) at 20°C, in Tris-HCl (25 mM, pH 7.5) containing 0.2 mM  $MgCl_2$  and 10, 30, 50 or 100 mM NaCl. The titration experiments were performed by incremental injections (each 200 or 300s) of 2.5  $\mu$ L aliquots of calixarene solutions (100, 150 or 200  $\mu$ M) in 300  $\mu$ L of a 8  $\mu$ M peptide solution contained in the reaction cell. The heat flow ( $\mu cal \times s^{-1}$ ) resulting from the interaction between the two partners was continuously recorded. The quantity of heat  $Q$  accompanying each injection was integrated over time and corrected for the heat of dilution measured in an independent experiment in which the calixarene solution was injected into the buffer alone. Instrument control, data acquisition, and analysis were done with the NanoAnalyze software provided by the manufacturer. Assuming 1:1 binding stoichiometry, the molar heat of binding  $\Delta H^0$  and the binding dissociation constant  $K_d$  were obtained by fitting the differential heat  $dQ/dX_{tot}$ <sup>106</sup> as a function of the total calixarene concentration ( $[Cal]_{tot}$ ) to equation (5):

$$\frac{1}{V_0 \left( \frac{dQ}{dX_{tot}} \right)} = \Delta H^0 \left( \frac{1}{2} + \frac{1 - \frac{(1+r)}{2} - X_r/2}{(X_r^2 - 2X_r(1-r) + (1+r)^2)^{1/2}} \right)$$

(5)

with  $1/r = [Cal]_{tot}/K_d$ ,  $X_r = X_{tot}/[Cal]_{tot}$  and  $X_{tot}$  is the peptide concentration in the reaction cell of volume  $V_0$ .

## Molecular Dynamics (MD) simulations

The ionization state of calixarenes was investigated by QUACPAC from OpenEye, version 1.7.0.2 (<https://www.eyesopen.com/quacpac>). Partial charges were then assigned at the am1-bcc level by *antechamber* from Amber12<sup>107,108</sup>. The full length NC from the NMR structure of its complex with a small molecule (PDB: 2M3Z)<sup>98</sup> was used as starting point in MD simulations after removal of the bound ligand. MD simulations were performed with Amber12. The ff12SB force field was used together with custom force field parameters for Zn-binding residues and coordinated Zn ions, as described previously<sup>17</sup>. A time step of 2 fs was used. C-H bonds were restrained by the SHAKE algorithm. For each system studied in this work, one molecule of NC and one molecule of calixarene were placed in a rectangular box of TIP3P water molecules at a random distance higher than 40 Å from each other. Neutrality of the system was achieved by adding the proper number of  $Na^+$  counter-ions. Each solvated system was then submitted to a two-step energy minimization. In the first step, water molecules were minimized around the frozen solute (NC + calixarene), while in the second step the solvated solute was energy minimized until convergence (rmsd = 0.05). Each system was then heated up to 300 K during 55 ps in the NVT ensemble and then equilibrated in density for 110 ps at constant pressure. After these steps, unrestrained MD trajectories were produced for 0.5  $\mu$ s in the NPT ensemble. Four replicas of MD trajectories of 0.5  $\mu$ s each were run to enhance statistical significance. Analysis of MD trajectories was performed with *cpptraj*<sup>109</sup>. Electrostatic surface potential was calculated by the APBS program<sup>110</sup>.

## Cell-based antiviral assay with replication-defective HIV pseudoparticles

1 This assay (described in supplementary information) is based  
2 on the infection of HeLa cells by replication-defective HIV  
3 particles pseudotyped with the Vesicular Stomatitis Virus  
4 glycoprotein<sup>11</sup> that bypass entry and mimic the early steps of  
5 the HIV virus infection cycle (post-entry to integration).

6 For each tested calixarene, the percentage of inhibition (%inh)  
7 was calculated with :

$$\%inh = \frac{(\text{Controlluciferasevalue}) - (\text{Sampleluciferasevalue})}{(\text{Controlluciferasevalue})} \times 100$$

(6)

12 Each concentration of compound was tested in sextuplicate.  
13 The reverse transcriptase inhibitor AZT (1  $\mu$ M) was used as a  
14 positive control. To determine the  $IC_{50}$  values, we plotted the  
15 percentage of inhibition against the calixarene concentration  
16 and fitted it with the dose-response equation (4).

17 In parallel to the infectivity test, the cytotoxicity of the  
18 molecules was quantified in a MTT assay (3-(4,5-  
19 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)<sup>12</sup>.  
20 Another plate of HeLa cells was thus prepared and treated in  
21 the same conditions as for the infectivity assay. 24 h after  
22 infection, the medium was replaced by a mix containing 100  
23  $\mu$ L of DMEM and 10  $\mu$ L of 12 mM MTT solution in PBS and  
24 cells were incubated during 4 h. In order to dissolve the  
25 insoluble purple formazan reduced by living cells, 85  $\mu$ L of  
26 the mix was replaced by 50  $\mu$ L of DMSO and gently shaken  
27 for 10 min. The absorbance was then measured at 540 nm in  
28 a spectrophotometer (Safas, Monaco) and expressed in  
29 percentage of cytotoxicity, using the DMEM + DMSO  
30 solution as the control.

### 31 Cell-based antiviral assay with replication 32 competent HIV-1 strains

33 The antiviral activity of calixarene CIP201 was evaluated by  
34 measuring its  $IC_{50}$  values against the HIV-1 wild-type  
35 reference strain NL4-3 and a panel of viruses with resistance  
36 mutations to approved HIV-1 drugs (supplementary data,  
37 Table S1) in a TZM-bl cell line based phenotypic assay<sup>13</sup>.  
38 TZM-bl cells are characterized by luciferase and  $\beta$ -  
39 galactosidase reporter genes integrated in the cell genome  
40 under the control of an HIV-1 LTR promoter. The expression  
41 of reporter genes is regulated by the viral Tat protein, which  
42 is produced following transcription of the integrated provirus.  
43 Since the NC viral protein is thought to be involved in both  
44 early and late events of HIV-1 life cycle, we adopted a re-  
45 infection assay, named BiCycle Assay, consisting in a first  
46 cycle of replication in the T-cell derived MT-2 cell line  
47 followed by an additional round of replication in TZM-bl  
48 cells. MT-2 cells were seeded at a concentration of 50,000  
49 cells/well in a 96-well plate and infected with the viruses at  
50 multiplicity of infection (M.O.I.) 0.1 in the presence of five-  
51 fold dilutions of the compounds. After 48-72 h, 50  $\mu$ L of  
52 supernatant from each well, containing the virus produced in  
53 the first round of infection, were used to infect TZM-bl cells  
54 seeded in a 96-plate well at a concentration of 30,000  
55 cells/well. Two days later, cells were lysed by adding 40  $\mu$ L  
56 of Glo Lysis Buffer (Promega) in each well for 5 min, then 40  
57  $\mu$ L of Bright-Glo Luciferase Reagent (Promega) were added  
58 to each well for relative luminescence units (RLU) counting  
59 using a Glo-Max Multi Detection System (Promega). RLU

60 values from each well were used to calculate the  $IC_{50}$  value of  
61 each compound using the GraphPad v6.0 software. The  
62 inhibition of the early events of viral replication was evaluated  
63 through a single cycle replication assay in TZM-bl cells,  
64 named MonoCycle Assay. The method consists in the  
65 infection of TZM-bl cells at M.O.I. 0.1 in presence of serial  
66 five-fold dilutions of the drugs, similarly to the first round of  
67 infection of BiCycle Assay. After 48 hours, RLU were  
68 counted and  $IC_{50}$  values were calculated using the GraphPad  
69 software. The reference drug-resistant viruses were obtained  
70 through the NIH AIDS Reagent Program (ARP,  
71 www.aidsreagent.org) and have been already characterized by  
72 the Phenosense Assay (Monogram Biosciences), considered  
73 as the reference assay for phenotypic investigation of HIV  
74 drug resistance due to its consistent application in clinical  
75 trials. The infectious clones were firstly transfected in 293LX  
76 cells and then expanded in MT-2 cells.

### 77 Impact of CIP201 in the production of viral 78 nucleic acid intermediates

79 The mechanism of action of calixarene CIP201 was evaluated  
80 through the quantification of viral nucleic acid intermediates  
81 produced during HIV-1 replication in cell culture. MT-2 cells  
82 were infected with NL4-3 wild-type strain at M.O.I 0.1 in  
83 presence of 100  $\mu$ M of CIP201, or the reference integrase  
84 inhibitor raltegravir, the protease inhibitor darunavir or the  
85 reverse transcriptase inhibitor rilpivirine, all at 2  $\mu$ M. The  
86 amount of viral cDNA produced after reverse transcription  
87 was quantified through a real time PCR targeting the LTR  
88 region at 16 h post infection, while 2-LTR circles and  
89 intracellular viral polyadenylated RNA transcripts were  
90 quantified with a specific real time protocol at 30 h post  
91 infection. Each of the three HIV-1 nucleic acids species was  
92 measured in two separate experiments and the values obtained  
93 with CIP201 were compared pairwise with the values  
94 obtained with each of the reference drugs considered, by  
95 Student t test.

### 96 SUPPLEMENTARY DATA

97 Calixarene synthesis and characterization, CIP200 and  
98 CIP201 do not eject zinc from NC(11-55), Binding of  
99 calixarenes to NC(11-55), Absence of interaction of CIP201  
100 with cTAR Molecular dynamics, Impairment of reverse  
101 transcription by CIP201, Effect of CIP201 on cell infection  
102 with the pseudotyped viruses, Evaluation of CIP201 in NSG  
103 humanized mice infected with HIV-1, Antiviral assay with  
104 replication-defective HIV pseudoparticles, Description of  
105 HIV-1 resistant strains.

### 106 FUNDING

107 This work was supported by the European Project THINPAD  
108 “Targeting the HIV-1 Nucleocapsid Protein to fight  
109 Antiretroviral Drug Resistance”, FP7 Grant Agreement  
110 601969, by the Agence Nationale de la Recherche (ANR) and  
111 the French Proteomic Infrastructure (ProFI, ANR-10-INBS-  
112 08-03) and by a PhD fellowship from Institut de Recherche  
113 Servier T. Botzanowski. YM is grateful to the Institut  
114 Universitaire de France (IUF) for support and providing  
115 additional time to be dedicated to research.

### 116 DEDICATION

1 Authors wish to dedicate this work to their friends and  
2 collaborators Prof. Maurizio Botta and Prof. Olga  
3 Zaporozhets, who passed away prematurely in 2019.

#### 4 CONFLICT OF INTERESTS

5 None declared

6

#### 7 REFERENCES

8

- 9 (1) Tang, M. W.; Shafer, R. W. HIV-1  
10 Antiretroviral Resistance: Scientific  
11 Principles and Clinical Applications.  
12 *Drugs* **2012**, *72* (9), e1–e25.  
13 [https://doi.org/10.2165/11633630-](https://doi.org/10.2165/11633630-000000000-00000)  
14 [000000000-00000](https://doi.org/10.2165/11633630-000000000-00000).
- 15 (2) Rein, A.; Henderson, L. E.; Levin, J. G.  
16 Nucleic-Acid-Chaperone Activity of  
17 Retroviral Nucleocapsid Proteins:  
18 Significance for Viral Replication.  
19 *Trends Biochem. Sci.* **1998**, *23* (8), 297–  
20 301.
- 21 (3) Levin, J. G.; Guo, J.; Rouzina, Ioulia;  
22 Musier-Forsyth, K. Nucleic Acid  
23 Chaperone Activity of HIV-1  
24 Nucleocapsid Protein: Critical Role in  
25 Reverse Transcription and Molecular  
26 Mechanism. In *Progress in Nucleic Acid*  
27 *Research and Molecular Biology*;  
28 Academic Press, 2005; Vol. 80, pp 217–  
29 286. [https://doi.org/10.1016/S0079-](https://doi.org/10.1016/S0079-6603(05)80006-6)  
30 [6603\(05\)80006-6](https://doi.org/10.1016/S0079-6603(05)80006-6).
- 31 (4) Levin, J. G.; Mitra, M.; Mascarenhas,  
32 A.; Musier-Forsyth, K. Role of HIV-1  
33 Nucleocapsid Protein in HIV-1 Reverse  
34 Transcription. *RNA Biol.* **2010**, *7* (6),  
35 754–774.  
36 <https://doi.org/10.4161/rna.7.6.14115>.
- 37 (5) Darlix, J.-L.; de Rocquigny, H.;  
38 Mauffret, O.; Mély, Y. Retrospective on  
39 the All-in-One Retroviral Nucleocapsid  
40 Protein. *Virus Res.* **2014**, *193*, 2–15.  
41 [https://doi.org/10.1016/j.virusres.2014.0](https://doi.org/10.1016/j.virusres.2014.05.011)  
42 [5.011](https://doi.org/10.1016/j.virusres.2014.05.011).
- 43 (6) De Rocquigny, H.; Gabus, C.; Vincent,  
44 A.; Fournié-Zaluski, M. C.; Roques, B.;  
45 Darlix, J. L. Viral RNA Annealing  
46 Activities of Human Immunodeficiency  
47 Virus Type 1 Nucleocapsid Protein  
48 Require Only Peptide Domains Outside  
49 the Zinc Fingers. *Proc. Natl. Acad. Sci.*  
50 *U. S. A.* **1992**, *89* (14), 6472–6476.  
51 (7) Bampi, C.; Jacquenet, S.; Lener, D.;  
52 Décimo, D.; Darlix, J.-L. The  
53 Chaperoning and Assistance Roles of  
54 the HIV-1 Nucleocapsid Protein in  
55 Proviral DNA Synthesis and  
56 Maintenance. *Int. J. Biochem. Cell Biol.*  
57 **2004**, *36* (9), 1668–1686.  
58 [https://doi.org/10.1016/j.biocel.2004.02.](https://doi.org/10.1016/j.biocel.2004.02.024)  
59 [024](https://doi.org/10.1016/j.biocel.2004.02.024).
- 60 (8) Mély, Y.; De Rocquigny, H.; Morellet,  
61 N.; Roques, B. P.; Gérard, D. Zinc  
62 Binding to the HIV-1 Nucleocapsid  
63 Protein: A Thermodynamic  
64 Investigation by Fluorescence  
65 Spectroscopy. *Biochemistry* **1996**, *35*  
66 (16), 5175–5182.  
67 <https://doi.org/10.1021/bi952587d>.
- 68 (9) Amarasinghe, G. K.; De Guzman, R. N.;  
69 Turner, R. B.; Chancellor, K. J.; Wu, Z.  
70 R.; Summers, M. F. NMR Structure of  
71 the HIV-1 Nucleocapsid Protein Bound  
72 to Stem-Loop SL2 of the Ψ-RNA  
73 Packaging Signal. Implications for  
74 Genome Recognition. *J. Mol. Biol.*  
75 **2000**, *301* (2), 491–511.  
76 <https://doi.org/10.1006/jmbi.2000.3979>.
- 77 (10) Beltz, H.; Clauss, C.; Piémont, E.;  
78 Ficheux, D.; Gorelick, R. J.; Roques, B.;  
79 Gabus, C.; Darlix, J.-L.; de Rocquigny,  
80 H.; Mély, Y. Structural Determinants of  
81 HIV-1 Nucleocapsid Protein for CTAR  
82 DNA Binding and Destabilization, and  
83 Correlation with Inhibition of Self-  
84 Primed DNA Synthesis. *J. Mol. Biol.*  
85 **2005**, *348* (5), 1113–1126.  
86 [https://doi.org/10.1016/j.jmb.2005.02.04](https://doi.org/10.1016/j.jmb.2005.02.042)  
87 [2](https://doi.org/10.1016/j.jmb.2005.02.042).
- 88 (11) Bourbigot, S.; Ramalanjaona, N.;  
89 Boudier, C.; Salgado, G. F. J.; Roques,  
90 B. P.; Mély, Y.; Bouaziz, S.; Morellet,  
91 N. How the HIV-1 Nucleocapsid Protein  
92 Binds and Destabilises the (–)Primer  
93 Binding Site During Reverse  
94 Transcription. *J. Mol. Biol.* **2008**, *383*  
95 (5), 1112–1128.  
96 [https://doi.org/10.1016/j.jmb.2008.08.04](https://doi.org/10.1016/j.jmb.2008.08.046)  
97 [6](https://doi.org/10.1016/j.jmb.2008.08.046).

- 1 (12) De Guzman, R. N.; Wu, Z. R.; Stalling,  
2 C. C.; Pappalardo, L.; Borer, P. N.;  
3 Summers, M. F. Structure of the HIV-1  
4 Nucleocapsid Protein Bound to the SL3  
5  $\Psi$ -RNA Recognition Element. *Science*  
6 **1998**, 279 (5349), 384–388.  
7 <https://doi.org/10.1126/science.279.534>  
8 9.384.
- 9 (13) Morellet, N.; Déméné, H.; Teilleux, V.;  
10 Huynh-Dinh, T.; de Rocquigny, H.;  
11 Fournié-Zaluski, M.-C.; Roques, B. P.  
12 Structure of the Complex between the  
13 HIV-1 Nucleocapsid Protein NCp7 and  
14 the Single-Stranded Pentanucleotide  
15 d(ACGCC)1. *J. Mol. Biol.* **1998**, 283  
16 (2), 419–434.  
17 <https://doi.org/10.1006/jmbi.1998.2098>.
- 18 (14) Spriggs, S.; Garyu, L.; Connor, R.;  
19 Summers, M. F. Potential Intra- and  
20 Intermolecular Interactions Involving  
21 the Unique-5' Region of the HIV-1 5'-  
22 UTR. *Biochemistry* **2008**, 47 (49),  
23 13064–13073.  
24 <https://doi.org/10.1021/bi8014373>.
- 25 (15) Lam, W. C.; Maki, A. H.; Casas-Finet,  
26 J. R.; Erickson, J. W.; Kane, B. P.;  
27 Sowder, R. C.; Henderson, L. E.  
28 Phosphorescence and Optically  
29 Detected Magnetic Resonance  
30 Investigation of the Binding of the  
31 Nucleocapsid Protein of the Human  
32 Immunodeficiency Virus Type 1 and  
33 Related Peptides to RNA. *Biochemistry*  
34 **1994**, 33 (35), 10693–10700.
- 35 (16) Mély, Y.; Piémont, E.; Sorinas-Jimeno,  
36 M.; de Rocquigny, H.; Jullian, N.;  
37 Morellet, N.; Roques, B. P.; Gérard, D.  
38 Structural and Dynamic  
39 Characterization of the Aromatic Amino  
40 Acids of the Human Immunodeficiency  
41 Virus Type I Nucleocapsid Protein Zinc  
42 Fingers and Their Involvement in  
43 Heterologous tRNA(Phe) Binding: A  
44 Steady-State and Time-Resolved  
45 Fluorescence Study. *Biophys. J.* **1993**,  
46 65 (4), 1513–1522.  
47 <https://doi.org/10.1016/S0006->  
48 3495(93)81222-0.
- 49 (17) Mori, M.; Dietrich, U.; Manetti, F.;  
50 Botta, M. Molecular Dynamics and DFT  
51 Study on HIV-1 Nucleocapsid Protein-7  
52 in Complex with Viral Genome. *J.*  
53 *Chem. Inf. Model.* **2010**, 50 (4), 638–  
54 650. <https://doi.org/10.1021/ci100070m>.
- 55 (18) Dorfman, T.; Luban, J.; Goff, S. P.;  
56 Haseltine, W. A.; Göttinger, H. G.  
57 Mapping of Functionally Important  
58 Residues of a Cysteine-Histidine Box in  
59 the Human Immunodeficiency Virus  
60 Type 1 Nucleocapsid Protein. *J. Virol.*  
61 **1993**, 67 (10), 6159–6169.
- 62 (19) Mori, M.; Kovalenko, L.; Lonnais, S.;  
63 Antaki, D.; Torbett, B. E.; Botta, M.;  
64 Mirambeau, G.; Mély, Y. Nucleocapsid  
65 Protein: A Desirable Target for Future  
66 Therapies Against HIV-1. In *The Future*  
67 *of HIV-1 Therapeutics*; Torbett, B. E.,  
68 Goodsell, D. S., Richman, D. D., Eds.;  
69 Current Topics in Microbiology and  
70 Immunology; Springer International  
71 Publishing, 2015; pp 53–92.  
72 [https://doi.org/10.1007/82\\_2015\\_433](https://doi.org/10.1007/82_2015_433).
- 73 (20) Sancineto, L.; Iraci, N.; Tabarrini, O.;  
74 Santi, C. NCp7: Targeting a  
75 Multitasking Protein for next-  
76 Generation Anti-HIV Drug  
77 Development Part 1: Covalent  
78 Inhibitors. *Drug Discov. Today* **2018**, 23  
79 (2), 260–271.  
80 <https://doi.org/10.1016/j.drudis.2017.10>.  
81 017.
- 82 (21) Iraci, N.; Tabarrini, O.; Santi, C.;  
83 Sancineto, L. NCp7: Targeting a  
84 Multitask Protein for next-Generation  
85 Anti-HIV Drug Development Part 2.  
86 Noncovalent Inhibitors and Nucleic  
87 Acid Binders. *Drug Discov. Today*  
88 **2018**, 23 (3), 687–695.  
89 <https://doi.org/10.1016/j.drudis.2018.01>.  
90 022.
- 91 (22) Gutsche, C. D. (1998) *Calixarenes*  
92 *Revisited, Monographs in*  
93 *Supramolecular Chemistry*.
- 94 (23) Böhmer, V. Calixarenes, Macrocycles  
95 with (Almost) Unlimited Possibilities.  
96 *Angew. Chem. Int. Ed. Engl.* **1995**, 34

- 1 (7), 713–745. 49  
2 <https://doi.org/10.1002/anie.199507131>. 50  
3 (24) Neri, P.; Sessler, J. L.; Wang, M.-X. 51 (33) D'acuarica, I.; Ghirga, F.; Quaglio, D.;  
4 *2016 Calixarenes and Beyond:* 52 Cerreto, A.; Ingallina, C.; Tafi, A.;  
5 *Switzerland.* 53 Botta, B. Molecular Recognition of  
6 (25) de Fatima, A.; Fernandes, S.; Sabino, A. 54 Natural Products by Resorc[4]Arene  
7 Calixarenes as New Platforms for Drug 55 Receptors. *Curr. Pharm. Des.* **2016**, *22*,  
8 Design. *Curr. Drug Discov. Technol.* 56 1715–1729.  
9 **2009**, *6* (2), 151–170. 57 (34) Giuliani, M.; Morbioli, I.; Sansone, F.;  
10 <https://doi.org/10.2174/1570163097884> 58 Casnati, A. Moulding Calixarenes for  
11 88302. 59 Biomacromolecule Targeting. *Chem.*  
12 (26) Biali, S. E. *In Calixarenes and Beyond;* 60 *Commun.* **2015**, *51* (75), 14140–14159.  
13 *Springer International Publishing:* 61 <https://doi.org/10.1039/C5CC05204A>.  
14 *Switzerland, 2016, Pp 75-93.* 62 (35) Yousaf, A.; Hamid, S. A.; Bunnori, N.  
15 (27) Guo, D.-S.; Liu, Y. Supramolecular 63 M.; Ishola, A. Applications of  
16 Chemistry of P-Sulfonatocalix[n]Arenes 64 Calixarenes in Cancer Chemotherapy:  
17 and Its Biological Applications. *Acc.* 65 Facts and Perspectives. *Drug Des.*  
18 *Chem. Res.* **2014**, *47* (7), 1925–1934. 66 *Devel. Ther.* **2015**, *9*, 2831–2838.  
19 <https://doi.org/10.1021/ar500009g>. 67 <https://doi.org/10.2147/DDDT.S83213>.  
20 (28) D'acuarica, I.; Ghirga, F.; Ingallina, 68 (36) Nimse, S. B.; Kim, T. Biological  
21 C.; Quaglio, D.; Zappia, G.; Uccello- 69 Applications of Functionalized  
22 Barretta, G.; Balzano, F.; Botta, B. *In* 70 Calixarenes. *Chem. Soc. Rev.* **2013**, *42*  
23 *Calixarenes and Beyond; Springer* 71 (1), 366–386.  
24 *International Publishing: Switzerland,* 72 <https://doi.org/10.1039/c2cs35233h>.  
25 *2016, Pp 175-193.* 73 (37) Hwang, K., M., Qi, Y. M., Liu, S., Y.,  
26 (29) Mutihac, L.; Lee, J. H.; Kim, J. S.; 74 Choy, W., Chen, J. (1994) Inhibition  
27 Vicens, J. Recognition of Amino Acids 75 and Treatment of Infection by  
28 by Functionalized Calixarenes. *Chem.* 76 Enveloped Virus with Calix(n)Arene  
29 *Soc. Rev.* **2011**, *40* (5), 2777–2796. 77 Compounds, In PCT Int. Appl. (Appl.,  
30 <https://doi.org/10.1039/C0CS00005A>. 78 P. I., Ed.), p 162, Genelabs  
31 (30) Perret, F.; Coleman, A. W. 79 Technologies, Inc, USA.  
32 Biochemistry of Anionic 80 (38) Harris, S. J. (1995) Preparation of  
33 Calix[n]Arenes. *Chem. Commun.* **2011**, 81 Calixarene-Based Compounds Having  
34 *47* (26), 7303–7319. 82 Antibacterial, Antifungal, Anticancer,  
35 <https://doi.org/10.1039/C1CC11541C>. 83 and Anti-HIV Activity, In PCT Int.  
36 (31) Mo, J.; Eggers, P. K.; Yuan, Z.; Raston, 84 Appl. (Appl., P. I., Ed.), p 148, Ire.  
37 C. L.; Lim, L. Y. Paclitaxel-Loaded 85 (39) Harris, S. J. (2002) Preparation of  
38 Phosphonated Calixarene Nanovesicles 86 Calixarenes as Anti-Viral Compounds,  
39 as a Modular Drug Delivery Platform. 87 In PCT Int. Appl. (Appl., P. I., Ed.), p  
40 *Sci. Rep.* **2016**, *6*, 23489. 88 44, Aids Care Pharma Limited, Ire.  
41 <https://doi.org/10.1038/srep23489>. 89 (40) Coveney, D., and Costello, B. (2005)  
42 (32) Rodik, R. V.; Klymchenko, A. S.; Jain, 90 Preparation of Alkylated Pyrogallol  
43 N.; Miroshnichenko, S. I.; Richert, L.; 91 Calixarene Type Compounds as Anti-  
44 Kalchenko, V. I.; Mély, Y. Virus-Sized 92 Viral Compounds, In U.S. Pat. Appl.  
45 DNA Nanoparticles for Gene Delivery 93 Publ. (Publ., U. S. P. A., Ed.), p 13,  
46 Based on Micelles of Cationic 94 Aids Care Pharma Limited, Ire.  
47 Calixarenes. *Chem. – Eur. J.* **2011**, *17* 95 (41) Srivastava, P.; Schito, M.; Fattah, R. J.;  
48 (20), 5526–5538. 96 Hara, T.; Hartman, T.; Buckheit Jr., R.  
97 W.; Turpin, J. A.; Inman, J. K.; Appella,

- 1 E. Optimization of Unique, Uncharged 50  
2 Thioesters as Inhibitors of HIV 51  
3 Replication. *Bioorg. Med. Chem.* **2004**, 52  
4 *12* (24), 6437–6450. 53  
5 <https://doi.org/10.1016/j.bmc.2004.09.0> 54 (48)  
6 32. 55  
7 (42) Mourer, M.; Psychogios, N.; Laumond, 56  
8 G.; Aubertin, A.-M.; Regnouf-de-Vains, 57  
9 J.-B. Synthesis and Anti-HIV 58  
10 Evaluation of Water-Soluble 59  
11 Calixarene-Based Bithiazolyl Podands. 60  
12 *Bioorg. Med. Chem.* **2010**, *18* (1), 36– 61  
13 45. 62  
14 <https://doi.org/10.1016/j.bmc.2009.11.0> 63  
15 16. 64  
16 (43) Tsou, L. K.; Dutschman, G. E.; Gullen, 65  
17 E. A.; Telpoukhovskaia, M.; Cheng, Y.- 66  
18 C.; Hamilton, A. D. Discovery of a 67  
19 Synthetic Dual Inhibitor of HIV and 68 (49)  
20 HCV Infection Based on a Tetrabutoxy- 69  
21 Calix[4]Arene Scaffold. *Bioorg. Med.* 70  
22 *Chem. Lett.* **2010**, *20* (7), 2137–2139. 71  
23 <https://doi.org/10.1016/j.bmcl.2010.02.0> 72  
24 43. 73  
25 (44) Luo, Z.-G.; Zhao, Y.; Ma, C.; Xu, X.- 74  
26 M.; Zhang, X.-M.; Huang, N.-Y.; He, 75 (50)  
27 H.-Q. Synthesis and Anti-Integrase 76  
28 Evaluation of Novel Calix[4]Arene 77  
29 Derivatives Containing the Triazolyl 78  
30 1,3-Diketo Moiety. *Chin. Chem. Lett.* 79  
31 **2014**, *25* (5), 737–740. 80  
32 <https://doi.org/10.1016/j.ccllet.2014.03.0> 81  
33 12. 82  
34 (45) Rodik, R. V.; Boyko, V. I.; Kalchenko, 83 (51)  
35 V. I. Calixarenes in Biotechnology and 84  
36 Bio-Medical Researches. In *Frontiers in* 85  
37 *Medicinal Chemistry*; Allen B. Reitz / 86  
38 Atta-ur-Rahman / M. Iqbal Choudhary, 87  
39 2016; Vol. 8, pp 206–301. 88  
40 (46) Luo, Z.; Zhao, Y.; Ma, C.; Li, Z.; Xu, 89  
41 X.; Hu, L.; Huang, N.; He, H. Synthesis, 90  
42 Biological Evaluation and Molecular 91  
43 Docking of Calix[4]Arene-Based B- 92  
44 Diketo Derivatives as HIV-1 Integrase 93  
45 Inhibitors. *Arch. Pharm. (Weinheim)* 94 (52)  
46 **2015**, *348* (3), 206–213. 95  
47 <https://doi.org/10.1002/ardp.201400390>. 96  
48 (47) Luo, Z.; Xu, X.; Zhang, X.; Hu, L. 97  
49 Development of Calixarenes, 98  
Cyclodextrins and Fullerenes as New  
Platforms for Anti-HIV Drug Design:  
An Overview. *Mini Rev. Med. Chem.*  
**2013**, *13* (8), 1160–1165.

- 1 Phosphatase Inhibitors. *Org. Biomol. Chem.* **2004**, 2 (21), 3162–3166.  
2 <https://doi.org/10.1039/B409526J>.  
3  
4 (53) Lugovskoy, E. V.; Gritsenko, P. G.;  
5 Koshel, T. A.; Koliesnik, I. O.;  
6 Cherenok, S. O.; Kalchenko, O. I.;  
7 Kalchenko, V. I.; Komisarenko, S. V.  
8 Calix[4]Arene Methylenebisphosphonic  
9 Acids as Inhibitors of Fibrin  
10 Polymerization. *FEBS J.* **2011**, 278 (8),  
11 1244–1251.  
12 [https://doi.org/10.1111/j.1742-](https://doi.org/10.1111/j.1742-4658.2011.08045.x)  
13 [4658.2011.08045.x](https://doi.org/10.1111/j.1742-4658.2011.08045.x).  
14 (54) Poon, D. T.; Wu, J.; Aldovini, A.  
15 Charged Amino Acid Residues of  
16 Human Immunodeficiency Virus Type 1  
17 Nucleocapsid P7 Protein Involved in  
18 RNA Packaging and Infectivity. *J.*  
19 *Virol.* **1996**, 70 (10), 6607–6616.  
20 (55) Wu, H.; Mitra, M.; Naufer, M. N.;  
21 McCauley, M. J.; Gorelick, R. J.;  
22 Rouzina, I.; Musier-Forsyth, K.;  
23 Williams, M. C. Differential  
24 Contribution of Basic Residues to HIV-  
25 1 Nucleocapsid Protein’s Nucleic Acid  
26 Chaperone Function and Retroviral  
27 Replication. *Nucleic Acids Res.* **2014**, 42  
28 (4), 2525–2537.  
29 <https://doi.org/10.1093/nar/gkt1227>.  
30 (56) Shvadchak, V.; Sanglier, S.; Rocle, S.;  
31 Villa, P.; Haiech, J.; Hibert, M.; Van  
32 Dorselaer, A.; Mély, Y.; de Rocquigny,  
33 H. Identification by High Throughput  
34 Screening of Small Compounds  
35 Inhibiting the Nucleic Acid  
36 Destabilization Activity of the HIV-1  
37 Nucleocapsid Protein. *Biochimie* **2009**,  
38 91 (7), 916–923.  
39 [https://doi.org/10.1016/j.biochi.2009.04.](https://doi.org/10.1016/j.biochi.2009.04.014)  
40 [014](https://doi.org/10.1016/j.biochi.2009.04.014).  
41 (57) Darlix, J.-L.; Godet, J.; Ivanyi-Nagy, R.;  
42 Fossé, P.; Mauffret, O.; Mély, Y.  
43 Flexible Nature and Specific Functions  
44 of the HIV-1 Nucleocapsid Protein. *J.*  
45 *Mol. Biol.* **2011**, 410 (4), 565–581.  
46 [https://doi.org/10.1016/j.jmb.2011.03.03](https://doi.org/10.1016/j.jmb.2011.03.037)  
47 [7](https://doi.org/10.1016/j.jmb.2011.03.037).  
48 (58) Stoylov, S. P.; Vuilleumier, C.;  
49 Stoylova, E.; De Rocquigny, H.;  
50 Roques, B. P.; Gérard, D.; Mély, Y.  
51 Ordered Aggregation of Ribonucleic  
52 Acids by the Human Immunodeficiency  
53 Virus Type 1 Nucleocapsid Protein.  
54 *Biopolymers* **1997**, 41 (3), 301–312.  
55 [https://doi.org/10.1002/\(SICI\)1097-](https://doi.org/10.1002/(SICI)1097-0282(199703)41:3<301::AID-BIP5>3.0.CO;2-W)  
56 [0282\(199703\)41:3<301::AID-](https://doi.org/10.1002/(SICI)1097-0282(199703)41:3<301::AID-BIP5>3.0.CO;2-W)  
57 [BIP5>3.0.CO;2-W](https://doi.org/10.1002/(SICI)1097-0282(199703)41:3<301::AID-BIP5>3.0.CO;2-W).  
58 (59) Bernacchi, S.; Stoylov, S.; Piémont, E.;  
59 Ficheux, D.; Roques, B. P.; Darlix, J. L.;  
60 Mély, Y. HIV-1 Nucleocapsid Protein  
61 Activates Transient Melting of Least  
62 Stable Parts of the Secondary Structure  
63 of TAR and Its Complementary  
64 Sequence1. *J. Mol. Biol.* **2002**, 317 (3),  
65 385–399.  
66 <https://doi.org/10.1006/jmbi.2002.5429>.  
67 (60) McGovern, R. E.; Fernandes, H.; Khan,  
68 A. R.; Power, N. P.; Crowley, P. B.  
69 Protein Camouflage in Cytochrome c–  
70 Calixarene Complexes. *Nat. Chem.*  
71 **2012**, 4 (7), 527–533.  
72 <https://doi.org/10.1038/nchem.1342>.  
73 (61) Godet, J.; de Rocquigny, H.; Raja, C.;  
74 Glasser, N.; Ficheux, D.; Darlix, J.-L.;  
75 Mély, Y. During the Early Phase of  
76 HIV-1 DNA Synthesis, Nucleocapsid  
77 Protein Directs Hybridization of the  
78 TAR Complementary Sequences via the  
79 Ends of Their Double-Stranded Stem. *J.*  
80 *Mol. Biol.* **2006**, 356 (5), 1180–1192.  
81 [https://doi.org/10.1016/j.jmb.2005.12.03](https://doi.org/10.1016/j.jmb.2005.12.038)  
82 [8](https://doi.org/10.1016/j.jmb.2005.12.038).  
83 (62) Vo, M.-N.; Barany, G.; Rouzina, I.;  
84 Musier-Forsyth, K. HIV-1 Nucleocapsid  
85 Protein Switches the Pathway of  
86 Transactivation Response Element  
87 RNA/DNA Annealing from Loop–Loop  
88 “Kissing” to “Zipper.” *J. Mol. Biol.*  
89 **2009**, 386 (3), 789–801.  
90 [https://doi.org/10.1016/j.jmb.2008.12.07](https://doi.org/10.1016/j.jmb.2008.12.070)  
91 [0](https://doi.org/10.1016/j.jmb.2008.12.070).  
92 (63) Avilov, S. V.; Boudier, C.; Gottikh, M.;  
93 Darlix, J.-L.; Mély, Y. Characterization  
94 of the Inhibition Mechanism of HIV-1  
95 Nucleocapsid Protein Chaperone  
96 Activities by Methylated  
97 Oligoribonucleotides. *Antimicrob.*  
98 *Agents Chemother.* **2012**, 56 (2), 1010–

- 1 1018.  
2 <https://doi.org/10.1128/AAC.05614-11>.  
3 (64) Vercruyse, T.; Basta, B.; Dehaen, W.;  
4 Humbert, N.; Balzarini, J.; Debaene, F.;  
5 Sanglier-Cianféroni, S.; Pannecouque,  
6 C.; Mély, Y.; Daelemans, D. A Phenyl-  
7 Thiadiazolylidene-Amine Derivative  
8 Ejects Zinc from Retroviral  
9 Nucleocapsid Zinc Fingers and  
10 Inactivates HIV Virions. *Retrovirology*  
11 **2012**, *9*, 95.  
12 <https://doi.org/10.1186/1742-4690-9-95>.  
13 (65) Mori, M.; Schult-Dietrich, P.;  
14 Szafarowicz, B.; Humbert, N.; Debaene,  
15 F.; Sanglier-Cianferani, S.; Dietrich, U.;  
16 Mély, Y.; Botta, M. Use of Virtual  
17 Screening for Discovering Antiretroviral  
18 Compounds Interacting with the HIV-1  
19 Nucleocapsid Protein. *Virus Res.* **2012**,  
20 *169* (2), 377–387.  
21 <https://doi.org/10.1016/j.virusres.2012.0>  
22 [5.011](https://doi.org/10.1016/j.virusres.2012.0).  
23 (66) Mori, M.; Nucci, A.; Lang, M. C. D.;  
24 Humbert, N.; Boudier, C.; Debaene, F.;  
25 Sanglier-Cianferani, S.; Catala, M.;  
26 Schult-Dietrich, P.; Dietrich, U.; et al.  
27 Functional and Structural  
28 Characterization of 2-Amino-4-  
29 Phenylthiazole Inhibitors of the HIV-1  
30 Nucleocapsid Protein with Antiviral  
31 Activity. *ACS Chem. Biol.* **2014**, *9* (9),  
32 1950–1955.  
33 <https://doi.org/10.1021/cb500316h>.  
34 (67) Record, M. T.; Lohman, T. M.; Haseth,  
35 P. de. Ion Effects on Ligand-Nucleic  
36 Acid Interactions. *J. Mol. Biol.* **1976**,  
37 *107* (2), 145–158.  
38 <https://doi.org/10.1016/S0022->  
39 [2836\(76\)80023-X](https://doi.org/10.1016/S0022-).  
40 (68) Mély, Y.; Rocquigny, H. de; Sorinas-  
41 Jimeno, M.; Keith, G.; Roques, B. P.;  
42 Marquet, R.; Gérard, D. Binding of the  
43 HIV-1 Nucleocapsid Protein to the  
44 Primer tRNA, In Vitro, Is Essentially  
45 Not Specific. *J. Biol. Chem.* **1995**, *270*  
46 (4), 1650–1656.  
47 <https://doi.org/10.1074/jbc.270.4.1650>.  
48 (69) Vuilleumier, C.; Bombarda, E.;  
49 Morellet, N.; Gérard, D.; Roques, B. P.;
- 50 Mély, Y. Nucleic Acid Sequence  
51 Discrimination by the HIV-1  
52 Nucleocapsid Protein NCp7: A  
53 Fluorescence Study. *Biochemistry* **1999**,  
54 *38* (51), 16816–16825.  
55 <https://doi.org/10.1021/bi991145p>.  
56 (70) Zielenkiewicz, W.; Marcinowicz, A.;  
57 Poznański, J.; Cherenok, S.; Kalchenko,  
58 V. Calorimetric, NMR, and UV  
59 Investigations of Aliphatic l-Amino  
60 Acids Complexation by Calix[4]Arene  
61 Bis-Hydroxymethylphosphous Acid. *J.*  
62 *Incl. Phenom. Macrocycl. Chem.* **2006**,  
63 *55* (1–2), 11–19.  
64 <https://doi.org/10.1007/s10847-005->  
65 [9012-y](https://doi.org/10.1007/s10847-005-).  
66 (71) Kalchenko, O.; Cherenok, S.;  
67 Yushchenko, O.; Kalchenko, V.  
68 Complexation of  
69 Calix[4]Arenehydroxymethylphosphoni  
70 c Acids with Amino Acids. Binding  
71 Constants Determination of the  
72 Complexes by HPLC Method. *J. Incl.*  
73 *Phenom. Macrocycl. Chem.* **2013**, *76*  
74 (1–2), 29–36.  
75 <https://doi.org/10.1007/s10847-012->  
76 [0169-x](https://doi.org/10.1007/s10847-012-).  
77 (72) Talotta, C.; Gaeta, C.; Neri, P. Endo-  
78 Complexation of Alkylammonium Ions  
79 by Calix[4]Arene Cavity: Facilitating  
80 Cation- $\pi$  Interactions through the  
81 Weakly Coordinating Anion Approach.  
82 *J. Org. Chem.* **2014**, *79* (20), 9842–  
83 9846.  
84 <https://doi.org/10.1021/jo5016689>.  
85 (73) Daze, K. D.; Hof, F. The Cation- $\pi$   
86 Interaction at Protein-Protein  
87 Interaction Interfaces: Developing and  
88 Learning from Synthetic Mimics of  
89 Proteins That Bind Methylated Lysines.  
90 *Acc. Chem. Res.* **2013**, *46* (4), 937–945.  
91 <https://doi.org/10.1021/ar300072g>.  
92 (74) Mori, M.; Manetti, F.; Botta, M.  
93 Predicting the Binding Mode of Known  
94 NCp7 Inhibitors To Facilitate the  
95 Design of Novel Modulators. *J. Chem.*  
96 *Inf. Model.* **2011**, *51* (2), 446–454.  
97 <https://doi.org/10.1021/ci100393m>.

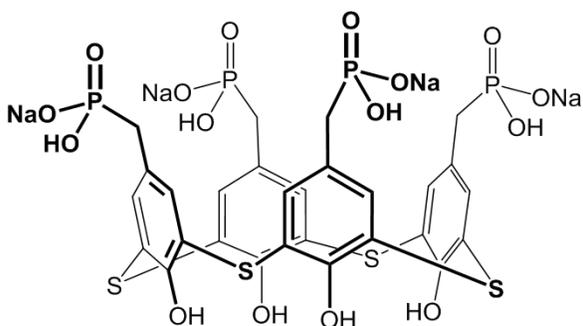
- 1 (75) Doyle, M. L.; Ladbury, J. E. 50 Provides Biochemical Profiling of  
2 Biocalorimetry 2 : applications of 51 Resistance Mutations in HIV-1 Gag and  
3 calorimetry in the biological sciences - 52 Protease. *Biochemistry* **2011**, *50* (20),  
4 Colby College Libraries 53 4371–4381.  
5 <http://link.colby.edu/portal/Biocalorimet>  
6 [ry-2--applications-of-calorimetry-in/q-](http://link.colby.edu/portal/Biocalorimet)  
7 [0LC2F9Ito/](http://link.colby.edu/portal/Biocalorimet) (accessed May 13, 2019). 54 <https://doi.org/10.1021/bi200031m>.  
8 (76) Sharma, K. K.; Przybilla, F.; Restle, T.; 55 (82) Cimarelli, A.; Sandin, S.; Höglund, S.;  
9 Boudier, C.; Godet, J.; Mély, Y. Reverse 56 Luban, J. Basic Residues in Human  
10 Transcriptase in Action: FRET-Based 57 Immunodeficiency Virus Type 1  
11 Assay for Monitoring Flipping and 58 Nucleocapsid Promote Virion Assembly  
12 Polymerase Activity in Real Time. *Anal.* 59 via Interaction with RNA. *J. Virol.*  
13 *Chem.* **2015**, *87* (15), 7690–7697. 60 **2000**, *74* (7), 3046–3057.  
14 <https://doi.org/10.1021/acs.analchem.5b>  
15 01126. 61 <https://doi.org/10.1128/JVI.74.7.3046->  
16 (77) Althaus, I. W.; Chou, J. J.; Gonzales, A. 62 3057.2000.  
17 J.; Deibel, M. R.; Chou, K. C.; Kezdy, 63 (83) Dussupt, V.; Sette, P.; Bello, N. F.;  
18 F. J.; Romero, D. L.; Aristoff, P. A.; 64 Javid, M. P.; Nagashima, K.; Bouamr,  
19 Tarpley, W. G.; Reusser, F. Steady-State 65 F. Basic Residues in the Nucleocapsid  
20 Kinetic Studies with the Non- 66 Domain of Gag Are Critical for Late  
21 Nucleoside HIV-1 Reverse 67 Events of HIV-1 Budding. *J. Virol.*  
22 Transcriptase Inhibitor U-87201E. *J.* 68 **2011**, *85* (5), 2304–2315.  
23 *Biol. Chem.* **1993**, *268* (9), 6119–6124. 69 <https://doi.org/10.1128/JVI.01562-10>.  
24 (78) Althaus, I. W.; Chou, J. J.; Gonzales, A. 70 (84) Rice, W. G.; Schaeffer, C. A.; Harten,  
25 J.; Deibel, M. R.; Chou, K. C.; Kezdy, 71 B.; Villinger, F.; South, T. L.; Summers,  
26 F. J.; Romero, D. L.; Palmer, J. R.; 72 M. F.; Henderson, L. E.; Bess, J. W.;  
27 Thomas, R. C. Kinetic Studies with the 73 Arthur, L. O.; McDougal, J. S.; et al.  
28 Non-Nucleoside HIV-1 Reverse 74 Inhibition of HIV-1 Infectivity by Zinc-  
29 Transcriptase Inhibitor U-88204E. 75 Ejecting Aromatic C-Nitroso  
30 *Biochemistry* **1993**, *32* (26), 6548–6554. 76 Compounds. *Nature* **1993**, *361* (6411),  
31 <https://doi.org/10.1021/bi00077a008>. 77 473–475.  
32 (79) Amand, M.; Iserentant, G.; Poli, A.; 78 <https://doi.org/10.1038/361473a0>.  
33 Sleiman, M.; Fievez, V.; Sanchez, I. P.; 79 (85) Rice, W. G.; Supko, J. G.; Malspeis, L.;  
34 Sauvageot, N.; Michel, T.; Aouali, N.; 80 Buckheit, R. W.; Clanton, D.; Bu, M.;  
35 Janji, B.; et al. Human 81 Graham, L.; Schaeffer, C. A.; Turpin, J.  
36 CD56dimCD16dim Cells As an 82 A.; Domagala, J.; et al. Inhibitors of  
37 Individualized Natural Killer Cell 83 HIV Nucleocapsid Protein Zinc Fingers  
38 Subset. *Front. Immunol.* **2017**, *8*. 84 as Candidates for the Treatment of  
39 <https://doi.org/10.3389/fimmu.2017.006>  
40 99. 85 AIDS. *Science* **1995**, *270* (5239), 1194–  
41 (80) De Rocquigny, H.; Shvadchak, V.; 86 1197.  
42 Avilov, S.; Dong, C. Z.; Dietrich, U.; 87 <https://doi.org/10.1126/science.270.523>  
43 Darlix, J.-L.; Mély, Y. Targeting the 88 9.1194.  
44 Viral Nucleocapsid Protein in Anti- 89 (86) Witvrouw, M.; Balzarini, J.;  
45 HIV-1 Therapy. *Mini Rev. Med. Chem.* 90 Pannecouque, C.; Jhaumeer-Laulloo, S.;  
46 **2008**, *8*, 24–35. 91 Esté, J. A.; Schols, D.; Cherepanov, P.;  
47 (81) Breuer, S.; Sepulveda, H.; Chen, Y.; 92 Schmit, J. C.; Debyser, Z.; Vandamme,  
48 Trotter, J.; Torbett, B. E. A Cleavage 93 A. M.; et al. SRR-SB3, a Disulfide-  
49 Enzyme-Cytometric Bead Array 94 Containing Macrolide That Inhibits a  
95 Late Stage of the Replicative Cycle of  
96 Human Immunodeficiency Virus.  
97 *Antimicrob. Agents Chemother.* **1997**,  
98 *41* (2), 262–268.

- 1 (87) Turpin, J. A.; Song, Y.; Inman, J. K.; 50  
2 Huang, M.; Wallqvist, A.; Maynard, A.; 51  
3 Covell, D. G.; Rice, W. G.; Appella, E. 52  
4 Synthesis and Biological Properties of 53  
5 Novel Pyridinioalkanoyl Thioesters 54  
6 (PATE) as Anti-HIV-1 Agents That 55  
7 Target the Viral Nucleocapsid Protein 56  
8 Zinc Fingers. *J. Med. Chem.* **1999**, *42* 57  
9 (1), 67–86. 58  
10 <https://doi.org/10.1021/jm9802517>. 59
- 11 (88) Bombarda, E.; Morellet, N.; Cherradi, 60  
12 H.; Spiess, B.; Bouaziz, S.; Grell, E.; 61  
13 Roques, B. P.; Mély, Y. Determination 62  
14 of the PKa of the Four Zn<sup>2+</sup>- 63  
15 Coordinating Residues of the Distal 64  
16 Finger Motif of the HIV-1 Nucleocapsid 65  
17 Protein: Consequences on the Binding 66  
18 of Zn<sup>2+</sup>. *J. Mol. Biol.* **2001**, *310* (3), 67  
19 659–672. 68  
20 <https://doi.org/10.1006/jmbi.2001.4770>. 69
- 21 (89) Huang, M.; Maynard, A.; Turpin, J. A.; 70  
22 Graham, L.; Janini, G. M.; Covell, D. 71  
23 G.; Rice, W. G. Anti-HIV Agents That 72  
24 Selectively Target Retroviral 73  
25 Nucleocapsid Protein Zinc Fingers 74  
26 without Affecting Cellular Zinc Finger 75  
27 Proteins. *J. Med. Chem.* **1998**, *41* (9), 76  
28 1371–1381. 77  
29 <https://doi.org/10.1021/jm9708543>. 78
- 30 (90) Jenkins, L. M. M.; Hara, T.; Durell, S. 79  
31 R.; Hayashi, R.; Inman, J. K.; Piquemal, 80  
32 J.-P.; Gresh, N.; Appella, E. Specificity 81  
33 of Acyl Transfer from 2- 82  
34 Mercaptobenzamide Thioesters to the 83  
35 HIV-1 Nucleocapsid Protein. *J. Am.* 84  
36 *Chem. Soc.* **2007**, *129* (36), 11067– 85  
37 11078. 86  
38 <https://doi.org/10.1021/ja071254o>. 87
- 39 (91) Jenkins, L. M. M.; Ott, D. E.; Hayashi, 88  
40 R.; Coren, L. V.; Wang, D.; Xu, Q.; 89  
41 Schito, M. L.; Inman, J. K.; Appella, D. 90  
42 H.; Appella, E. Small-Molecule 91  
43 Inactivation of HIV-1 NCp7 by 92  
44 Repetitive Intracellular Acyl Transfer. 93  
45 *Nat. Chem. Biol.* **2010**, *6* (12), 887–889. 94  
46 <https://doi.org/10.1038/nchembio.456>. 95
- 47 (92) Rice, W. G.; Baker, D. C.; Schaeffer, C. 96  
48 A.; Graham, L.; Bu, M.; Terpening, S.; 97  
49 Clanton, D.; Schultz, R.; Bader, J. P.; 98  
Buckheit, R. W.; et al. Inhibition of  
Multiple Phases of Human  
Immunodeficiency Virus Type 1  
Replication by a Dithiane Compound  
That Attacks the Conserved Zinc  
Fingers of Retroviral Nucleocapsid  
Proteins. *Antimicrob. Agents  
Chemother.* **1997**, *41* (2), 419–426.
- (93) Rice, W. G.; Turpin, J. A.; Huang, M.;  
Clanton, D.; Buckheit, R. W.; Covell, D.  
G.; Wallqvist, A.; McDonnell, N. B.;  
DeGuzman, R. N.; Summers, M. F.; et  
al. Azodicarbonamide Inhibits HIV-1  
Replication by Targeting the  
Nucleocapsid Protein. *Nat. Med.* **1997**, *3*  
(3), 341–345.  
<https://doi.org/10.1038/nm0397-341>.
- (94) Pannecouque, C.; Szafarowicz, B.;  
Volkova, N.; Bakulev, V.; Dehaen, W.;  
Mély, Y.; Daelemans, D. Inhibition of  
HIV-1 Replication by a Bis-  
Thiadiazolbenzene-1,2-Diamine That  
Chelates Zinc Ions from Retroviral  
Nucleocapsid Zinc Fingers. *Antimicrob.  
Agents Chemother.* **2010**, *54* (4), 1461–  
1468.  
<https://doi.org/10.1128/AAC.01671-09>.
- (95) Goebel, F. D.; Hemmer, R.; Schmitt, J.-  
C.; Bogner, J. R.; de Clercq, E.;  
Witvrouw, M.; Pannecouque, C.;  
Valeyev, R.; Vandeveld, M.; Margery,  
H.; et al. Phase I/II Dose Escalation and  
Randomized Withdrawal Study with  
Add-on Azodicarbonamide in Patients  
Failing on Current Antiretroviral  
Therapy. *AIDS* **2001**, *15*, 33–45.
- (96) Breuer, S.; Chang, M. W.; Yuan, J.;  
Torbett, B. E. Identification of HIV-1  
Inhibitors Targeting the Nucleocapsid  
Protein. *J. Med. Chem.* **2012**, *55* (11),  
4968–4977.  
<https://doi.org/10.1021/jm201442t>.
- (97) Kim, M.-J.; Kim, S. H.; Park, J. A.; Yu,  
K. L.; Jang, S. I.; Kim, B. S.; Lee, E. S.;  
You, J. C. Identification and  
Characterization of a New Type of  
Inhibitor against the Human  
Immunodeficiency Virus Type-1  
Nucleocapsid Protein. *Retrovirology*

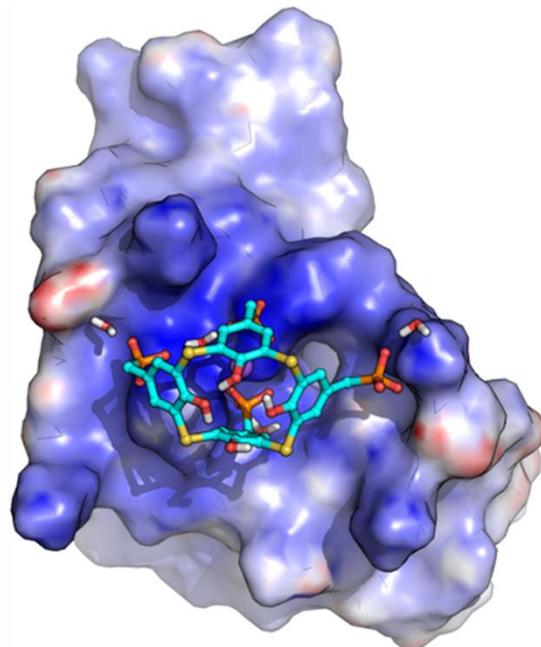


- 1 Trajectory Data. *J. Chem. Theory*  
2 *Comput.* **2013**, 9 (7), 3084–3095.  
3 <https://doi.org/10.1021/ct400341p>.  
4 (110) Dolinsky, T. J.; Nielsen, J. E.;  
5 McCammon, J. A.; Baker, N. A.  
6 PDB2PQR: An Automated Pipeline for  
7 the Setup of Poisson–Boltzmann  
8 Electrostatics Calculations. *Nucleic*  
9 *Acids Res.* **2004**, 32 (suppl\_2), W665–  
10 W667.  
11 <https://doi.org/10.1093/nar/gkh381>.  
12 (111) Dull, T.; Zufferey, R.; Kelly, M.;  
13 Mandel, R. J.; Nguyen, M.; Trono, D.;  
14 Naldini, L. A Third-Generation  
15 Lentivirus Vector with a Conditional  
16 Packaging System. *J. Virol.* **1998**, 72  
17 (11), 8463–8471.
- 18 (112) Mosmann, T. Rapid Colorimetric Assay  
19 for Cellular Growth and Survival:  
20 Application to Proliferation and  
21 Cytotoxicity Assays. *J. Immunol.*  
22 *Methods* **1983**, 65 (1), 55–63.  
23 [https://doi.org/10.1016/0022-](https://doi.org/10.1016/0022-1759(83)90303-4)  
24 [1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4).  
25 (113) Saladini, F.; Giannini, A.; Boccuto, A.;  
26 Vicenti, I.; Zazzi, M. Agreement  
27 between an In-House Replication  
28 Competent and a Reference Replication  
29 Defective Recombinant Virus Assay for  
30 Measuring Phenotypic Resistance to  
31 HIV-1 Protease, Reverse Transcriptase,  
32 and Integrase Inhibitors. *J. Clin. Lab.*  
33 *Anal.* **2017**, n/a-n/a.  
34 <https://doi.org/10.1002/jcla.22206>.  
35

### Calixarene CIP201



### Binding model of CIP201 to the HIV-1 nucleocapsid protein NC



$K_d \approx 1 \mu\text{M}$   $\Rightarrow$  blocks NC binding to nucleic acids  $\Rightarrow$  antiviral activity ( $IC_{50} \approx 1 \mu\text{M}$ ) with low cytotoxicity ( $CC_{50} > 5 \text{ mM}$ ) on HIV-1 reference and resistant viral strains.

2

3