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Tetrahedron 71 (2015) 3020–3026

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Polyphenolic C-glucosidic ellagitannins present in oak-aged wine inhibit HIV-1 nucleocapsid protein

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ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form 31 October 2014
Accepted 16 January 2015
Available online 22 January 2015

Keywords:
Polyphenols
Ellagitannins
Acutissimins
NCp7
TAR

ABSTRACT

HIV-1 nucleocapsid protein (NC) is a nucleic acid chaperone implicated in several steps of the virus replication cycle and an attractive new target for drug development. In reverse transcription, NC destabilizes nucleic acid secondary structures and catalyzes the annealing of HIV-1 TAR RNA to its DNA copy (cTAR) to form the heteroduplex TAR/cTAR. A screening program led to the identification of the plant polyphenols acutissimins A and B as potent inhibitors of NC in different assays. These two flavano-ellagitannins, which are found in wine aged in oak barrels, exhibited different mechanisms of protein inhibition and higher potency relatively to their epimers, epiacutissimins A and B, and to simpler structures notably representing hydrolytic fragments and metabolites therefrom.

1. Introduction

The urgent need for new anti-HIV drugs is a global concern. Side effects and the emergence of drug resistance have limited the therapeutic usefulness of existing drugs, and new targets are actively explored. An emerging and promising target is represented by HIV-1 nucleocapsid protein NCp7 (NC), a nucleic acid-binding protein generated during virion budding by the proteolytic cleavage of the Gag precursor. NC is responsible for maturation of the dimeric RNA, and plays a role in essentially every step of the replication cycle, from reverse transcription and DNA integration to packaging and assembly. Furthermore, the strict conservation of the motifs responsible for NC activity, together with the fact that most of the known mutations render the virus non-infectious, raises the possibility that HIV-1 will be unable to generate mutants resistant to anti-NC drugs.[1,2]

NC is a relatively small (55 amino acid residues) and highly basic protein.[3] Upon interaction with RNA its flexible N-terminal domain forms a 3_{10}-helix,[4] while its core is constituted by two highly conserved zinc finger domains (i.e., the N-term. ZF1 and the C-term. ZF2). According to NMR analysis,[5] the fingers fold into very tight, rigid loops that form a hydrophobic pocket comprising the aromatic residues Trp37 and Phe16. The abundance of highly charged basic residues is responsible for the protein's broad nucleic acid-binding activity,[6] whereas its intrinsic conformational flexibility and the hydrophobic nature of ZF residues confer the ability to bind specific nucleic acid structures.[7,8] In particular, specific binding is driven by the formation of relatively strong stacking interactions between the aromatic residues in the second position of each ZF (Phe16 in ZF1 and Trp37 in ZF2) and exposed unpaired purines, especially guanosines.[9] In this way, the N-term helix can penetrate the major groove of DNA/RNA hairpin structures to establish non-specific electrostatic interactions with the phosphodiester backbone,[7] while the ZF domains engage in highly specific interactions with the exposed loops of these elements of secondary structure.[4] As a result of this dual binding mode, NC can catalyze the folding and re-folding of nucleic acids by lowering the energy barrier for dissociation and re-formation of base pairs, promoting the transient unpairing of bases within helical structures and making them available for re-annealing in alternative combinations.[10–12] Neither the destabilizing nor the aggregating NC activities rely on ATP hydrolysis.[12,13]

NC is required during strand transfer in HIV-1 reverse transcription: the protein catalyzes the melting and annealing of complementary regions of the viral genome during the complex

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http://dx.doi.org/10.1016/j.tet.2015.01.035

series of events that culminate in the synthesis of a linear double-stranded DNA copy of the viral RNA genome. Minus strand transfer involves the transactivation response element (TAR), an RNA structure contained in the repeat regions of the long terminal repeats (LTR) of the HIV genome, and cTAR, its complementary DNA sequence. Although thermodynamically favored, their annealing does not occur extensively in the absence of NC, since both TAR and cTAR are highly structured regions whose apical parts are defined by a stem-bulge-loop structure (Fig. 1). Their stable secondary structures hamper strand transfer and decrease the efficiency of DNA synthesis by the viral reverse transcriptase (RT).[3,14] In contrast, NC protein destabilizes the stem-loop structures and promotes the formation of the TAR/cTAR hybrid that is required for RT elongation and synthesis of the DNA copy of HIV genome.[15] Interestingly, Tat, another HIV viral protein targeting TAR, has been recently described as a nucleic acid annealer, and shown to promote TAR/cTAR annealing during reverse trasnscription.[16,17]

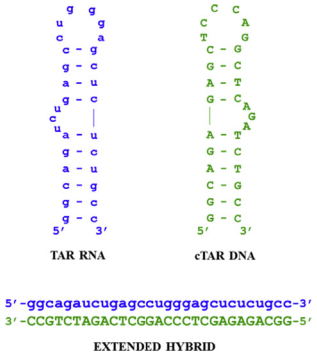

Fig. 1. Sequence and secondary structure of oligonucleotides replicating TAR RNA and cTAR DNA, which were employed in our assays. Their annealing can lead to an extended TAR/cTAR heteroduplex.

In light of these observations, the inhibition of NC's chaperone activity on TAR and cTAR could potentially decrease the efficiency of strand transfer and eventually prevent the completion of reverse transcription. Any agent capable of interfering with these functions would provide a new effective approach for inhibiting the replication cycle of HIV-1. Although no approved drug is currently available, different classes have been proposed as possible anti-NC therapeutics. Agents capable of inducing zinc ejection have been studied extensively, but clinical trials evidenced severe side effects.[2] Intercalators, such as actinomycin D, have manifested the ability to stabilize dynamic nucleic acid structures, which results in partial indirect inhibition of NC functions.[18] A series of direct antagonists of NC-binding activity were also identified, which contain a (poly)hydroxylated xanthenyl ring system.[19] In this case, the hydroxyl groups were shown to be essential for protein binding, possibly through the formation of relatively stable H-bonds with its core domain.

Searching for new inhibitors, we analyzed a library of over two hundred molecules belonging to different chemical classes.[20] The assay tested their ability to inhibit NC in vitro by evaluating the IC_{50} of stem melting inhibition (Fig. 2). The study identified two classes of compounds consisting of the intercalating anthraquinones developed in-house to enhance their nucleic acid-binding properties[20] and the *C*-glucosidic ellagitannins described in this report.

The inclusion of these plant polyphenols in our screening was prompted by the structural similarities with the (poly)hydroxylated xanthenyl compounds proposed as possible NC inhibitors.[19] The *C*-glucosidic ellagitannins in this study are hydrosoluble polyphenolic compounds occurring notably in fagaceous hardwoods such as *Quercus* (oak) and *Castanea* (chestnut) species. The most naturally abundant representatives of this class of ellagitannins are vescalagin (**1**) and its C1 epimer castalagin (**2**).[21–23] In these compounds,

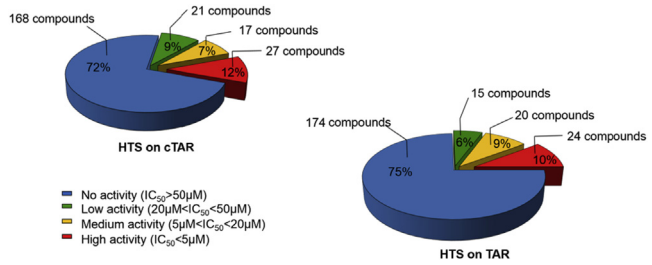

Fig. 2. Results of high throughput screening (HTS) experiments aimed at the identification of putative inhibitors of NC melting of TAR and cTAR.

the usual ellagitannin glucopyranose core is replaced by an open-chain glucose unit, which is rarely encountered in nature. This structure stems from the formation of a *C*-aryl glucosidic bond between the glucose C1 center and the galloyl-derived ring esterified at the glucose O2 position.[24,25] The C1-linked O2-galloyl unit is part of a terarylic nonahydroxyterphenoyl (NHTP) unit, which is additionally esterified at the glucose O3 and O5 positions. A biarylic hexahydroxydiphenoyl (HHDP) unit at the glucose O4 and O6 positions completes the structure of **1** and **2** (Fig. 3). These stereochemically well-defined biarylic and terarylic units are part of 10- and 11-membered rings that confer a relative rigidity and well-organized shape to the overall globular structure of these compounds, which make them particularly well-suited for interacting with protein targets. Both of these ellagitannins occur in wine as the result of the aging of this beverage in oak-made barrels. Vescalagin (**1**) does react with grape-derived wine flavan-3-ols such as (epi)catechin (**3**) to form the flavano-ellagitannins (epi)acutissimins A and B (**4** and **5**) (Fig. 3).[26,27] In this study, six wine ellagitannins (i.e., **1**, **2**, **4a**/**b**, and **5a**/**b**)[26–29] and their hydrolytic metabolites were evaluated for their capacity to inhibit NC activity. The study included also vescalin (**6**) and castalin (**7**), two minor *C*-glucosidic ellagitannins lacking the HHDP unit at the glucose O4 and O6 positions; catechin (**3a**); ellagic acid (**9**), the primary metabolite of the hydrolytic cleavage of the 4,6-HHDP unit of **1** and **2**;

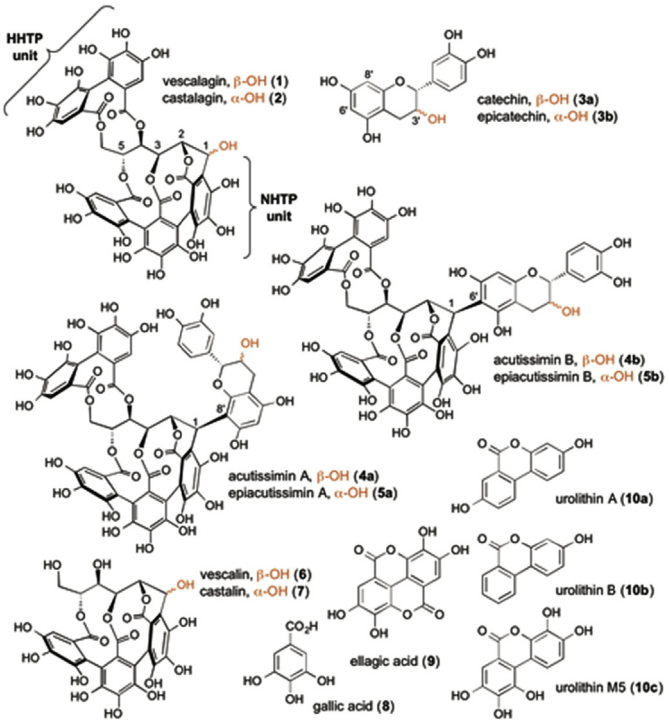

Fig. 3. Structures of the *C*-glucosidic ellagitannins and related (poly)phenolic compounds analyzed in the NC-inhibition screen.

known ellagic acid metabolites, such as the urolithins A, B and M5 (**10a**–**c**);[30–32] and gallic acid (**8**) (Fig. 3).

Two different assays employing recombinant full-length NC, TAR RNA, and its complementary cTAR sequence were performed to identify inhibitors of NC. The first assay, amenable to HTS format, relied on fluorescently labeled constructs to monitor the melting of their double-stranded stems. The second instead analyzed the complete chaperoning cycle involved in strand transfer (e.g., melting of stable stem-loop structures followed by annealing into an hybrid heteroduplex). Acutissimin A (**4a**) was found to be the most potent inhibitor of the series tested here. Lower activity was found for acutissimin B (**4b**), which was further reduced in both epiacutissimins (**5a** and **5b**), thus indicating a selectivity in the recognition of the protein target rather than non-specific effects. Binding of acutissimins A and B (**4a**, **4b**) and of epiacutissimin A (**5a**) to NC was further confirmed by mass spectrometry. All of the other compounds shown in Fig. 3 were less active or inactive, with a notable exception represented by ellagic acid (**9**), which however exhibited a slightly different mechanism. In comparison with other reported inhibitors[2,19,20,33–36] the acutissimins exhibit a remarkable anti-NC action in vitro, with a mechanism of action different from that of intercalators and zinc-ejectors.

2. Results and discussion

2.1. Inhibition of NC-mediated stem melting of TAR and cTAR

The helix destabilization (or stem melting) activity of NC has been exploited to set up a high throughput screening (HTS) to identify in vitro inhibitors of NC.[20,33] The assay is based on doubly labeled oligonucleotides that manifest an increase of fluorescence as a result of NC's helix destabilizing activity. As depicted in Fig. 4, protein binding can induce dissociation of the lower half of the stem, which increases the distance between fluorophore and quencher placed at the two ends of the construct.

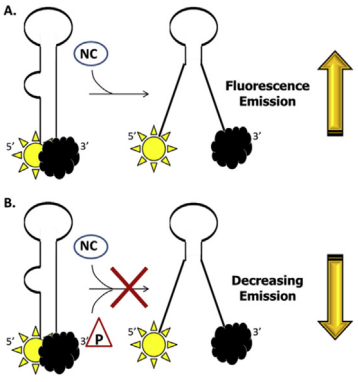

Fig. 4. Schematic representation of the NC-induced stem melting assay. The reporter construct was labeled at the 5′- and 3′-end with a fluorophore (yellow) and quencher (dark), respectively. (A). The destabilization of nucleic acid secondary structure (i.e., stem melting) was evaluated by measuring the fluorescence of the reporter, which was expected to increase with the distance between donor and acceptor. (B) Addition of inhibitor (P) prevented NC-induced melting, which resulted in dose-dependent decreases of the fluorescence signal over the control. The assay was readily adapted to an HTS format that enabled the rapid identification and ranking of possible hits.

After preliminary controls ensured the absence of direct quenching activity by the compounds under investigation, their ability to inhibit stem melting was evaluated in the presence of recombinant HIV-1 NC (Fig. 4B). The results in Table 1 report the half maximal inhibitory concentration (i.e., IC_{50}) of helix destabilization mediated by NC, respectively, on TAR (RNA) and cTAR (DNA). Acutissimin A (**4a**), a water-soluble flavano-ellagitannin in which the C8′ center of catechin is connected to the C1 center of

Table 1
Inhibition of NC-induced helix destabilization

	Helix destabilization IC_{50}[a] (μM)	
Compound	TAR	cTAR
Vescalagin (**1**)	8.42±0.43	1.50±0.57
Castalagin (**2**)	9.39±0.84	7.24±0.26
Catechin (**3a**)	>100	>100
Acutissimin A (**4a**)	1.46±0.02	1.18±0.18
Acutissimin B (**4b**)	0.81±0.13	0.95±0.06
Epiacutissimin A (**5a**)	0.44±0.03	1.18±0.13
Epiacutissimin B (**5b**)	3.82±0.40	0.54±0.01
Vescalin (**6**)	>100	>100
Castalin (**7**)	>100	>100
Gallic acid (**8**)	>100	>100
Ellagic acid (**9**)	66±1	38±1
Urolithin A (**10a**)	>100	>100
Urolithin B (**10b**)	>100	54 ±1
Urolithin M5 (**10c**)	43±2	44±2

[a] Data are averages±SEM of three independent experiments.

vescalagin (**1**, Fig. 3) was found to be among the most active compounds with equivalent potency against TAR and cTAR stem melting. Its α-epimer derived from epicatechin, epiacutissimin A (**5a**), manifested very potent helix destabilization and exhibited preferential inhibition of TAR melting. Acutissimin B (**4b**), in which the C6′ center of catechin is connected to the C1 center of vescalagin, was also a positive hit on both substrates, while epiacutissimin B (**5b**), the α-epimer of **4b** (Fig. 3), was active as cTAR melting inhibitor but much less potent on TAR (Table 1).

Control experiments were carried out to ensure that the activity of acutissimins, among the most potent inhibitors in the series, was not the result of undesirable NC precipitation. Considering that protein precipitation is one of the classic criteria for evaluating the interaction of polyphenols with cognate proteins,[37] assays were conducted after different incubation intervals, while examining the samples for possible signs of precipitation. The TAR reporter was monitored in three separate experiments conducted 5 min apart in the presence of increasing amounts of **4a**. The respective curves shown in Fig. S1 (see Supplementary data) were perfectly superimposable and did not display the typical signal scatter that is a hallmark of protein precipitation (personal observation). Additional controls were performed to verify whether the potent inhibition displayed by acutissimins was just a general trait shared with other related *C*-glucosidic ellagitannins, or a very distinctive feature of these specific molecules. When vescalagin (**1**) and castalagin (**2**) were submitted to the stem-melting assay, they both exhibited much lower activity on TAR melting, but only weak interference with cTAR (DNA) melting. Catechin (**3a**) was totally inactive. Vescalin (**6**) and castalin (**7**), which derive from hydrolysis of vescalagin and castalagin, respectively, were also totally inactive. Ellagic acid (**9**), which can be released by hydrolytic cleavage of the 4,6-HHDP unit of both vescalagin and castalagin,[26] exhibited inhibitory properties that were quite weak, albeit more pronounced when cTAR was the reporter. The study included also three urolithins known as gut metabolites of **9**, urolithin A (**10a**), urolithin B (**10b**), and the pentahydroxylated urolithin known as M5 (**10c**).[32] The urolithins **10b** and **10c** exhibited limited inhibition, comparable to that of **9**, whereas **10a** was totally inactive. Gallic acid (**8**) showed no activity (Table 1).

2.2. Acutissimins inhibit NC-mediated annealing of TAR and cTAR

These preliminary experiments clearly suggested that the simplification of the structure of acutissimins to its constitutive

components may lead to significant loss of activity, pointing to a possible mechanism of structure-specific inhibition. Among the two acutissimins and their epimers, a few significant differences could be noted in the potency of stem-melting inhibition, with epiacutissimin B (**5b**) being the least potent of the four related compounds on TAR melting. This observation prompted further evaluation of these inhibitors against other important aspects of NC's chaperone activity. In the case of TAR and cTAR, helix destabilization is followed by strand annealing and eventual formation of a stable TAR/cTAR heteroduplex.[12,38] Therefore, monitoring the formation of dimeric species in the presence of protein offers the opportunity to assess the inhibitory properties of the various hits in the context of the entire structure remodeling process mediated by NC.

For this reason, we implemented the Nucleocapsid Annealing Mediated Electrophoresis (NAME) assay to further characterize acutissimins activity. Based on the different electrophoretic mobility of individual nucleic acids structures, NAME does not require fluorescent labeling of oligonucleotide substrates, but rely instead on the direct electrophoretic analysis of folded TAR and cTAR constructs, readily differentiated from one another and from the double-stranded TAR/cTAR hybrid produced by NC catalysis.[20,39] In selected experiments, the acutissimins were preincubated for 15 min with full-length recombinant NC, mixed with unlabeled/prefolded TAR and cTAR, and finally incubated for an additional 15-min interval (NC-preincubation mode).

The results shown in Fig. 5 clearly demonstrated the ability of NC to mediate the complete formation of annealed TAR/cTAR heteroduplex in the absence of acutissimins (lanes 4 and 10). In contrast, inhibition of chaperone activity was evidenced by the dose-dependent decrease of annealed heteroduplex in the presence of **4a** and **4b** (lanes 5–9 and 11–15), in parallel with the detection of its individual cTAR and TAR component. The inhibitor **4a** displayed greater potency than the corresponding **4b** species. This subtle difference of activity could be possibly ascribed to the different positions of their catechin units, which are connected to the vescalagin core either through the C6′ (**4b**) or C8′ (**4a**) center.

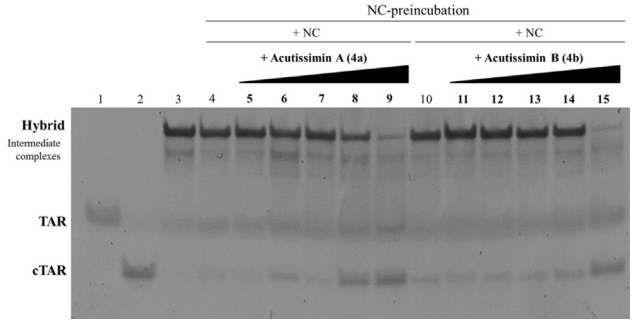

Fig. 5. Nucleocapsid Annealing Mediated Electrophoresis (NAME) assay completed in NC-preincubation mode (see Experimental section). Inhibition effects were evaluated in the presence of increasing amounts of acutissimin A (**4a**) or acutissimin B (**4b**). The full-length recombinant NC protein (8 μM) was preincubated with increasing concentrations (0, 1, 5, 10, 20, 50 μM final) of each acutissimin for 15 min at room temperature (lanes 4–9 and 10–15). TAR (1 μM) and cTAR (1 μM), folded separately and then mixed together, were incubated with the NC–acutissimin solutions for 15 min at room temperature. TAR (lane 1), cTAR (lane 2), and the hybrid TAR/cTAR (lane 3) were used as controls.

In other experiments, the acutissimins were preincubated with recombinant NC prior to mixing with prefolded constructs and final incubation before analysis (oligo-preincubation mode, see Fig. S2). Indeed, changing the mixing order while keeping overall incubation time constant was expected to reveal possible variations introduced by the different affinity toward the protein or nucleic acid structures. At the same time, the assays helped rank the relative potencies of a set of closely related compounds in slowing down the very efficient annealing activity of the protein. Under the selected conditions, it was possible to calculate the inhibitory concentration (IC_{50}) for the NC-mediated annealing of TAR to cTAR, reported in Table 2.

Table 2
NC annealing inhibition by NAME assay

Compound	Annealing activity IC_{50}[a] (μM)	
	NC-preincubation	Oligo-preincubation
Vescalagin (**1**)	>50	>50
Castalagin (**2**)	>50	47.4±0.3
Acutissimin A (**4a**)	26.4±0.8	21.5±0.3
Acutissimin B (**4b**)	34.6±0.7	33.1±0.5
Epiacutissimin A (**5a**)	>50	31.2±2.0
Epiacutissimin B (**5b**)	>50	>50
Ellagic acid (**9**)	40.8±2.1	>50
Urolithin B (**10b**)	>50	>50
Urolithin M5 (**10c**)	>50	>50

[a] Values are the mean±SEM of three independent experiments.

Examined in either mode, acutissimins A (**4a**) and to a lower extent acutissimin B (**4b**) exhibited similar inhibitory potency, whereas the other inhibitors proved less efficient in slowing NC-mediated annealing. The behavior exhibited by such compounds was consistent with the possible presence of distinctive molecular interactions within the NC–oligo complexes. The fact that epiacutissimin A (**5a**), the epimer of **4a**, was significantly influenced by oligo-preincubation (Table 2 and Fig. S3) suggests that extensive contact may take place between the nucleic acid substrates and this ellagitannin, perhaps through H-bonding and/or π-stacking. Interestingly, ellagic acid (**9**) proved to be relatively active in the NAME assays. However, unlike the other ellagitannins tested, its activity was higher upon preincubation with protein, thus indicating a different mode of interaction with the nucleic acids-protein complex (Table 2). In contrast, epiacutissimin B (**5b**) was not influenced by the incubation mode (Table 2). This compound displayed no activity within the range of concentrations surveyed by NAME (i.e., up to 50 μM), in spite of its identification by the fluorescence-based assay depicted in Fig. 4. Although we cannot exclude that **5b** may represent a false positive obtained by the fluorescence assay, we believe that its interaction with the NC–oligo complex may lead to only partial inhibition of chaperoning activity, in contrast with the observations afforded by the other acutissimins.

2.3. Acutissimins possess direct NC-binding activity

A careful comparison of the results provided by the different approaches revealed subtle discrepancies in their conclusions. Both NC-mediated melting and annealing assays consistently recognized the same acutissimin structures (i.e., **4a** and **4b**) as the most potent inhibitors in the study. In contrast, the assays showed some disagreement on the potency of the respective epimeric structures. Indeed, **5a** was found to be a good annealing inhibitor when preincubated with oligonucleotides, whereas its melting inhibition capabilities were markedly different when TAR or cTAR were considered. Similarly, the weak annealing inhibitor **5b** displayed very low activity against TAR melting, but could be easily ranked as the most potent compound for cTAR melting inhibition. Some of these discrepancies could be explained by the possibility that the fluorescence-based assay may overestimate the inhibitory activity, while the NAME approach may provide a more faithful

representation. At the same time, the differences observed when NAME experiments were completed, suggested that the mechanism of inhibition may involve direct binding of acutissimins to the NC protein.

This hypothesis was tested by using electrospray ionization mass spectrometry (ESI-MS) to evaluate the binding properties of the species under consideration. This technique has been previously shown capable of detecting intact zinc-bound NC, as well as its non-covalent complexes with various RNA substrates.[40–42] The possible formation of NC–acutissimin complexes was monitored by adding increasing amounts of ligand to a fixed concentration of protein (see Experimental section). A preliminary determination of the initial protein afforded an experimental mass of 6488.910 Da, which matches very closely the monoisotopic value of 6488.906 Da calculated from the sequence and including two bound Zn(II) ions (not shown).[40] Samples containing the different ligands afforded different results, as shown by the representative spectra in Fig. 6. In fact, a 1:1 complex between NC and acutissimin A was readily detected with an experimental mass of 7695.011 Da, which matches the monoisotopic value of 7695.040 Da calculated from the sequence and elemental composition of the ligand (Fig. 6a). The resolution achieved in these experiments allowed us to fully resolve the isotopic distribution of the detected species, revealing the charge state of individual ions and making data interpretation unambiguous.

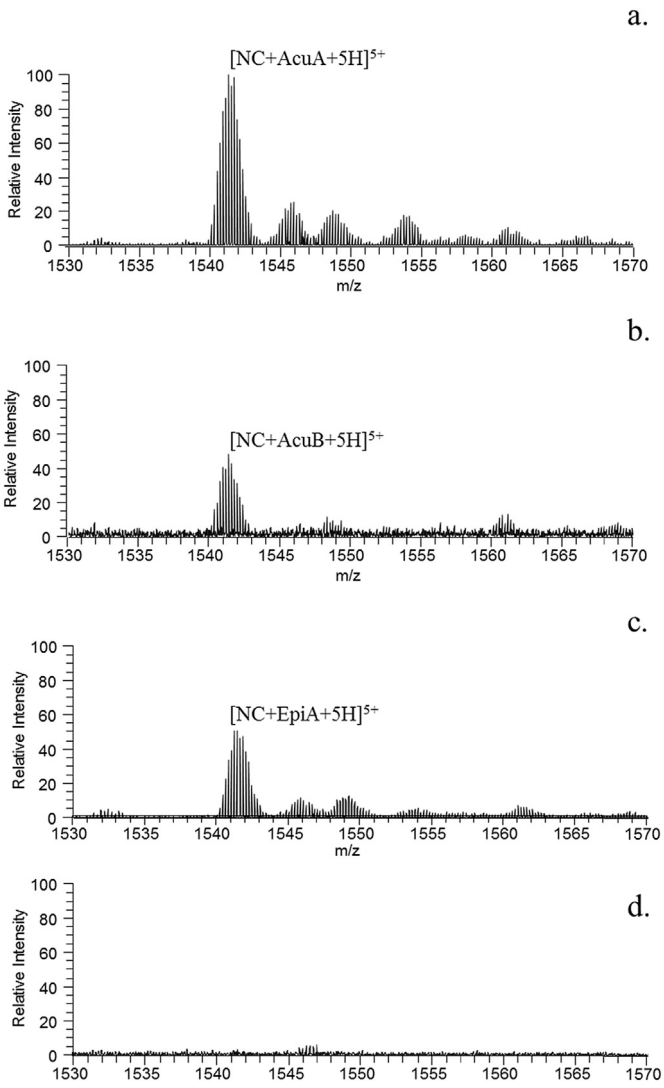

Fig. 6. Representative ESI-MS spectra of samples containing NC and (a) acutissimin A, (b) acutissimin B, (c) epiacutissimin A, and (d) epiacutissimin B (see Experimental section). For the sake of clarity, only the region containing the complex is shown. The weaker signals near the main peak correspond to typical sodium adducts.

In similar fashion, 1:1 complexes were detected also for acutissimin B and epiacutissimin A (Fig. 6b and c), respectively, whereas no complex was observed for epiacutissimin B (Fig. 6d). It is important to note that these epimeric compounds share the same elemental composition and, thus, were detected with identical masses. In all cases, the mass of the ellagitannin–NC complex matched those calculated by including two Zn(II) ions, thus ruling out zinc ejection as a possible mechanism of inhibition. The possibility that multiple ligand equivalents may bind to the protein at the same time was tested by increasing the amount of ellagitannin in solution. Under these conditions, however, no additional binding was observed. Furthermore, the abundance of the 1:1 complexes did not appear to increase as the concentration of ligand was risen up to 10:1 ligand to protein ratio (not shown). A direct comparison of the observed abundances suggested a putative **4a**>**4b**≅**5a** relative scale of binding affinities, which matched the ranking order of the annealing inhibition experiments. The fact that no binding activity was observed for epiacutissimin B (**5b**) contradicted the cTAR melting experiments and suggested that a strong DNA interaction may be the source of those results. Further experiments are in progress to answer to this question.

3. Conclusion

Our screening program has positively identified several ellagitannins as putative in vitro inhibitors of HIV-1 NC activity. Among this class of plant polyphenols present in wine aged in oak barrels, acutissimins have emerged as potent inhibitors of melting and annealing capabilities, as revealed by assays involving the TAR/cTAR system. In particular, acutissimin A (**4a**) was the most active compound identified by NAME assays, while its α-epimer epiacutissimin A (**5a**) was weaker, consistent with the lower binding affinity toward NC detected by ESI-MS experiments. The regioisomer acutissimin B (**4b**) was slightly less active than acutissimin A (**4a**), while its α-epimer epiacutissimin B (**5b**) was unable to bind NC and inactive in the annealing inhibition assay. Although strong evidence was obtained of direct interactions between **4a**, **4b**, **5a** and NC in its zinc-bound form, no binding was observed for **5b** under the selected experimental conditions. The fact that this compound induced melting inhibition when DNA oligonucleotides were used as substrates suggests that this polyphenol may establish direct interactions with the nucleic acids. Further studies are underway to identify the molecular determinants that confer to these compounds their excellent ability to interfere with NC chaperoning of TAR and cTAR.

In this direction, it is important to consider that the constituting units of the acutissimin structures and closely related compounds are simpler polyphenols, which are also present in wine, but were found to possess only limited or no activity whatsoever. The activity of vescalagin (**1**) and its α-epimer castalagin (**2**) was negligible in NAME assays, while vescalin (**6**), castalin (**7**), and catechin (**3a**) were found inactive against NC, thus providing further proof of the important role played by the higher structural complexity, including overall size and shape, in the recognition of the protein target. For this reason, it is not surprising that acutissimins have been shown to induce specific inhibition of other nucleic acid-binding proteins, such as the human topoisomerase IIα.[26,43]

Ellagic acid (**9**), the bislactone produced from the hydrolytic cleavage of ellagitannin HHDP units, was also identified among the hits and confirmed to inhibit NC-mediated annealing. In contrast with the other compounds, NAME assays showed that the inhibitory activity of this simpler but not water-soluble polyphenol was negatively affected by preincubation with nucleic acids. This

small and planar structure is perhaps better suited to interact directly with the hydrophobic cavity of NC, possibly by stacking with the aromatic side chains in the zinc fingers. Interestingly, the monolactonic urolithin metabolites of ellagic acid were also found to possess no activity by our tests.

The low concentrations of acutissimins in wine (0.30–0.40 mg/L)[28] clearly rule out any claim that wine might serve as an alternative remedy for HIV. Our study employed in vitro approaches that aimed at recognizing active hits amenable to further development in drug discovery programs. The outcome indicated that these natural structures constitute valuable scaffolds for NC protein inhibition, which are distinct in molecular complexity from other classes of molecules that have been proposed as possible anti-NC agents. Further studies are underway to investigate their activity in infected cells to fully evaluate their antiviral potential. We hope that this study will foster the interest in plant polyphenols as a source of inspiration for the discovery of new NC inhibitors.

4. Experimental section

4.1. Materials

The four acutissimins were hemisynthesized from (−)-vescalagin (**1**) and (+)-catechin (**3a**) or (−)epicatechin (**3b**), as previously described.[26,27] (−)-Vescalin (**6**) and (+)-castalin (**7**) were prepared by acidic hydrolysis of (−)-vescalagin (**1**) and (−)-castalagin (**2**), as previously described.[26] (+)-Catechin (**3a**), ellagic acid (**9**), and gallic acid (**8**) were purchased from Sigma–Aldrich, while urolithins were a kind gift of Prof. Zagotto (University of Padova).[44,45]

All oligonucleotides were synthesized by Metabion International AG (Martinsried, Germany) and stored at −20 °C in 10 mM Tris–HCl, pH 7.5. Dilutions were made in DEPC-treated water (Ambion). TAR is the 29-mer RNA sequence 5′-GGCAGAUCUGAGCCUGGGAGCUCUCUGCC-3′ and cTAR is its DNA complementary sequence 5′-GGCAGAGAGCTCCCAGGCTCAGATCTGCC-3′. When specified, TAR and cTAR were labeled at 5′ and 3′ ends, respectively, by the fluorophore 5-carboxyfluorescein (FAM) and the dark quencher 4-(4′-dimethylaminophenylazo)benzoic acid (Dabcyl).

The full-length recombinant NC protein was obtained as previously reported.[46] The protein concentration was determined on a UV–vis Spectrophotometer Lambda 20, PerkinElmer, using an extinction coefficient at 280 nm of 6410 M^{-1} cm^{-1}.

4.2. Inhibition of NC-induced stem melting (helix destabilization)

The stem melting assay (or high throughput screening—HTS) was performed to identify inhibitors of NC chaperone activity both on TAR and cTAR. We used a microplate reader VictorIII (PerkinElmer) with 485 and 535 nm as excitation and emission wavelengths. 5′-FAM and 3′-DAB modified TAR or cTAR (each 1 μM) were folded in TNMg (Tris–HCl 10 mM, NaCl 20 mM, $Mg(ClO_4)_2$ 1 mM pH 7.5): the oligonucleotides were denatured at 95 °C for 5 min and then left to cool to room temperature in order to assume their stem-bulge-loop structure. cTAR or TAR was then diluted to 0.1 μM in TN (Tris–HCl 10 mM, NaCl 20 mM pH 7.5). Increasing concentrations of compound (0, 0.1, 0.5, 1, 5, 10, 50, 100 μM final) were incubated with 0.1 μM cTAR or TAR in each well. Finally, NC 0.8 μM (molar ratio oligos/NC=1:8) was added to each sample. The plate was read three times with a delay of 1 min one reading from the other, unless differently specified. The experimental data were fitted as reported and the IC_{50} value was calculated for each compound.[20] Each experiment was performed in triplicate to calculate a standard deviation of the IC_{50} value.

4.3. Inhibition of TAR/cTAR annealing by polyphenols

Nucleocapsid Annealing Mediated Electrophoresis (NAME) assay was developed to investigate the ability of compounds to impair the biological activity of the full-length NC protein, monitoring the annealing of TAR with cTAR. TAR, cTAR, and the hybrid TAR/cTAR (each 1 μM) each folded in TNMg (Tris–HCl 10 mM, NaCl 20 mM, $Mg(ClO_4)_2$ 1 mM pH 7.5) were used as controls: the oligonucleotides were denatured at 95 °C for 5 min and then left to cool to room temperature in order to assume their stem-bulge-loop (TAR and cTAR) or double-stranded (hybrid TAR/cTAR) structure. To evaluate the inhibition of NC-mediated TAR/cTAR hybrid formation two different assay formats were exploited.

4.3.1. NC-preincubation. The full-length recombinant NC protein (8 μM) was preincubated with increasing concentrations (0, 1, 5, 10, 20, 50 μM compound final concentrations) of each polyphenol for 15 min at room temperature. TAR (1 μM) and cTAR (1 μM), folded separately as described above and then mixed together, were incubated with the NC–polyphenol solutions for 15 min at room temperature. Gel Loading Buffer containing SDS (GLB_{SDS}: Tris–HCl 100 mM, EDTA 4 mM, 50% w/v glycerol, 2% w/v SDS, 0.05% w/v bromophenol blue), was added to the samples, that were then kept on ice and finally resolved on a 12% native PAGE (acrylamide/bisacrylamide=19:1), run in TBE buffer (Tris 89 mM, boric acid 89 mM, and EDTA 2 mM, pH 8) for 3 h at 200 V.

4.3.2. Oligo-preincubation. TAR (1 μM) and cTAR (1 μM) were folded separately as described above and then individually preincubated with increasing concentrations of compound (each oligo with 0, 1, 5, 10, 20, 50 μM compound final concentrations) for 15 min at room temperature, then mixed together and added of NC solution (8 μM) and incubated for other 15 min at room temperature. Gel Loading Buffer containing SDS (GLB_{SDS}: Tris–HCl 100 mM, EDTA 4 mM, 50% w/v glycerol, 2% w/v SDS, 0.05% w/v bromophenol blue), was added to the samples, that were then kept on ice and finally resolved on a 12% native PAGE (acrylamide/bisacrylamide=19:1), run in TBE buffer (Tris 89 mM, boric acid 89 mM, and EDTA 2 mM, pH 8) for 3 h at 200 V.

After electrophoresis, the gels were stained with SybrGreen II and detected on a Geliance 600 Imaging System (PerkinElmer). In both cases, the IC_{50} (concentration of the compound required to inhibit the hybrid formation by half) was calculated by the quantification (using GeneTools software from PerkinElmer) of the percentage of the hybrid formation.

4.4. Acutissimins–NC binding analysis

Samples for the binding studies were prepared by mixing appropriate volumes of stock solution of NC (2 μM final) with each compound in 150 mM ammonium acetate (pH 7.5). The final mixtures contained up to a 10:1 compound/NC molar ratio. In order to ensure the binding equilibrium in solution before analysis, the samples were incubated for 10 min at room temperature. Control experiments were performed by using a solution of NC protein in 150 mM ammonium. All samples were analyzed by direct infusion ESI on a Thermo Fisher Scientific (West Palm Beach, CA) LTQ-Orbitrap Velos mass spectrometer. The analyses were performed in nanoflow ESI mode by using quartz emitters produced in-house by a Sutter Instruments Co. (Novato, CA) P2000 laser pipette puller. Up to 7 μL samples were loaded onto each emitter by using a gel-loader pipette tip. A stainless steel wire was inserted in the back-end of the emitter and used to supply an ionizing voltage ranged around 0.8–1.1 kV. Source temperature and desolvation conditions were adjusted by closely monitoring the incidence of ammonium adducts and water clusters, with typical source temperature of

200 °C. Data were processed by using Xcalibur 2.1 software by Thermo Scientific.

Acknowledgements

We thank Dr. Matteo Scalabrin (The RNA Institute, SUNY Albany, NY, USA) for his help in obtaining the recombinant NC protein. This work was supported financially by MIUR, Progetti di Ricerca di Interesse Nazionale (Grant 2010W2KM5L_006), by Ministero degli Affari Esteri e Cooperazione Internazionale (MAE, Unità per la cooperazione scientifica e tecnologica bilaterale e multilaterale – Grant PGR00171), and by the Conseil Interprofessionnel du Vin de Bordeaux (CIVB), Bordeaux, France.

Supplementary data

Fig. S1 shows the activity curve resulting from the NC-induced stem destabilization of TAR in the presence of increasing amount of acutissimin A (**4a**) for three experiments conducted 5 min apart. Fig. S2 shows the NAME assay with acutissimins A and B (**4a** and **4b**). Fig S3 shows the effect of epiacutissimin A (**5a**) on NC-mediated annealing in the oligo-preincubation and NC-preincubation modes. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2015.01.035.

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