Supplementary Materials

Design, Synthesis and Biological Evaluation of TAR and cTAR binders as HIV-1 Nucleocapsid Inhibitors

Alice Sosic[a], Francesco Frecentese[b], Elisa Perissutti[b], Laura Sinigaglia[a], Vincenzo Santagada[b], Giuseppe Caliendo[b], Elisa Magli[b], Antonio Ciano[b], Giuseppe Zagotto[a], Cristina Parolin[c], Barbara Gatto[a]*

[a]Dipartimento di Scienze del Farmaco, via Marzolo 5, 35131, Padova, Italy

[b]Dipartimento di Farmacia, Università degli Studi di Napoli "Federico II", via D. Montesano 49, 80131, Napoli, Italy

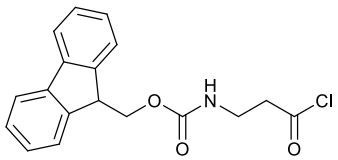
[c]Dipartimento di Biologia, via Gabelli, 63, 35131 Padova, Italy

Experimentals

General Materials and Methods: All reagents were purchased from the Aldrich-Sigma Chemical Company and were used without purification. Solvents were analytical reagent grade or higher purity and were used as supplied. All reactions were performed in standard Pyrex glassware.
Microwave reactions were carried out in sealed vessels using a microwave oven (ETHOS 1600, Milestone) especially designed for organic synthesis. The temperature of the stirred reaction mixtures was monitored by a microwave-transparent fluoroptic probe inserted into the solution. Irradiation time and power were monitored with the "easyWAVE" software package. Thin layer chromatography was performed on Macherey-Nagel silica gel 50 plates with fluorescent indicator and the plates were visualized with UV light (254 nm). A Buchi R-114 rotavapor was utilized for the removal of the solvents in vacuo. Analytical RP-HPLC was performed with a Vydac C_{18}-column (5 μm, 4.6 x 250 mm, spherical) employing the following solvents: A: 100% acetonitrile in 0.1% TFA, B: 100% H_2O in 0.1% TFA. RP-HPLC purifications were performed on a Waters Delta Prep 4000 system equipped with a Waters 484 multi-wavelength detector on a Vydac C18 column (15–20 mm, 22x5000 mm) adopting the same gradient used for the analytical determinations. The operational flow rate was 30 mL/min. Capacity factor (K') of the final compounds were assessed by analytical RP-HPLC using both Vydac C_{18} column (5 mm, 4.6x250 mm) and Beckman C_{18} column (5 mm, 4.6x250 mm) employing the following conditions: linear gradient from 10% to 70% A over 35 min on the Vydac column (system 1) and 20 to 80% A over 35 min on the Beckman C18 column (system 2), UV detection at 220 nm, flow rate 1 mL/min). The column was connected to a Rheodyne model 7725 injector, a Waters 600 HPLC system, a Waters 486 tunable absorbance detector, and a Waters 746 chart recorder.
The structures were verified spectroscopically by proton ^{1}H-NMR, ^{13}C-NMR and MS. Spectra were recorded on Varian Mercury Plus 400 instrument. Chemical shifts are given as δ and are referred to Me_4Si as internal standard. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), bs (broad singlet), d (doublet), dd (double doublet), t (triplet), m (multiplet). Mass spectra were performed on Applyed Biosystem API 2000 triple-quadrupole mass spectrometry.

Synthetic Procedures

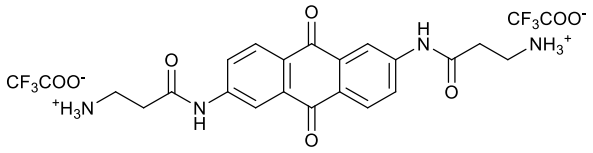
(9H-fluoren-9-yl)methyl 3-chloro-3-oxopropylcarbamate (3; Fmoc-β-Alanine-Cl)



Commercially available Fmoc-β-alanine-OH (Aldrich®, 3.5g, 11.25 mmol) was dissolved in anhydrous dichloromethane (40 mL) in a two-neck flask. An excess of $SOCl_2$ (6 mL; 82.25mmol) was added dropwise. The mixture was transferred into a sealed vessel and heated by microwave irradiation at 50°C (300W power) for 8 minutes. Solvent was evaporated under reduced pressure

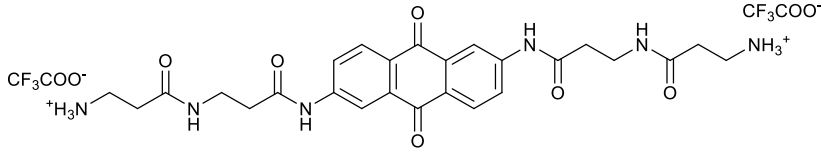
and the residue was purified by crystallization from n-hexane affording 3.59 g of pure **3** as a white solid.
Yield: 97%.
^{1}H-NMR, ^{13}C-NMR and MS were consistent to that already reported.[1]

N,N'-(9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(3-aminopropanamide)-bis-trifluoroacetate (4)



Fmoc-β-Ala-Cl (**3**, 3.19 g, 9.7 mmol) and pyridine (638 µL, 7.9 mmol) were slowly added to a solution of 2,6-diaminoanthraquinone (**2**, 175 mg, 0.97 mmol) in DMF (40 mL). Reaction mixture was stirred at room temperature for 24 hours. Solvent was removed by reduced pressure distillation and the residue was purified by crystallization from ethyl acetate obtaining 2,6-bis[N-(3-Fmoc-amino)-propionamide]anthracene-9,10-dione (2,6-Fmoc-β-Ala-anthraquinone) as an orange powder (695 mg, 0.84 mmol, yield 87%) used in the next step without any further purification.
The obtained compound was reacted with a 33% N,N-diethylamine solution in THF (30 mL) for 2 hours. Solvent was again distilled off under reduced pressure and the residue was finally reacted with a solution of trifluoroacetic acid in water (9:1, v/v, 20 mL) for 1 hour. Reaction mixture was then added with diethyl ether (30 mL) and the obtained precipitate was collected by centrifugation and dried obtaining 460 mg of intermediate **4** as an intense orange solid.
Yield 78% (overall).
^{1}H-NMR, ^{13}C-NMR and MS were consistent to that already reported.[2]

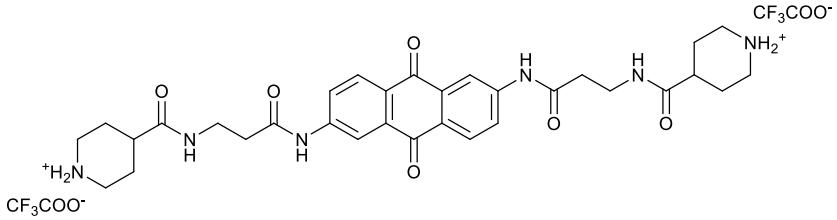
N,N'-(9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(3-(3aminopropanamido)propanamide)-bis-trifluoroacetate (5a)



Intermediate **4** (203 mg, 0.33 mmol) was dissolved in dry DMF (15 mL), and DMAP (443 mg, 3.63 mmol), Fmoc-β-Alanine-OH (1.03 g, 3.3 mmol), and HBTU (1.25 g, 3.3 mmol) were added. The resulting solution was stirred at room temperature for 24 hours, then poured in Et_2O and centrifuged. The solid obtained was washed with Et_2O and the product (2,6-Fmoc-β-Alanine-β-Alanine anthraquinone) was dried in vacuo and used in the next step without any further purification.
To remove the Fmoc- protecting group, the solid thus obtained was reacted with a 33% N,N-diethylamine solution in THF (30 mL) for 2 hours, poured in Et_2O and centrifuged. The solid obtained was washed with Et_2O and water and then dried in vacuo.
Subsequently, the obtained solid was reacted with a solution of trifluoroacetic acid in water (9:1, v/v, 20 mL) for 1 hour and then poured in Et_2O. The resulting suspension was centrifuged. The solid obtained was washed with Et_2O and dried in vacuo. Purification by preparative RP-HPLC afforded the pure compound **5a** as an intense orange solid.
Yield: 66%. Orange solid. K' (HPLC): 14.5 (system 1), 13.2 (system 2).

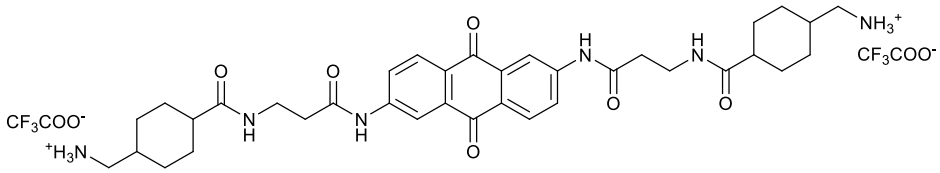
^{1}H-NMR (DMSO-d_6, 400MHz): δ 10.68(s, 2H), 8.47 (d, J=1.6 Hz, 2H), 8.27 (t, J=5.2, 2H), 8.13 (d, J=8.4, 2H), 8.03 (dd, J=8.4 and 1.6 Hz, 2H), 7.78 (bs, 6H), 3.38 (m, 4H), 2.98 (m, 4H), 2.59 (t, J=6.4 Hz, 4H), 2.43 (t, J=6.8 Hz, 4H).
^{13}C-NMR (DMSO-d_6, 400MHz): δ 182.04, 171.21, 170.14, 145.38, 135.03, 129.19, 128.65, 124.09, 116.51, 37.03, 36.00, 35.53, 32.68
ESI-MS: 522.9 [M + H]+; 262.0 [M + 2H]++

N,N'-(3,3'-(9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl)bis(3-oxopropane-3,1-diyl))dipiperidine-4-carboxamide-bis-trifluoroacetate (5b)



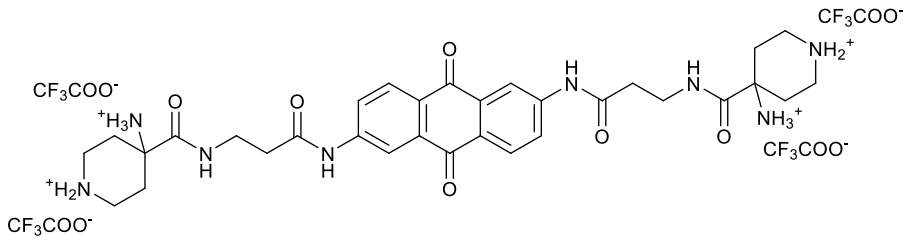
The compound was obtained with the same coupling procedure adopted for **5a,** starting from intermediate **4** and 1-Fmoc-piperidine-4-carboxylic acid (Fmoc-Inp-OH).
Yield: 77%. Orange solid. K' (HPLC): 5.3 (system 1), 4.8 (system 2).
^{1}H-NMR (DMSO-d_6, 400MHz): δ 10.63(s, 2H), 8.62 (t, J=5.0 Hz, 2H), 8.46 (d, J=1.8 Hz, 2H), 8.14 (d, J=8.4, 2H), 8.07 (bs, 4H), 8.02 (dd, J=8.4 and 1.8 Hz, 2H), 3.38 (m, 4H), 3.24 (m, 4H), 2.85 (m, 4H), 2.57 (t, J=6.4 Hz, 4H), 2.39 (m, 2H), 1.77 (m, 4H), 1.69 (m, 4H)
^{13}C-NMR (DMSO-d_6, 400MHz): δ 182.06, 173.81, 171.24, 145.41, 135.07, 129.19, 128.67, 124.13, 116.55, 43.17, 39.33, 37.23, 35.65, 25.85
ESI-MS: 603.3 [M + H]+; 302.1 [M + 2H]++

N,N'-(3,3'-(9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl)bis(3-oxopropane-3,1-diyl))bis(4-(aminomethyl)cyclohexanecarboxamide)-bis-trifluoroacetate (5c)



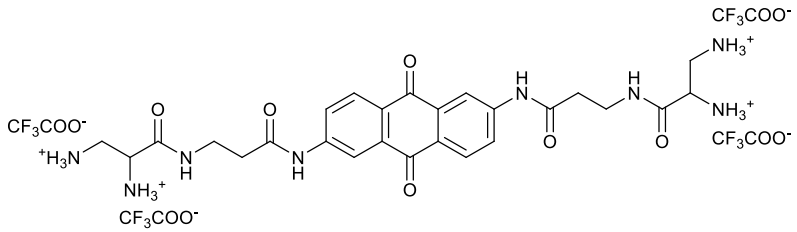
The compound was obtained with the same coupling procedure adopted for **5a,** starting from intermediate **4** and 4-(Fmoc-aminomethyl)cyclohexanecarboxylic acid (N-Fmoc-tranexamic acid).
Yield: 62%. Orange solid. K' (HPLC): 5.9 (system 1), 5.1 (system 2).
^{1}H-NMR (DMSO-d_6, 400MHz): δ 10.62 (s, 2H), 8.46 (d, J=1.8 Hz, 2H), 8.13 (d, J=8.4, 2H), 8.01 (dd, J=8.4 and 1.8 Hz, 2H), 7.91 (t, J=5.2 Hz, 2H), 7.76 (bs, 6H), 3.33 (m, 4H), 2.62 (t, J=6.4 Hz, 4H), 2.54 (s, 4H), 2.03 (t, J=1.7 Hz, 2H), 1.75 (m, 2H), 1.29 (m, 8H), 0.88 (m, 8H)
^{13}C-NMR (DMSO-d_6, 400MHz): δ 182.03, 175.72, 171.28, 145.39, 135.02, 129.17, 128.62, 124.07, 116.49, 44.99, 44.09, 37.32, 35.76, 35,50, 29.56, 29.04
ESI-MS: 659.1 [M + H]+; 330.2 [M + 2H]++

N,N'-(3,3'-(9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl)bis(3-oxopropane-3,1-diyl))bis(4-aminopiperidine-4-carboxamide)-tetratrifluoroacetate (5d)



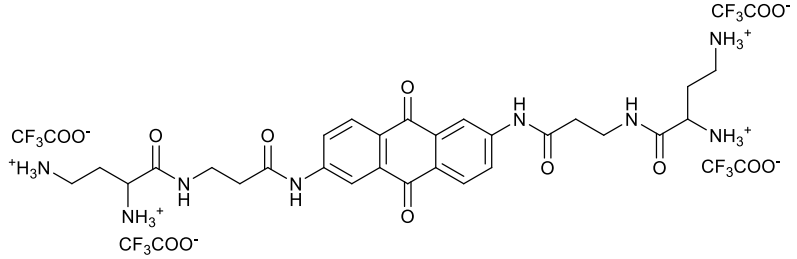
The compound was obtained with the same coupling procedure adopted for **5a,** starting from intermediate **4** and 1-Fmoc-4-(Fmoc-amino)-piperidine-4-carboxylic acid (Fmoc-Pip(Fmoc)-OH).
Yield: 66%. Orange solid. K' (HPLC): 4.4 (system 1), 3.9 (system 2).
^{1}H-NMR (DMSO-d_6, 400MHz): δ 10.73(s, 2H), 9.01 (bs, 4H), 8.71 (bs, 6H), 8.54 (t, J=5.2 Hz, 2H), 8.45 (d, J=1.8 Hz, 2H), 8.14 (d, J=8.8, 2H), 8.02 (dd, J=8.4 and 1.8 Hz, 2H), 3.48 (m, 4H), 3.24 (m, 4H), 2.85 (m, 4H), 2.65 (t, J=5.6 Hz, 4H), 2.40 (m, 4H), 1.96 (m, 4H)
^{13}C-NMR (DMSO-d_6, 400MHz): δ 182.06, 171.01, 168.86, 145.38, 135.05, 129.20, 128.69, 124.19, 116.60, 56.22, 43.19, 36.52, 35.75, 28.86
ESI-MS: 633.4 [M + H]+; 317.2 [M + 2H]++

N,N'-(3,3'-(9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl)bis(3-oxopropane-3,1-diyl))bis(2,3-diaminopropanamide)-tetratrifluoroacetate (5e)



The compound was obtained with the same coupling procedure adopted for **5a,** starting from intermediate **4** and N-alpha-Fmoc-N-beta-Boc-L-diaminopropionic acid (Fmoc-Dap(Boc)-OH).
Yield: 83%. Orange solid. K' (HPLC): 4.5 (system 1), 3.9 (system 2).
^{1}H-NMR (DMSO-d_6, 400MHz): δ 10.75 (s, 2H), 8.72 (t, J=5.4Hz, 2H), 8.49 (d, J=1.6 Hz, 2H), 8.41 (bs, 6H), 8.20 (bs, 6H), 8.15 (d, J=8.4, 2H), 8.03 (dd, J=8.4 and 1.6 Hz, 2H), 4.07 (m, 2H), 3.46 (m, 4H), 3.18 (m, 4H), 2.66 (t, J=4.8Hz, 4H).
^{13}C-NMR (DMSO-d_6, 400MHz): δ 181.90, 171.06, 165.95, 145.35, 134.93, 129.32, 128.52, 124.27, 116.44, 50.93, 40.15, 36.34, 35,64.
ESI-MS: 553.5 [M + H]+; 277.2 [M + 2H]++

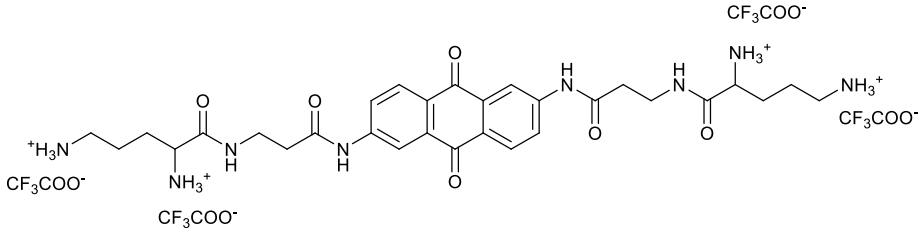
N,N'-(3,3'-(9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl)bis(3-oxopropane-3,1-diyl))bis(2,4-diaminobutanamide)-tetratrifluoroacetate (5f)



The compound was obtained with the same coupling procedure adopted for **5a,** starting from intermediate **4** and N-alpha-Fmoc-N-gamma-Boc-L-diaminobutyric acid (Fmoc-Dab(Boc)-OH).
Yield: 78%. Orange solid. K' (HPLC): 4.4 (system 1), 3.8 (system 2).

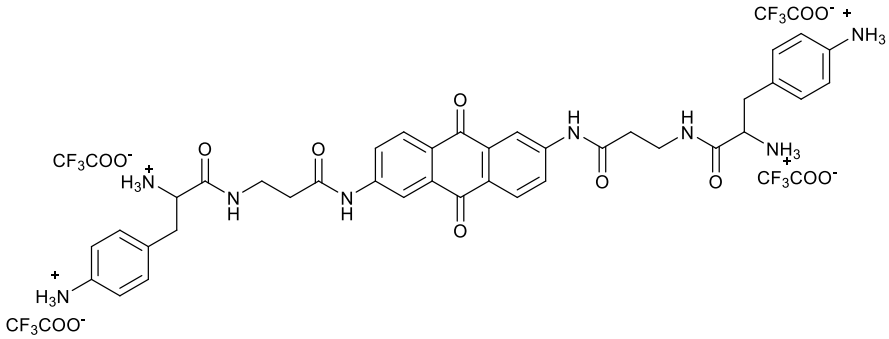
^{1}H-NMR (DMSO-d_6, 400MHz): δ 10.76 (s, 2H), 8.70 (t, J=5.2Hz, 2H), 8.48 (d, J=2.0 Hz, 2H), 8.31 (bs, 6H), 8.20 (bs, 6H), 8.15 (d, J=8.8, 2H), 8.02 (dd, J=8.8 and 2.0 Hz, 2H), 7.94 (bs, 6H), 3.84 (m, 2H), 3.45 (m, 4H), 2.83 (m, 4H), 2.65 (t, J=4.8Hz, 4H), 1.97 (m, 4H).
^{13}C-NMR (DMSO-d_6, 400MHz): δ 182.06, 171.00, 168.40, 145.35, 135.06, 129.26, 128.56, 124.12, 116.51, 50.59, 36.59, 35.75, 35.57, 29.61
ESI-MS: 581.4 [M + H]+; 291.3 [M + 2H]++

N,N'-(3,3'-(9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl)bis(3-oxopropane-3,1-diyl))bis(2,5-diaminopentanamide)-tetratrifluoroacetate (5g)



The compound was obtained with the same coupling procedure adopted for **5a,** starting from intermediate **4** and N-alpha-Fmoc-N-delta-Boc-L-ornithine (Fmoc-Orn(Boc)-OH).
Yield: 82%. Orange solid. K' (HPLC): 4.5 (system 1), 3.9 (system 2).
^{1}H-NMR (DMSO-d_6, 400MHz): δ 10.83 (s, 2H), 8.70 (t, J=5.2Hz, 2H), 8.52 (d, J=2.0 Hz, 2H), 8.25 (bs, 6H), 8.15 (d, J=8.8, 2H), 8.04 (dd, J=8.8 and 2.0 Hz, 2H), 7.90 (bs, 6H), 3.75 (m, 2H), 3.45 (m, 4H), 2.74 (m, 4H), 2.65 (t, J=4.8Hz, 4H), 1.68 (m, 4H), 1.56 (m, 4H).
^{13}C-NMR (DMSO-d_6, 400MHz): δ 182.07, 171.05, 169.03, 145.37, 135.06, 129.22, 128.70, 124.11, 116.51, 52.18, 38.85, 36.77, 35.58, 28.81, 23.18
ESI-MS: 609.4 [M + H]+; 305.4 [M + 2H]++

N,N'-(3,3'-(9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl)bis(3-oxopropane-3,1-diyl))bis(2-amino-3-(4-aminophenyl)propanamide)-tetratrifluoroacetate (5h)



The compound was obtained with the same coupling procedure adopted for **5a,** starting from intermediate **4** and Fmoc-4-(Boc-amino)-L-phenylalanine (Fmoc-4-(NH-Boc)-OH).
Yield: 72%. Orange solid. K' (HPLC): 4.6 (system 1), 4.2 (system 2).
^{1}H-NMR (DMSO-d_6, 400MHz): δ 10.75(s, 2H), 8.57 (t, J=5.2Hz, 2H), 8.50 (d, J=1.8 Hz, 2H), 8.16 (d, J=8.8, 2H), 8.14 (bs, 6H), 8.04 (dd, J=8.4 and 1.8 Hz, 2H), 7.92 (bs, 6H), 7.18 (d, J=5.6, 4H), 7.06 (d, J=5.6, 4H), 3.93 (m, 4H), 3.38 (m, 4H), 2.95 (m, 2H), 2.57 (t, J=5.6 Hz, 4H)
^{13}C-NMR (DMSO-d_6, 400MHz): δ 182.06, 170.96, 168.62, 158.75, 145.36, 135.07, 132.31, 131.22, 129.19, 128.73, 124.16, 120.95, 116.58, 54.14, 37.11, 36.67, 35.53
ESI-MS: 705.4 [M + H]+; 353.3 [M + 2H]++

Materials

All oligonucleotides were synthesized by Metabion International AG (Martinsried, Germany) and stored at -20°C in 10mM Tris-HCl, 1mM EDTA pH 8.0. 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 a kind gift of Prof. Daniele Fabris (The RNA Institute, University at Albany, State University of NY).[3, 4]

Determination of helix stabilization by Thermal Melting Assay

The ability of each compound to bind nucleic acids was measured by the increase of melting temperature of the oligonucleotide in the presence of anthraquinones essentially as described [5]. Melting temperature (T*m*) is the temperature at which 50% molecules of oligonucleotide are denatured. TAR (RNA) and cTAR (DNA) were both 5'-labelled with FAM and 3'-labelled with Dabcyl. TAR/cTAR duplex was obtained by the annealing of labelled TAR and cTAR. Before the analysis of melting profiles we analysed the best conditions to obtain the complete TAR/cTAR annealing employing two different protocols (Fast or Slow annealing): the extent of annealing was verified by gel electrophoresis (Figure S1).

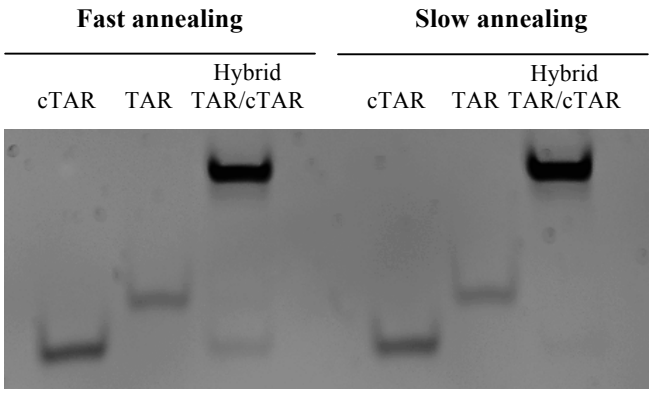


Figure S1: TAR/cTAR hybrid formation. TAR and cTAR (each 1µM) were loaded as controls. The TAR/cTAR hybrid was formed, in TNMg (Tris-HCl 10mM, NaCl 20mM, $Mg(ClO_4)_2$ 1mM pH 7.5) using the fast annealing procedure, on the left, and the slow annealing procedure, on the right. Electrophoresis on a 20% polyacrylamide gel at r.t. in TBE (Tris-HCl 89mM, Borate 89mM, EDTA 2mM). After electrophoresis nucleic acids on the gel were stained with SybrGreen II and detected on a Geliance 600 Imaging System (PerkinElmer).

In buffer TNMg (Tris-HCl 10mM, NaCl 20mM, $Mg(ClO_4)_2$ 1mM pH 7.5) the TAR/cTAR hybrid is completely formed (Figure S1) with both procedures: the fast annealing, in which the oligonucleotides, denatured for 5 minutes at 95°C, were immediately placed at room temperature; the slow annealing, which consisted in the denaturation of oligonucleotides at 95 °C for 5 minutes after what the samples were left to cool gradually to room temperature. As shown in Figure S1, the slow annealing in TNMg is the best procedure to obtain the complete TAR/cTAR hybrid formation.

Therefore, each oligo i.e. TAR, cTAR or annealed TAR/cTAR, were folded at 10µM in TNMg

(Tris-HCl 10mM, NaCl 20mM, $Mg(ClO_4)_2$ 1mM pH 7.5) and then diluted to 1µM concentration in ETN (EDTA 1mM, Tris HCl 12 mM, NaCl 24mM, pH 7.5). In each microplate well the nucleic acid solutions were mixed with the anthraquinones solution to the final concentrations 1, 5, 10 and 100µM. Nucleic acid solutions without compound were used to measure the reference value for T*m* (Table S1). The random dsDNA sequence control was obtained annealing FAM-GTGAGATACCGACAGAGG (FAM-random) with CCTCTGTCGTGATCTCAC-DAB (DAB-Random). Melting protocol consisted in a melting phase, in which the temperature increases from 25°C to 99°C C in 1h (0.02°C/s). Fluorescence emission of FAM was read with Light Cycler 480 II (Roche) with emission at λ=510 nm and was correlated to the melting temperature of the oligonucleotide. The Tm value was mathematically derived from the thermal denaturing profile using LC480 software. Δ*Tm* was calculated using the following equation: $\Delta Tm = Tm_2 - Tm_1$, where Tm_2 and Tm_1 are the T*m* values measured testing the oligonucleotides (or the hybrid) in the presence and in the absence of compound respectively.

Table S1: T*m* reference values (Tm_1) of TAR, cTAR and TAR/cTAR hybrid.

	Tm_1[a] (°C)
TAR	69.30±0.52
cTAR	53.80±0.28
TAR/cTAR hybrid	69.40±0.18
Random dsDNA	49.87±0.71

[a] values are the mean of three experiments performed in triplicate at [nucleic acid]=1µM

Melting experiments were conducted also to compare relative affinity of anthraquinones to annealed double stranded DNA of random sequence (dsDNA), used as a control. The stem of cTAR DNA and the annealed dsDNA are organized in "B" conformation. In Figure S2 we report the relative stabilisation of each oligo expressed as Δ*Tm* toward the random dsDNA and the cTAR, to highlight the different behaviour of anthraquinones and the preferential binding of 5f and 5g to the bulge-loop structure.

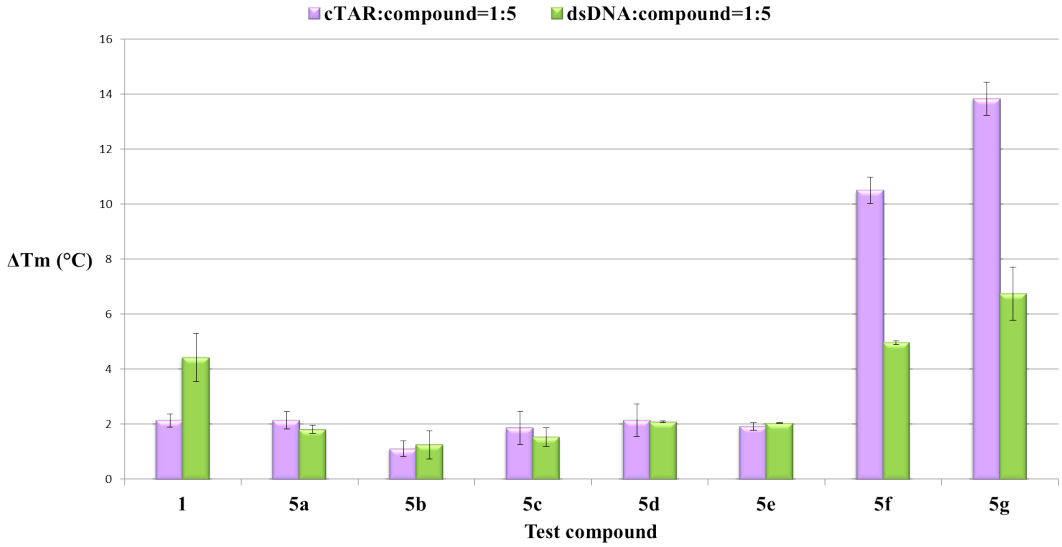


Figure S2: Variation of the melting temperature (Δ*Tm*) of cTAR and annealed random dsDNA upon addition of 2,6-disubstituted anthraquinones. Values reported were experimentally determined at [AQ] = 5 µM in Tris-HCl 10mM, NaCl 20mM, $Mg(ClO_4)_2$ 1mM pH 7.5

Determination of NC-induced helix destabilization by Fluorescence Quenching Assay

The screening of potential inhibitors of the NC-induced TAR and cTAR melting was performed employing an high throughput screening recently described that exploits the ability of NC to melt the oligonucleotide stem increasing the distance between the two ends.[6] TAR and cTAR were doubly end-labeled with a fluorophore (5'-FAM) and a quencher (3'-Dabcyl): the proximity of TAR (or cTAR) ends induces a strong fluorescence quenching. When NC melts the lower half of the nucleic acid stem, a raise of FAM fluorescence can be observed. The full-length recombinant NC protein resulted active on both the bulge-loop structures TAR and cTAR (see Figure S3A for TAR and B for cTAR). We verified preliminarily that none of the tested compounds would affect the fluorescence of the doubly-labeled nucleic acids.

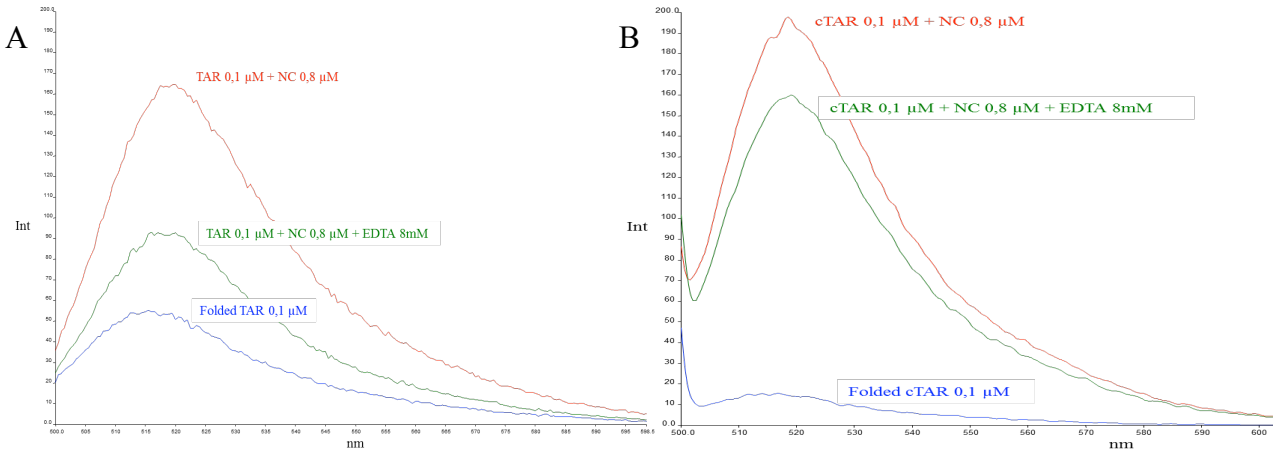


Figure S3: Fluorescence Quenching Assay: analysis of the helix destabilization activity of the full-length NC protein on TAR (A) and on cTAR (B). Fluorescence spectra of 0.1µM 3'-DAB and 5'-FAM modified TAR or cTAR in TNMg (Tris 10 mM, NaCl 20 mM, $Mg(ClO_4)_2$ 1 mM, pH 7.5) in absence (blue line) and in presence (red line) of NC; after adding an excess of EDTA (green line). Spectra were corrected for buffer fluorescence.

Our assays were performed using the full-length recombinant NC: the correct conditions, in terms of relative ratio of protein and nucleic acid, were thoroughly analyzed. The Fluorescence Quenching Assay was carried out using the microplate reader VictorIII (Perkin Elmer): increasing concentrations, from 0 to 1.1 µM, of NC were added to 0.1 µM of folded TAR or cTAR in each well. Since the full-length NC protein possesses several activities in addition to the melting of nucleic acids stem-loop structures, the better relative ratio nucleic acid/NC resulted to be 1/8 to have the higher helix destabilization activity but to avoid aggregation of oligonucleotides, in agreement with what indicated in literature.[7, 8]

Determination of inhibition of NC-induced helix destabilization by anthraquinones

To evaluate the concentrations of the compounds necessary to inhibit the helix destabilization of TAR and cTAR by NC, we used a microplate reader VictorIII (Perkin Elmer) with 485 and 535nm as excitation and emission wavelengths. 5'-FAM and 3'-DAB modified TAR or cTAR (1µM) were folded in TNMg (Tris-HCl 10mM, NaCl 20mM, $Mg(ClO_4)_2$ 0.2mM pH 7.5): the oligonucleotides were denatured at 95°C for 5 minutes and then left to cool to room temperature in order to assume

the stem-loop structure. cTAR or TAR was then diluted to 0.1µM in TN (Tris-HCl 10mM, NaCl 20mM pH 7.5). Increasing concentrations of compound (0, 0.1, 0.5, 1, 5, 10, 50, 100µM) were incubated with 0.1µM cTAR or TAR in each well. Finally, NC 0.8µM (molar ratio oligo/NC=1/8) was added to each sample. The plate was read three times with a delay of 1 minute one reading from the other. The experimental data were fitted as reported and the IC_{50} value was calculated for each compound.[6] Each experiment was performed in triplicate to calculate a standard deviation of the IC_{50} value.

Inhibition of the NC-induced TAR/cTAR annealing

NC possesses several activities in addition to the melting of nucleic acids stem-loop structures described above: one additional activity is the annealing between nucleic acid strands: the protein is positively charged and lowers the electrostatic barrier of the annealing reaction and increases the rate at which two separate complementary sequences comes together.[9] This competence is connected with the helix destabilization activity and with the ability of the protein to aggregate nucleic acids (due to its unstructured N-terminal region). The analysis of the TAR/cTAR annealing mediated by NC, under conditions previously identified by fluorescence assays, was investigated by polyacrilammide gel electrophoresis (Nucleocapsid Annealing Mediated Electrophoresis - NAME - assay).

TAR, cTAR and the hybrid TAR/cTAR (each 1µM) each folded in TNMg (Tris-HCl 10mM, NaCl 20mM, $Mg(ClO_4)_2$ 1mM pH 7.5) were used as controls: the oligonucleotides were denatured at 95°C for 5 minutes and then left to cool to room temperature in order to assume their stem-loop (TAR and cTAR) or double-stranded (hybrid TAR/cTAR) structure. The annealing reaction of TAR to cTAR was then performed both in the absence and in the presence of NC: folded TAR and cTAR were incubated with NC (oligos/NC=1/8) for 3 hours at r.t. or at 37°C (Figure S4). The samples were added of denaturing Gel Loading Buffer (Tris-HCl 100mM, EDTA 4mM, 50% w/v glycerol, 2% w/v SDS, 0.05% w/v bromophenol blue).

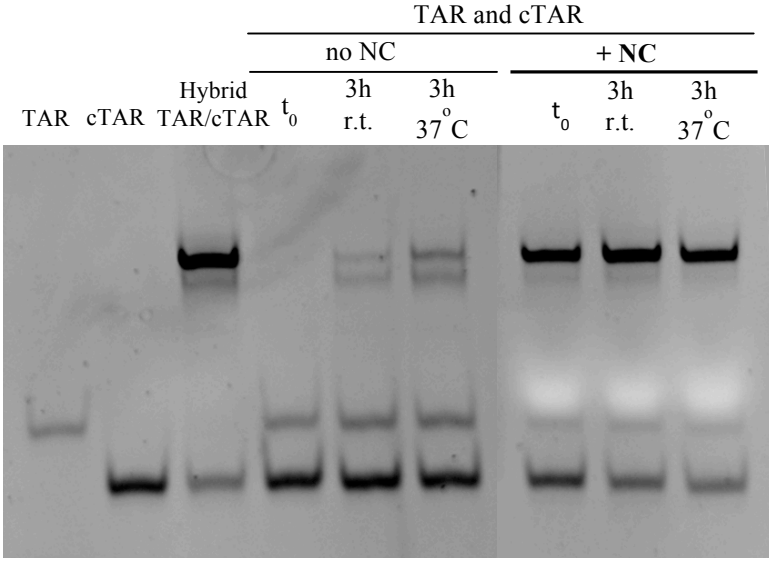


Figure S4: TAR/cTAR annealing reaction in the presence of the full-length recombinant NC protein. Folded TAR, cTAR and the hybrid TAR/cTAR (each 1µM) were used as controls; the annealing of TAR to cTAR was performed both in the absence and in the presence of NC: folded TAR and cTAR were incubated with NC

(oligos/NC=1/8) for 3 hours at r.t. or at 37°C; the reactions immediately stopped (t_0) were also loaded into the gel (lines 4 and 7). All the samples were added of denaturing Gel Loading Buffer. Electrophoresis on a 20% polyacrylamide gel at r.t. in TBE (Tris-HCl 89mM, Borate 89mM, EDTA 2mM). After electrophoresis nucleic acids on the gel were stained with SybrGreen II and detected on a Geliance 600 Imaging System (PerkinElmer).

It is clear from the above gel that NC, as expected catalyzes very efficiently the annealing of the two complementary nucleic acids into the double stranded hybrid, as seen from the appearance of the lower mobility band already at t_0 when NC is present.

To verify the effects of anthraquinones in this model, the test compounds were incubated with the protein for a short time to prevent nucleic acids aggregation and precipitation. Using the molar ratio oligos/NC of 1/8 (same conditions of helix destabilization assay), we identified as 15 minutes the shortest time necessary to completely form the hybrid (data not shown). Hence, folded TAR and cTAR were incubated separately with increasing concentrations of compound (0, 1, 10, 50, 100µM) for 15 minutes at room temperature, then mixed and incubated with NC 8µM (oligos/NC=1/8) for 15 minutes at room temperature. The samples were added of denaturing Gel Loading Buffer, kept steady on ice and finally loaded into the gel. The samples were analyzed by electrophoresis on a 12% polyacrylamide gel at room temperature in TBE (Tris-HCl 89mM, Borate 89mM, EDTA 2mM). After electrophoresis nucleic acids on the gel were stained with SybrGreen II and detected on a Geliance 600 Imaging System (PerkinElmer).

Cytotoxicity assay

The human Jurkat cell line were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U of penicillin per mL and 100 µg of streptomycin per mL (all from GIBCO). The cytotoxicity of the compounds was based on the cell viability measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells ($5x10^3$ per well) were cultured in triplicate in a 96-well plate in the absence or presence of various concentrations of the test compounds. After 72 h of incubation at 37°C, MTT was added to each well, and the mixture was incubated for 4 h before the solubilization with DMSO. The plates were then read at 540 nm with a Microplate Reader (BioRad).

Effect on anti-HIV-infected cells

The human T-lymphoid Jurkat cell lines was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100U of penicillin per ml and 100 µg of streptomycin per mL (Life Biotechnologies). HIV-1 virus stock was produced by transient transfection of Jurkat cells with the pSVC21 plasmid containing the infectious HXB2 molecular clone of HIV-1 using the DEAE-dextran method as described[10] and stored at –80°C until use. Viral titre was measured as 50% tissue culture infective doses per ml ($TCID_{50}$/ml) by the end-point dilution method of Reed-Muench.[11] The compounds effect against acute HIV-1 infection was based on the inhibition of reverse transcriptase (RT) activity in Jurkat cell culture supernatants. Jurkat cells ($1x10^6$ cells) were incubated with virus stock at a multiplicities of infection (m.o.i.) of 0.01 50% tissue culture infective dose/cell. After 2 h of incubation at 37°C, the cultures were washed twice and cultured in the absence or in the presence of various concentrations of the test

compounds, performing a 50-100% medium change every three-four days. Virus replication after infection was monitored by measuring RT activity in cell-free culture supernatants.[12]

The antiviral activity of 5g was evaluated with a transcomplementation assay with the envelope glycoprotein by means of a defective HIV-1 genome containing the chloramphenicol acetiltransferase (CAT) gene under the transcriptional control of the 5'LTR. This assay measures the CAT activity in the lysates of the target cells, thus providing a quantitative assessment of the abilities of the cells to support the early phases of the HIV-1 life cycle.[13] No effects were observed up to 50 micromolar for 5g.

Uptake studies and coefficient partition measurement

Uptake experiments were conducted with slight modification of the method described.[14] Briefly, Jurkat cells ($2{,}5x10^6$/well) were incubated in HBSS (GIBCO) at the concentration of 100μM. After three hours of incubation at 37°C, cells were separated from the extracellular solution by centrifugation through a water-impermeable silicone-oil barrier (density, 1,029 g/cm^3) in a microcentrifuge tube. The cell pellet was lysed overnight with 1 mL of 0.1 M glycine-HCl buffer (pH 3.0) and then the samples were centrifuged for 5 min at 5,600 x g. The amount of compound in the lysis supernatant was determined by absorbance at 360 nm ($\varepsilon = 4900\ M^{-1}cm^{-1}$).

The RP-HPLC technique, commonly used to correlate the hydrophobicity of the compounds with their retention time, was used to measure the partition coefficient $LogP_{OW}$, replacing the usual shake flask method. The RP-HPLC method is based on the linear relationship between the values $LogP_{OW}$ and the Logk'. The k' factor is defined as: $k'=(t_{compound}-t_0)/t_0$, where $t_{compound}$ is the elution time of retained peak and t_0 the elution time of an unretained peak.

The analysis was performed with a C18 Symmetry column (Waters; 5 μm, 300 Å, 250 x 4.60 nm); the mobile phase used is a mix 50% H_2O (with 0.1%TFA) and 50% Methanol, eluting at 1 mL/min. To calibrate the system, the retention time of six compounds (thiourea, 2-butanone, acetophenone, nitrobenzene, benzene and naphthalene) with known $LogP_{OW}$ was measured and plotted: in abscissa the Logk' and in ordinate the $LogP_{OW}$. The resulting tritation line was used to value the $LogP_{OW}$ of all of our compounds. The related $LogP_{OW}$ values are reported in the Table S2.

Table S2: Experimentl logP pf test compounds.

Compound	$LogP_{OW}$
5a	-2.982
5b	-2.913
5c	-2.306
5d	-2.913
5e	-2.555
5f	-2.553
5g	-2.512
5h	-2.511

REFERENCES

1. G. Zagotto, A. Ricci, E. Vasquez, A. Sandoli, S. Benedetti, M. Palumbo and C. Sissi, *Bioconjug Chem*, 2011, 22, 2126-2135.
2. G. Zagotto, C. Sissi, L. Lucatello, C. Pivetta, S. A. Cadamuro, K. R. Fox, S. Neidle and M. Palumbo, *J Med Chem*, 2008, 51, 5566-5574.
3. K. B. Turner, A. S. Kohlway, N. A. Hagan and D. Fabris, *Biopolymers*, 2009, 91, 283-296.
4. N. Hagan and D. Fabris, *Biochemistry*, 2003, 42, 10736-10745.
5. A. Gianoncelli, S. Basili, M. Scalabrin, A. Sosic, S. Moro, G. Zagotto, M. Palumbo, N. Gresh and B. Gatto, *ChemMedChem*, 2010, 5, 1080-1091.
6. V. Shvadchak, S. Sanglier, S. Rocle, P. Villa, J. Haiech, M. Hibert, A. Van Dorsselaer, Y. Mély and H. de Rocquigny, *Biochimie*, 2009, 91, 916-923.
7. H. Beltz, C. Clauss, E. Piemont, D. Ficheux, R. J. Gorelick, B. Roques, C. Gabus, J. L. Darlix, H. de Rocquigny and Y. Mely, *J Mol Biol*, 2005, 348, 1113-1126.
8. I. Kanevsky, F. Chaminade, Y. Chen, J. Godet, B. Rene, J. L. Darlix, Y. Mely, O. Mauffret and P. Fosse, *Nucleic Acids Res*, 2011, 39, 8148-8162.
9. J. G. Levin, J. Guo, I. Rouzina and K. Musier-Forsyth, *Prog Nucleic Acid Res Mol Biol*, 2005, 80, 217-286.
10. B. R. Cullen, *Methods Enzymol*, 1987, 152, 684-703.
11. E. Jawetz, J. L. Melnick and E. A. Adelberg, *Serologic diagnosis and immunologic detection of virus infection*, Lange Medical Publications, Los Altos, California, 1980.
12. H. M. Rho, B. Poiesz, F. Ruscetti and R. C. Gallo, *Virology*, 1981, 112, 355-360.
13. C. Parolin, B. Gatto, C. Del Vecchio, T. Pecere, E. Tramontano, V. Cecchetti, A. Fravolini, S. Masiero, M. Palumbo and G. Palù, *Antimicrob Agents Chemother*, 2003, 47, 889-896.
14. G. Manfroni, B. Gatto, O. Tabarrini, S. Sabatini, V. Cecchetti, G. Giaretta, C. Parolin, C. D. Vecchio, A. Calistri, M. Palumbo and A. Fravolini, *Bioorg Med Chem Letters*, 2009, 19, 714-717.