

Multiple *in Vitro* Inhibition of HIV-1 Proteins by 2,6-Dipeptidyl-anthraquinone Conjugates Targeting the PBS RNA

Elia Gamba, Alice Sosic,* Irene Saccone, Elisa Magli, Francesco Frecentese, and Barbara Gatto


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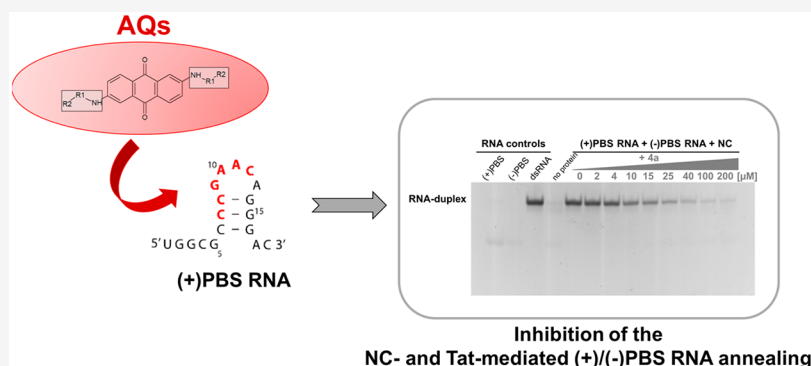
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ABSTRACT: We recently reported a series of 2,6-dipeptidyl-anthraquinone conjugates (Aqs) as Trans-Activation Response element (TAR) RNA-binding agents able to inhibit *in vitro* the HIV-1 nucleocapsid (NC) protein-mediated processes. Because NC is a highly adaptable nucleic acid chaperone assisting several crucial steps along reverse transcription, in this study we investigate the ability of Aqs to interact with other virus-derived nucleic acid structures thus potentially inhibiting multiple NC functions. Focusing on the HIV-1 Primer Binding Site (PBS) RNA sequence, we demonstrate that properly substituted dipeptidyl-anthraquinone conjugates efficiently inhibit the NC-mediated primer annealing in the low micromolar range. Similarly, we extended the analysis to the HIV-1 trans-activator of transcription (Tat) peptide, which has been recently shown to mimic the annealer functions of NC upon interacting with the same nucleic acid regulatory sequences. Our results highlight how RNA-targeting agents can act as multimode inhibitors of key viral proteins affecting their chaperone activity in reverse transcription processes.

KEYWORDS: *Tat protein, NC protein, RNA-targeted agents, antivirals, HIV-1*

Antiretroviral therapy exploits a great variety of drugs that efficiently control HIV-1 infection, but the onset of resistance demands the identification of novel pharmacological targets that are less prone to mutate during chronic treatment.¹ Recently, a great effort has been made looking for strategies to inhibit the HIV-1 nucleocapsid protein (NC), a nucleic acid chaperone that catalyzes many steps of the viral replication process, supporting the activity of the reverse transcriptase (RT) and participating in the late step of viral particle maturation.^{2,3} The strict conservation and multiple roles that NC plays throughout the entire viral replication cycles make this protein an attractive and promising target against HIV.⁴

Even if different classes of molecules have been proposed as anti-NC agents,⁵ drug-candidates interfering with NC functions are still missing. In the attempt to identify small molecules able to impair NC binding/annealing activities, we recently focused our work on dipeptidyl-anthraquinone conjugates (Aqs), nucleic acid binding agents acting as threading intercalators able to recognize and stabilize the dynamic structures of nucleic acids which are substrate of NC, thus impairing the expected nucleic acid rearrangements

catalyzed by the viral protein.^{6–8} These Aqs are characterized by a common anthraquinone scaffold and two side chains at opposite sides of the polycyclic aromatic nucleus: the peptidyl-side chains are built combining a first amino acid referred as “linker” (R1) and a second amino acid acting as a cationic terminal residue (R2), whose function is to facilitate the interaction with the nucleic acid phosphate backbone. We therefore designed a chemical library including four series of analogues identified by the R1 linkers (β -alanine, glycine, L- or D-alanine) and different R2 residues and reported their *in vitro* activity as potent inhibitors of the NC-mediated TAR/cTAR annealing, a suitable *in vitro* model of the minus-strand transfer event during the HIV-1 reverse transcription process.^{6–8} A

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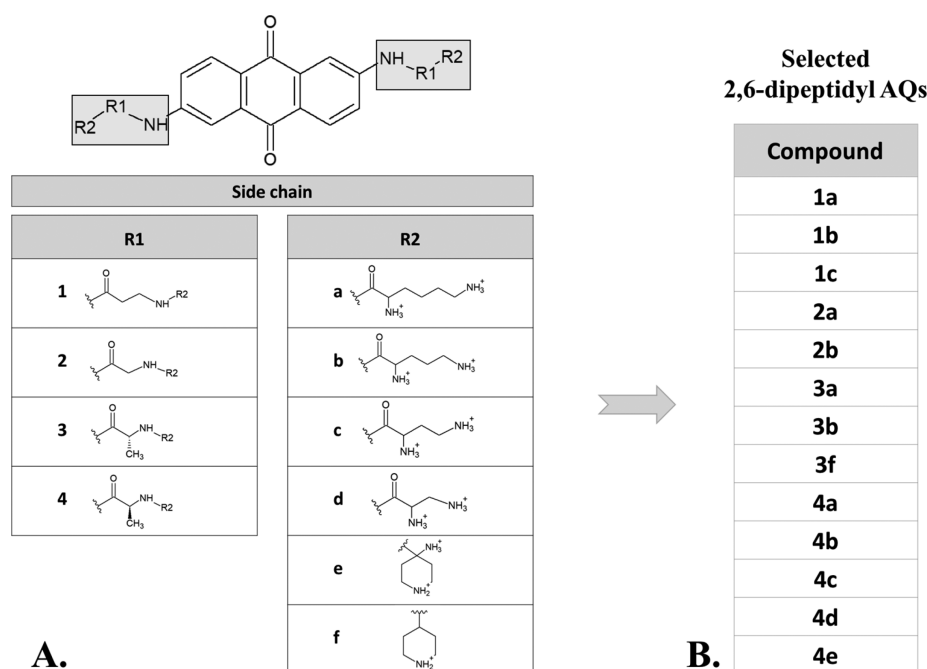


Figure 1. (A) Schematic structures of the 2,6-dipeptidyl-anthraquinone conjugates. Side chain composition identifies each compound: R1 linkers (β -alanine, glycine, L- or D-alanine) are marked by a number, whereas a letter is specific for the R2 terminal residues. (B) On the right are reported the 2,6-dipeptidyl-anthraquinone conjugates analyzed in this study.^{6–8,11}

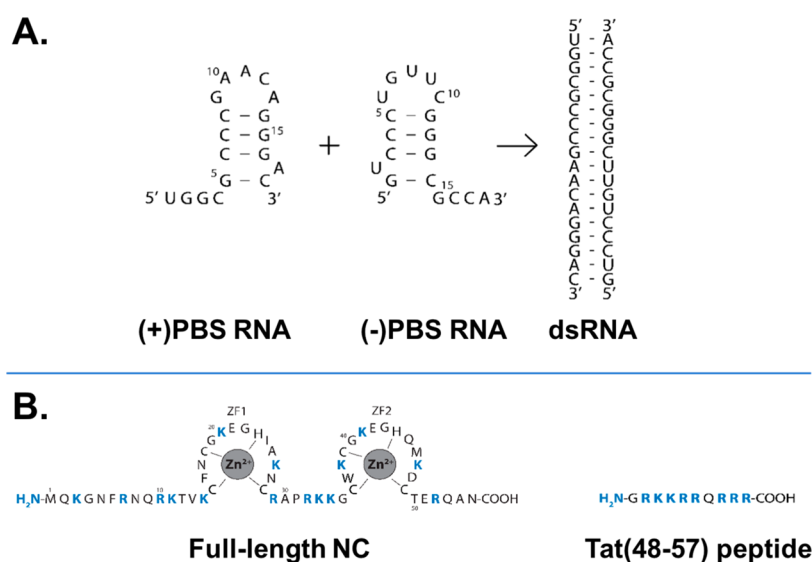


Figure 2. (A) Sequence and secondary structure of the (+)PBS RNA and of the complementary (–)PBS RNA constructs employed in this study. Heat-refolding or protein-mediated annealing can lead to the formation of the RNA-duplex (dsRNA). (B) Sequences of the full-length NC protein and Tat(48–57) peptide. Basic amino acids are highlighted in blue.

schematic representation of AQs chemical structure is shown in Figure 1A.

Interestingly, the structure–activity relationship (SAR) of these NC inhibitors allowed identification of positive hits targeting preferentially the Trans-Activation Response element (TAR), an essential RNA substrate of NC, and we demonstrated that TAR binding is a key event in their molecular mechanisms of NC inhibition.^{6,8,9} The studies highlighted the crucial role of the highly basic nature of the terminal (R2) residues as well as the flexibility and stereochemistry of the R1 linker connecting them to the aromatic nucleus to achieve the desired binding and stabilization of the

RNA substrate.^{6,8} Moreover, these TAR-binding agents displayed the ability to hamper specific interactions of TAR with a peptide mimicking the HIV-1 trans-activator of transcription (Tat) factor,^{8,9} opening the possibility to consider AQs as multitarget agents acting against different viral proteins through specific interaction with the common TAR RNA substrate. RNA-binding small molecules are an important and highly challenging area of therapeutic research.¹⁰ Our findings prompted us to further investigate selected 2,6-dipeptidyl anthraquinones as RNA-specific ligands.

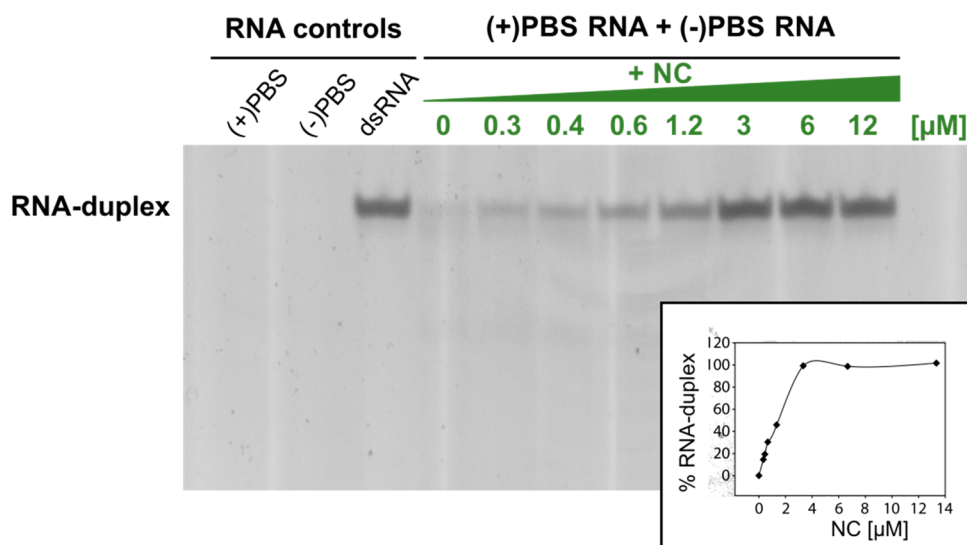


Figure 3. RNA annealer-mediated primer annealing (RAMPA) assay completed in the presence of the full-length NC protein. Folded (+)PBS and (−)PBS RNA, each 1 μ M, were incubated with increasing concentrations of NC (0, 0.3, 0.4, 0.6, 1.2, 3, 6, and 12 μ M) for 10 min at room temperature. Monomeric (+)PBS RNA, (−)PBS RNA, and the RNA-duplex (dsRNA) annealed by thermal denaturation were used as controls. In all the experiments, the bands corresponding to monomeric RNA sequences are not stained intensely enough to be unambiguously displayed. (+)PBS and (−)PBS RNA sequences were always barely detected in the gel system by SYBR Green II, probably due to the limited number of base-pairs characterizing their secondary structure. Reactions were therefore evaluated by following the RNA-duplex species. Band quantification was always performed considering the band corresponding to the RNA-duplex (see [Experimental Details](#) in the Supporting Information). Inset: optical density quantification of bands corresponding to the RNA-duplex species at increasing NC concentrations.

Since many RNA regulatory sequences in the HIV-1 genome are locked up into stable secondary structure requiring the NC chaperone activity, we propose that AQs may target several of these nucleic acid structures, potentially inhibiting NC at multiple levels. To test this hypothesis, we moved our attention to the Primer Binding Site (PBS) RNA sequence. In the early steps of reverse transcription, the primer tRNA^{Lys3} must be annealed to the complementary PBS sequence of the viral RNA genome. This RNA–RNA annealing allows the minus-strand synthesis, a step not occurring in the absence of NC.¹² Annealing involves a stretch of 18 nucleotides in the tRNA^{Lys3} 3′-acceptor stem, with the same sequence as (−)PBS RNA, forming an RNA-duplex with PBS, (+)PBS RNA, that is selectively used as primer by the HIV-1 RT.¹² The stable secondary structure of (+)PBS RNA, similarly to TAR RNA, is the target of the chaperone activity of NC.¹³ In this study, we therefore selected from our chemical library of AQs a cluster of 2,6-di-peptidyl-anthraquinone conjugates ([Figure 1B](#)) to explore their putative ability to inhibit NC-mediated (+)/(−)PBS RNA annealing *in vitro*. Interestingly, the placement of tRNA^{Lys3} primer onto viral RNA is also promoted by the HIV-1 trans-activator of transcription (Tat) protein.^{14,15} This protein is thought to support the annealing activities of NC during reverse transcription, thus earning itself the definition of nucleic acids annealer.^{16,17} For this reason, we developed an assay relying on the annealer activity of both NC and Tat. We named it “RNA annealer-mediated primer annealing” (RAMPA) assay, since it allows us to employ either NC or Tat to mediate the annealing of the (+)PBS RNA construct with its complementary sequence (−)PBS RNA, leading to the formation of the annealed RNA-duplex (dsRNA) as shown in [Figure 2](#). In RAMPA assay, the analysis of the (+)/(−)PBS RNA annealing reaction can be investigated by native polyacrylamide gel electrophoresis (PAGE).

We employed the RAMPA assay to assess the ability of AQs to interfere with the annealer-mediated (+)/(−)PBS RNA annealing *in vitro*. Preliminarily, we optimized the RAMPA assay with NC. Analysis of the (+)PBS RNA secondary structure by UV-absorbance and circular dichroism melting/annealing experiments, reported in [Figure S1](#) (Supporting Information), confirmed the predicted secondary structure of the RNA construct consistent with the 4-base pair stem reported in [Figure 2](#). In the absence of the annealer proteins, spontaneous RNA-duplex formation occurred rather slowly with less than 40% of RNA-duplex formed after incubation at room temperature up to 60 min ([Figure S2](#), Supporting Information), indicating that (+)PBS and (−)PBS RNA are kinetically stable into monomeric structures under the condition used. The (+)/(−)PBS RNA annealing reaction was instead extremely accelerated by the presence of the full-length NC protein. A representative RAMPA assay is reported in [Figure 3](#), showing that complete RNA–RNA annealing was achieved after 10 min in the presence of 3 μ M NC, i.e. at 12 nucleotide (nt):NC molar ratio. As an additional control experiment, we performed RAMPA assay in the presence of APO-NC (a zinc-less version of NC prepared as detailed in the [Supporting Information](#)). The results are reported in [Figure S3](#) (Supporting Information) and showed that the APO-NC possesses a similar annealer activity compared to the zinc-bound protein. These results confirmed that even in the absence of folded Zn fingers the APO-NC mutant preserves an efficient annealing activity, while the role of properly folded NC Zn fingers is critical to guide the extended duplex nucleation through loop–loop interactions.^{18–21}

Then, we set up the RAMPA assay to assess the Tat-mediated (+)/(−)PBS RNA annealing. We employed the Tat(48–57) peptide ([Figure 2](#)), corresponding to the essential Tat basic domain responsible for the nucleic acid chaperone activity.¹⁴ Similarly to NC, Tat(48–57) was able to

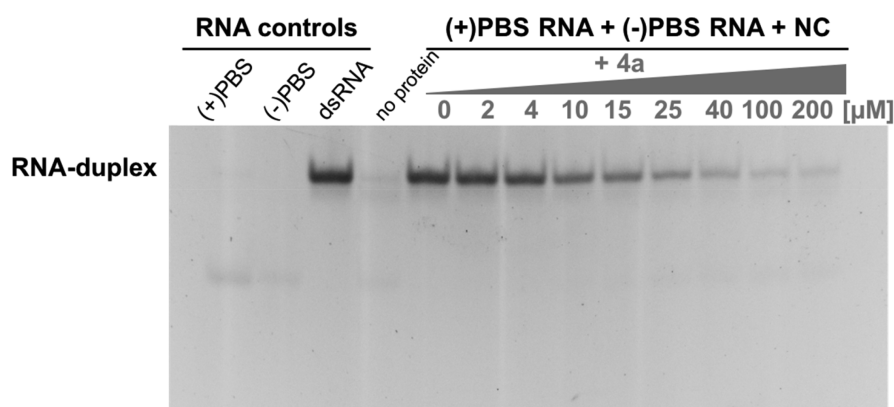


Figure 4. Inhibition of the (+)/(−)PBS RNA NC-mediated annealing reaction by compound **4a**. RNA annealer-mediated primer annealing (RAMPA) assay was completed in the presence of the full-length NC. Inhibition effects were evaluated in the presence of increasing amounts of compound **4a**. Folded PBS sequences, each 1 μM , were incubated with increasing concentrations of compound (0, 2, 4, 10, 15, 25, 40, 100, and 200 μM) for 15 min at room temperature. (+)PBS and (−)PBS RNA samples were then mixed and incubated with NC 3 μM for 10 min at room temperature. (+)PBS RNA, (−)PBS RNA, and the RNA-duplex (dsRNA) annealed by thermal denaturation were used as controls. (+)PBS and (−)PBS RNA were incubated also in the absence of the annealer protein (no protein).

dramatically speed up the reaction with a complete annealing of the two RNAs achieved at 6 μM peptide concentration, i.e. Six nt:Tat(48–57) molar ratio, indicating that the basic peptide is about 2-fold less efficient compared to NC as nucleic acid annealer in our RAMPA assay (Figure S4, Supporting Information).

Having defined a suitable platform to analyze *in vitro* either the NC- or Tat-mediated (+)/(−)PBS RNA annealing step, we proceeded to evaluate the inhibitory activity of the selected 2,6-di-peptidyl-anthraquinone conjugates. RAMPA assay was therefore performed with all selected compounds to evaluate their inhibitory activity on either NC- or Tat-mediated annealing of (+)/(−)PBS RNA. A representative RAMPA assay conducted in the presence of NC and compound **4a** is shown in Figure 4. The gradual disappearing of the RNA-duplex band clearly indicates inhibition of the annealer-mediated annealing in the presence of increasing concentration of compound. Experiments for all compounds are reported in Figures S5 and S6 (Supporting Information).

Quantification of the band corresponding to the annealer-induced RNA-duplex was assessed to calculate the IC_{50} as a measure of the ability of each compound to slow down the annealing activity of either NC or Tat(48–57) under the selected experimental conditions. Results are reported in Table 1.

The obtained results allowed for the direct comparison of the inhibitory activity of each compound toward the two different viral proteins. When NC was examined, Aqs with 11-atom linear-aliphatic side chain and two ionizable groups (**1b**, **2a**, and **4a**) resulted in the most active inhibitors, with **4a** as the best among all the series (IC_{50} value: $12.5 \pm 1.5 \mu\text{M}$, Table 1). Slightly lower inhibitory activity was observed for 10-atom linear side chain compounds (**1c**, **2b**, **3b**, and **4b**); the activity drastically decreased by further shortening the side chain length as observed with compounds **4c** and **4d**. Similarly, inhibition decreased with excessive lengthening of the side chain (12-atom length, **1a**). Side chain chirality also played a major role in the NC-inhibition as highlighted by the conjugates bearing the D- or L-alanine linker. Comparing the activity of the D- and L-stereoisomers having the same terminal residue (**3a** to **4a** and **3b** to **4b**), the non-natural linker configuration (D-Ala) enhances the inhibition of NC activity.

Table 1. Side Chain Features and IC_{50} Values Observed for the Inhibition of NC- and Tat(48–57)-Mediated Annealing of (+)PBS and (−)PBS RNA Constructs (RAMPA Assay)

Side chain	Compound	NC IC_{50} [μM] ^a	Tat(48–57) IC_{50} [μM] ^a
linear, 12-atom length	1a	85 ± 2	>100
linear, 11-atom length	1b	20 ± 2	20 ± 1
	2a	23 ± 1	18 ± 1
	3a	54 ± 8	48 ± 9
	4a	12 ± 2	15 ± 1
linear, 10-atom length	1c	30 ± 3	24 ± 3
	2b	30 ± 2	34 ± 1
	3b	55 ± 5	54 ± 6
	4b	21 ± 1	16 ± 1
linear, 9- or 8-atoms length	4c	88 ± 2	>100
	4d	54 ± 1	45 ± 1
cyclic, bulky	4e	>200	>200
	3f	>200	>200

^aEach value represents the mean and standard error of the mean (SEM) obtained from triplicate experiments.

Interestingly, Aqs displayed the same behavior in inhibiting the NC-mediated TAR/cTAR annealing step.⁸ When RAMPA assay was performed employing Tat(48–57) as annealer, the activity profile of the tested Aqs mirrored the one observed for NC (Table 1). These results highlighted side chain length and linker configuration as the major structural determinants affecting the Aqs inhibitory activity of both the NC- and Tat-mediated (+)/(−)PBS RNA-annealing. Hence, Aqs displayed the same potency scale when testing NC and Tat RNA-annealing properties, suggesting that their mechanism of action is based on their RNA-directed targeting.

Unambiguous evidence of Aqs direct binding to the (+)PBS and (−)PBS RNA constructs was assessed by employing electrospray ionization mass spectrometry (ESI-MS) under non-denaturing conditions.^{22–24} Compound-RNA binding experiments were performed for all selected compounds in the presence of either (+)PBS or (−)PBS construct. Representative spectra of **4a** interactions with (+)PBS and (−)PBS RNA are reported in Figure S8 (Supporting Information). All compounds were able to bind both RNA

sequences and form a 1:1 complex, offering a direct observation of the RNA binding abilities of these compounds. Binding of a second AQ molecule (2:1 binding stoichiometry) was observed only for **1b**, **2a**, and **4a**. The observation of a 2:1 complex in the presence of the free substrate suggested that the two binding sites share similar affinities with no binding cooperativity.^{6,7,9} Quantification of the different species peaks reveals the accurate partitioning between free and bound forms in solution, which was used to calculate the relative binding affinity of the various compounds for the different RNA substrates, employing the percentage of bound substrate observed in each spectrum.⁶ A graphical representation of the obtained results is reported in Figure S9. Most compounds showed higher affinity for (+)PBS RNA rather than for the (−)PBS RNA sequence. This can be related to a folding structure of (+)PBS that is more favorable for drug binding and to a higher content in purine bases that can offer stronger stacking interactions with the anthraquinone scaffold of AQs. When looking at the structural requirements of AQs that maximize binding to the RNAs, a striking correlation was observed with the SAR obtained by RAMPA assay. The correlation between the inhibitory activity of NC-mediated (+)/(−)PBS RNA annealing and AQs binding affinity for (+)PBS construct is plotted in Figure 5.

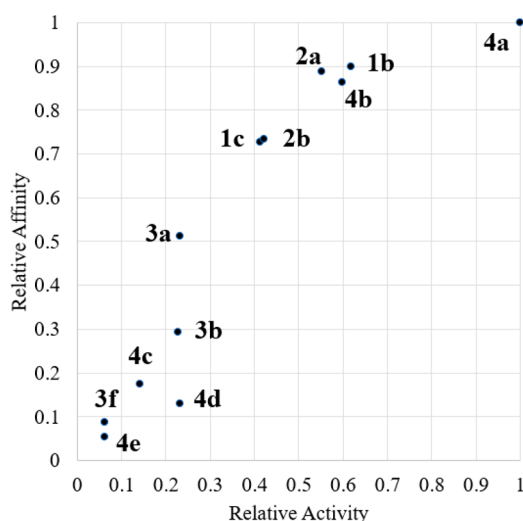


Figure 5. Correlation between the inhibitory activity of NC-mediated (+)/(−)PBS RNA annealing and AQs binding affinity for (+)PBS construct. The relative activity in the x-axis was calculated from the data reported in Table 1 by normalizing the IC_{50} values of compounds to the value of the best inhibitor compound **4a**, obtained by the RAMPA assay conducted with NC. The relative affinity in the y-axis was calculated from the data reported in Figure S9 by normalizing the fraction bound of compounds to the value of the strongest binder compound **4a**, obtained by ESI-MS binding experiments conducted with (+)PBS construct. A clear correlation was observed between the AQs inhibitory activity and their affinity for the RNA substrate.

Compounds with 11-atom side chain length (**1b**, **2a**, and **4a**) proved to be the best binders, followed by shorter analogues (compounds **1c**, **2b**, and **4b**). Affinity was strongly impaired by further shortening the cationic side chains to 9- and 8-atoms (compounds **4c** and **4d**), by L-conformation of the peptide linker (e.g., compound **3a**) and by bulky terminal residues such as those in compounds **4e** and **3f**. Interestingly, we previously reported analogous SAR when testing the same AQs in the

NC-mediated TAR/cTAR annealing process: compounds **1b**, **2a**, and **4a** proved to be the most potent NC inhibitors of our chemical library with comparable activity in the micromolar range, while compound **3f** was inactive.^{6–8} Also in the TAR system, we observed a strict correlation between NC inhibition and binding affinity to TAR construct.^{6,8} These observations demonstrate that RNA binding is a key event in AQs molecular mechanisms of proteins inhibition.

Further investigations were performed to identify the location of the AQs binding sites on the (+)PBS RNA construct. Compound **4a** was selected because it resulted to be the most potent NC and Tat inhibitor and the strongest (+)PBS binder to perform a footprinting experiment by MS. The effects of **4a** complexation on the gas-phase fragmentation of the (+)PBS structure were investigated by comparing the results provided by tandem MS (MS/MS) of free and bound RNA construct. The free (+)PBS substrate produced the typical fragmentation pattern expected from this type of nucleic acid,^{25,26} as shown in Figure S10 (Supporting Information). Complete sequence coverage obtained under the selected experimental conditions offered no evidence of possible fragmentation inhibition that could be ascribed to base-pairing between the stem complementary strands. Collision induced activation of the 1:1 complex of **4a** with (+)PBS induced fragmentation of the oligonucleotide backbone (Figure 6A). A distinctive gap in the fragmentation pattern of (+)PBS corresponding to nucleotides C7–C12 (Figure 6B) indicates a protective effect on the 5'-side of the hairpin loop structure by the compound (Figure 6C). These results show that **4a** preferentially binds to dynamic regions in the RNA structure such as the apical loop of the (+)PBS structure, which can better accommodate both stacking of the anthraquinone scaffold with base pairs and electrostatic interaction between the cationic terminal residues with the phosphate backbone.

In conclusion, this study led to the identification of a promising set of 2,6-dipeptidyl-anthraquinone conjugates able to inhibit NC- and Tat-mediated primer annealing in the low micromolar range. By exploring their SAR, we demonstrated that AQs' mechanism of action resides in their binding with good affinity to the (+)PBS and (−)PBS RNA sequences which are shared substrates of both NC and Tat proteins. Hence, the selected AQs revealed their dual activity opening the possibility to consider them as multitarget inhibitors. In addition, the most potent AQs in the PBS system resulted to be highly active as NC inhibitors also in the previously analyzed TAR system,^{6–8} highlighting their putative ability to impair NC activities at multiple levels. These findings indicate that AQs potentially exert a pleiotropic antiviral activity by targeting important RNA regulatory sequences while inhibiting the activity of multiple virus chaperones. NC and Tat are multifunctional proteins involved in crucial steps of the HIV-1 life cycle, both acting through interaction with viral RNA domains. Based on this, we strongly believe that agents capable of interfering with those critical steps are expected to block HIV-1 replication at multiple stages. This multimode mechanism of action represents a valuable approach that may help prevent the onset of drug resistance by targeting multiple and conserved structures within the HIV-1 genome. Although further investigations are needed, additional studies are underway to evaluate the AQs' interactions with other viral RNA domains that possess dynamic structure similar to TAR and PBS RNA, such as the stem loops in the psi-RNA region,

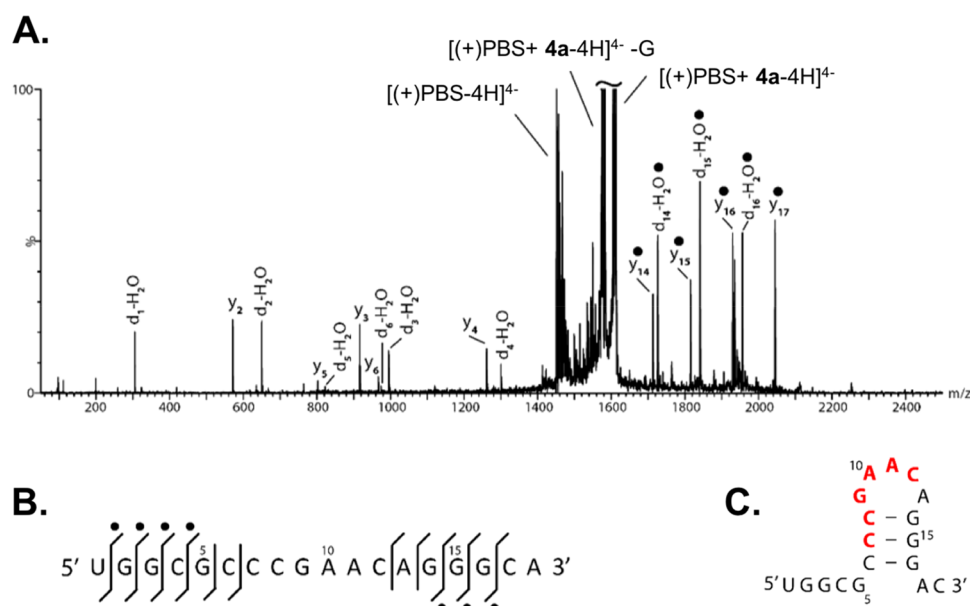


Figure 6. (A) MS/MS spectrum of (+)PBS+4a assembly obtained in negative ion mode. Characteristic ion series are labeled according to standard nomenclature.^{25,26} $[(+)\text{PBS}+4\text{a-4H}]^{4-}$ indicates the precursor ion. (B) Fragmentation pattern is summarized on the (+)PBS sequence. d-H₂O and y-series ions were observed. Fragment ions containing bound ligand were labeled with a dot. (C) Binding of 4a to (+)PBS displayed protection of the C7:C12 sequence (marked in red in the cartoon), corresponding to the loop region at the 5' side of the RNA construct.

potentially interfering with additional NC- and/or Tat-mediated viral processes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.9b00682>.

Detailed experimental procedures and supplementary figures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Alice Sosic – Dipartimento di Scienze del Farmaco, Università di Padova, 35131 Padova, Italy; orcid.org/0000-0002-1070-1869; Phone: +390498275691; Email: alice.sosic@unipd.it; Fax: +390498275366

Authors

Elia Gamba – Dipartimento di Scienze del Farmaco, Università di Padova, 35131 Padova, Italy

Irene Saccone – Dipartimento di Farmacia, Università degli Studi di Napoli “Federico II”, 80131 Napoli, Italy

Elisa Magli – Dipartimento di Farmacia, Università degli Studi di Napoli “Federico II”, 80131 Napoli, Italy

Francesco Frecentese – Dipartimento di Farmacia, Università degli Studi di Napoli “Federico II”, 80131 Napoli, Italy

Barbara Gatto – Dipartimento di Scienze del Farmaco, Università di Padova, 35131 Padova, Italy; orcid.org/0000-0001-9465-6913

Complete contact information is available at:

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

This work is in memory of Prof. Maurizio Botta, who inspired generations of young medicinal chemists with his dedication to research.

■ ABBREVIATIONS

HIV, human immunodeficiency virus; NC, nucleocapsid protein; Tat, trans-activator of transcription; TAR, trans-activation response element; PBS, primer binding site; Lys3, tRNA^{Lys3}; dsRNA, RNA duplex; AQs, peptidyl-anthraquinone conjugates; RT, reverse transcriptase; SAR, structure–activity relationship; RAMPA, RNA annealer-mediated primer annealing; IC₅₀, 50% inhibitory concentration; ESI-MS, electrospray ionization mass spectrometry

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