# Supporting Information

# Multifaceted aspects of HIV-1 nucleocapsid inhibition by TAR-targeting peptidyl-anthraquinones bearing terminal aromatic moieties

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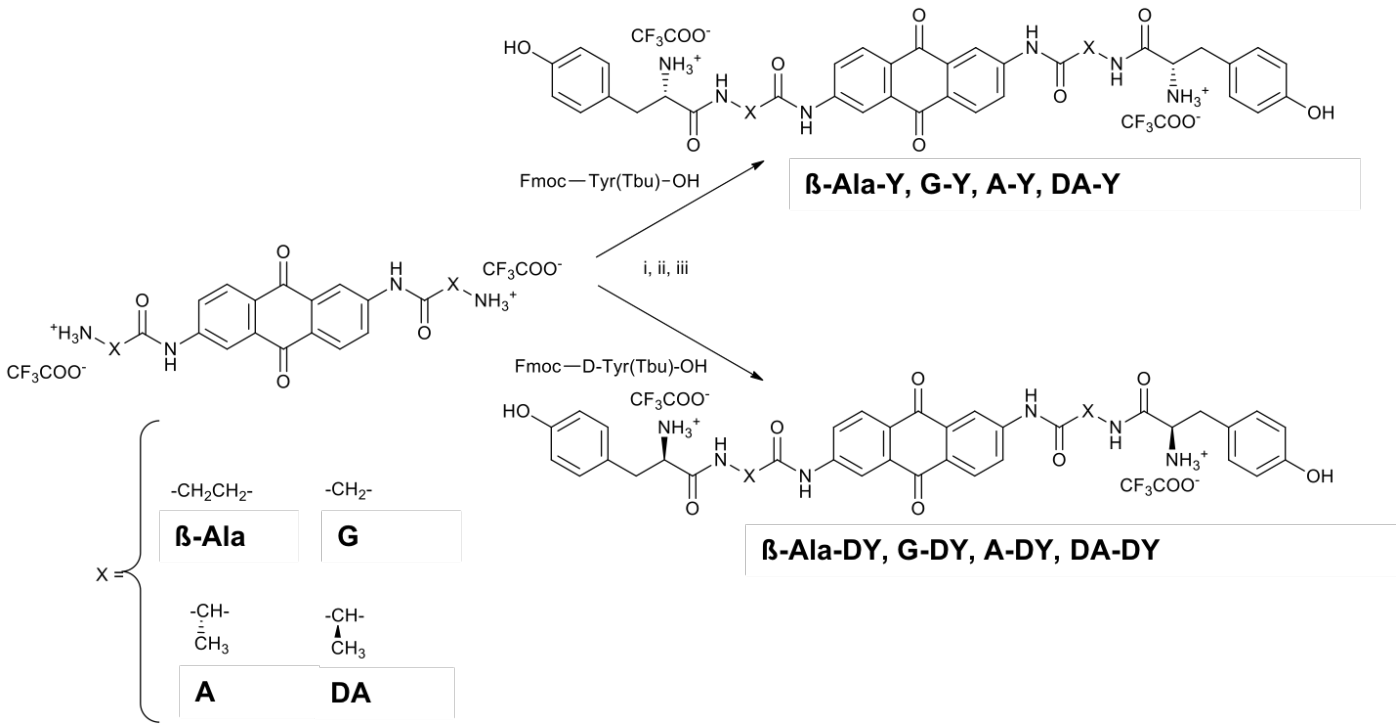
**Scheme S1. General synthetic pathway for the preparation of the anthraquinone derivatives included in the study and their characterization.**
Reagents and conditions: (i) HBTU, DMAP, DMF; (ii) 33% N,N-diethylamine, THF; (iii) TFA/$H_2O$.




**(2S,2'S)-N,N'-(((9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl))bis(3-oxopropane-3,1-diyl))bis(2-amino-3-(4-hydroxyphenyl)propanamide)-bis-trifluoroacetate (ß-Ala-Y)**

N,N'-(9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(3-aminopropanamide)-bis-trifluoroacetate (**ß-Ala**, 128 mg, 0.21 mmol) was dissolved in dry DMF (9 mL), and DMAP (282 mg, 2.31 mmol), Fmoc-Tyr(Tbu)-OH (965 mg, 2.1 mmol), and HBTU (796 mg, 2.1 mmol) were added. The resulting solution was stirred at room temperature for 24 hours, then poured into $Et_2O$ and centrifuged. The solid obtained was washed with $Et_2O$ and the final product was dried in vacuo and used in the next step without any further purification. In order to remove the Fmoc- protecting group, the solid obtained was reacted with a 33% diethylamine solution in THF (20 mL) for 2 hours, poured into $Et_2O$ 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, 10 mL) for 1 hour and then poured into $Et_2O$. 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 **ß-Ala-Y** as an intense orange solid. Yield: 68%. Orange solid. K'(HPLC): 4.6. ESI-MS: 707.4 $[M + H]^+$; 354.4 $[M + 2H]^{++}$.

**(2S,2'S)-N,N'-(((9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl))bis(2-oxoethane-2,1-diyl))bis(2-amino-3-(4-hydroxyphenyl)propanamide)-bis-trifluoroacetate (G-Y)**

The compound was obtained with the same coupling procedure adopted for **ß-Ala-Y**, starting from **G** and Fmoc-Tyr(Tbu)-OH. Yield: 64%. Orange solid. K'(HPLC): 4.7. ESI-MS: 679.4 $[M + H]^+$; 340.3 $[M + 2H]^{++}$.

**(2S,2'S)-N,N'-((2S,2'S)-((9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis-(azanediyl))bis(1-oxopropane-2,1-diyl))bis(2-amino-3-(4-hydroxyphenyl)-propanamide)-bis-trifluoroacetate (A-Y)**

The compound was obtained with the same coupling procedure adopted for **ß-Ala-Y**, starting from **A** and Fmoc-Tyr(Tbu)-OH. Yield: 56%. Orange solid. K′ (HPLC): 4.3. ESI-MS: 707.4 $[M + H]^{+}$; 354.2 $[M + 2H]^{++}$.

**(2S,2'S)-N,N'-((2R,2'R)-((9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis-(azanediyl))bis(1-oxopropane-2,1-diyl))bis(2-amino-3-(4-hydroxyphenyl)-propanamide)-bis-trifluoroacetate (DA-Y)**

The compound was obtained with the same coupling procedure adopted for **ß-Ala-Y**, starting from **DA** and Fmoc-Tyr(Tbu)-OH. Yield: 62%. Orange solid. K′ (HPLC): 4.4. ESI-MS: 707.6 $[M + H]^{+}$; 354.5 $[M + 2H]^{++}$.

**(2R,2'R)-N,N'-(((9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl))bis(3-oxopropane-3,1-diyl))bis(2-amino-3-(4-hydroxyphenyl)propanamide)-bis-trifluoroacetate (ß-Ala-DY)**

The compound was obtained with the same coupling procedure adopted for **ß-Ala-Y**, starting from **ß-Ala** and Fmoc-D-Tyr(Tbu)-OH. Yield: 59%. Orange solid. K′(HPLC): 4.6. ESI-MS: 707.3 $[M + H]^{+}$; 354.3 $[M + 2H]^{++}$.

**(2R,2'R)-N,N'-(((9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis(azanediyl))bis(2-oxoethane-2,1-diyl))bis(2-amino-3-(4-hydroxyphenyl)propanamide)-bis-trifluoroacetate (G-DY)**

The compound was obtained with the same coupling procedure adopted for **ß-Ala-Y**, starting from **G** and Fmoc-D-Tyr(Tbu)-OH. Yield: 55%. Orange solid. K′(HPLC): 4.6. ESI-MS: 679.3 $[M + H]^{+}$; 340.3 $[M + 2H]^{++}$.

**(2R,2'R)-N,N'-((2S,2'S)-((9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis-(azanediyl))bis(1-oxopropane-2,1-diyl))bis(2-amino-3-(4-hydroxyphenyl)-propanamide)-bis-trifluoroacetate (A-DY)**

The compound was obtained with the same coupling procedure adopted for **ß-Ala-Y**, starting from **A** and Fmoc-D-Tyr(Tbu)-OH. Yield: 54%. Orange solid. K′ (HPLC): 4.3. ESI-MS: 707.3 $[M + H]^{+}$; 354.3 $[M + 2H]^{++}$.

**(2R,2'R)-N,N'-((2R,2'R)-((9,10-dioxo-9,10-dihydroanthracene-2,6-diyl)bis-(azanediyl))bis(1-oxopropane-2,1-diyl))bis(2-amino-3-(4-hydroxyphenyl)-propanamide)-bis-trifluoroacetate (DA-DY)**

The compound was obtained with the same coupling procedure adopted for **ß-Ala-Y**, starting from **DA** and Fmoc-D-Tyr(Tbu)-OH. Yield: 49%. Orange solid. K′ (HPLC): 4.4. ESI-MS: 707.4 $[M + H]^{+}$; 354.5 $[M + 2H]^{++}$.

**Figure S1. Dose-response curves resulting from the NC-mediated melting in the presence of 2,6-AQs on TAR substrate.**

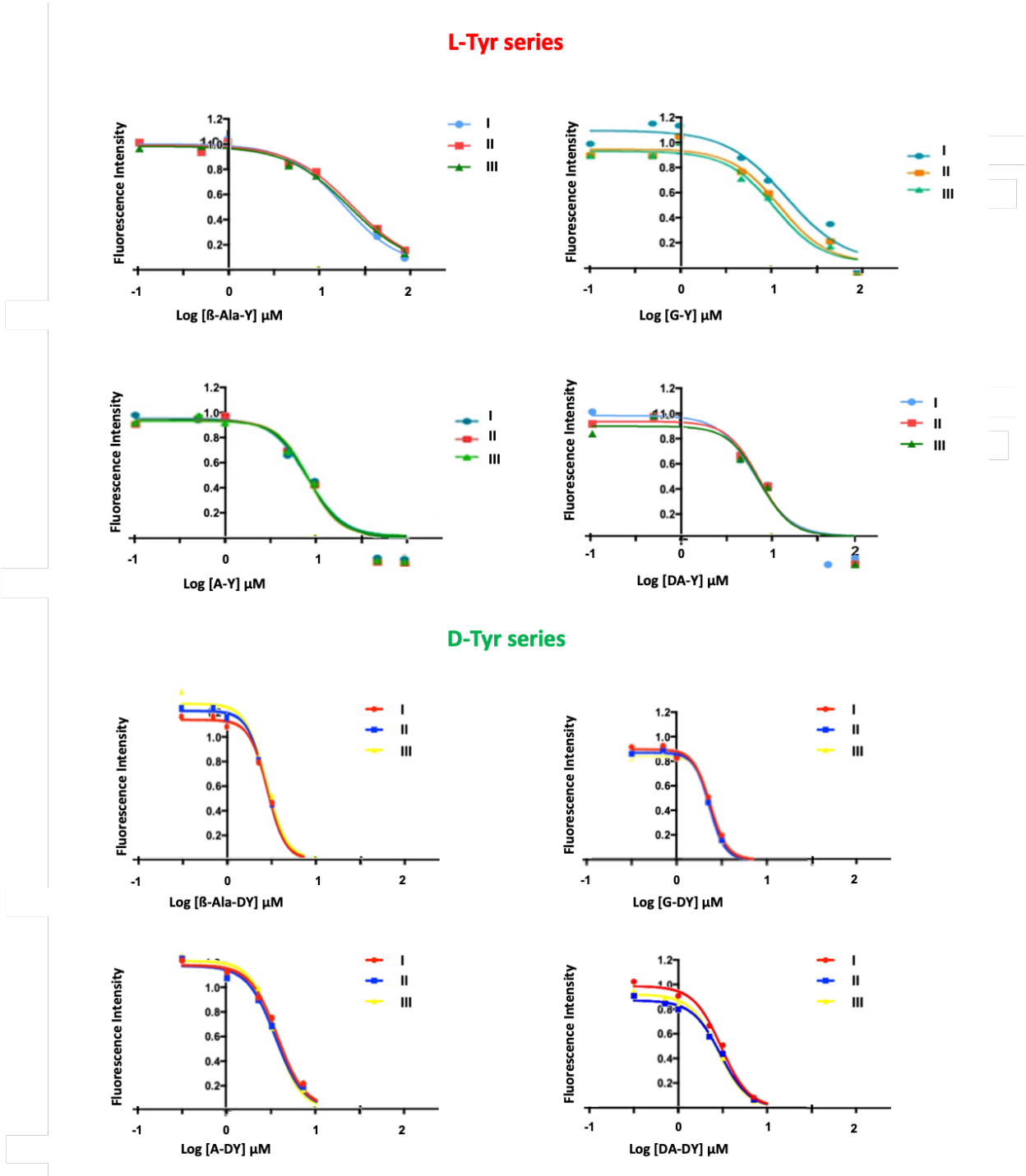


**Table S1. Inhibition of NC-mediated melting of cTAR by AQ-AA$_1$-L-Tyr (red columns) and AQ-AA$_1$-D-Tyr (green columns).**

| AQ-AA$_1$-L-Tyr | IC$_{50}$ cTAR (μM) | AQ-AA$_1$-D-Tyr | IC$_{50}$ cTAR (μM) |
|---|---|---|---|
| **ß-Ala-Y** | 19.4 ± 0.40 | **ß-Ala-DY** | 8.17 ± 0.53 |
| **G-Y** | 15.2 ± 1.49 | **G-DY** | 5.14 ± 0.28 |
| **A-Y** | 10.7 ± 0.31 | **A-DY** | 10.4 ± 1.73 |
| **DA-Y** | 8.85 ± 0.51 | **DA-DY** | 10.2 ± 0.86 |

**Figure S2. Representative ESI-MS spectra of samples obtained by mixing TAR with compounds of the L-Tyr and D-Tyr series.** In the figure are reported the ESI-MS spectra acquired in negative ion mode of samples obtained by mixing 1 μM of TAR with 10 μM of (**A**) **ß-Ala-Y**, (**B**) **G-Y**, (**C**) **A-Y**, (**D**) **DA-Y**, (**E**) **ß-Ala-DY**, (**F**) **G-DY**, (**G**) **A-DY**, (**H**) **DA-DY** in 150 mM ammonium acetate. Symbol ■ corresponds to TAR RNA substrate and the red ● to the ligand. The stoichiometry of bound species is graphically represented in each spectrum. Lower intensity signals near free/bound species consist of typical sodium and ammonium adducts.

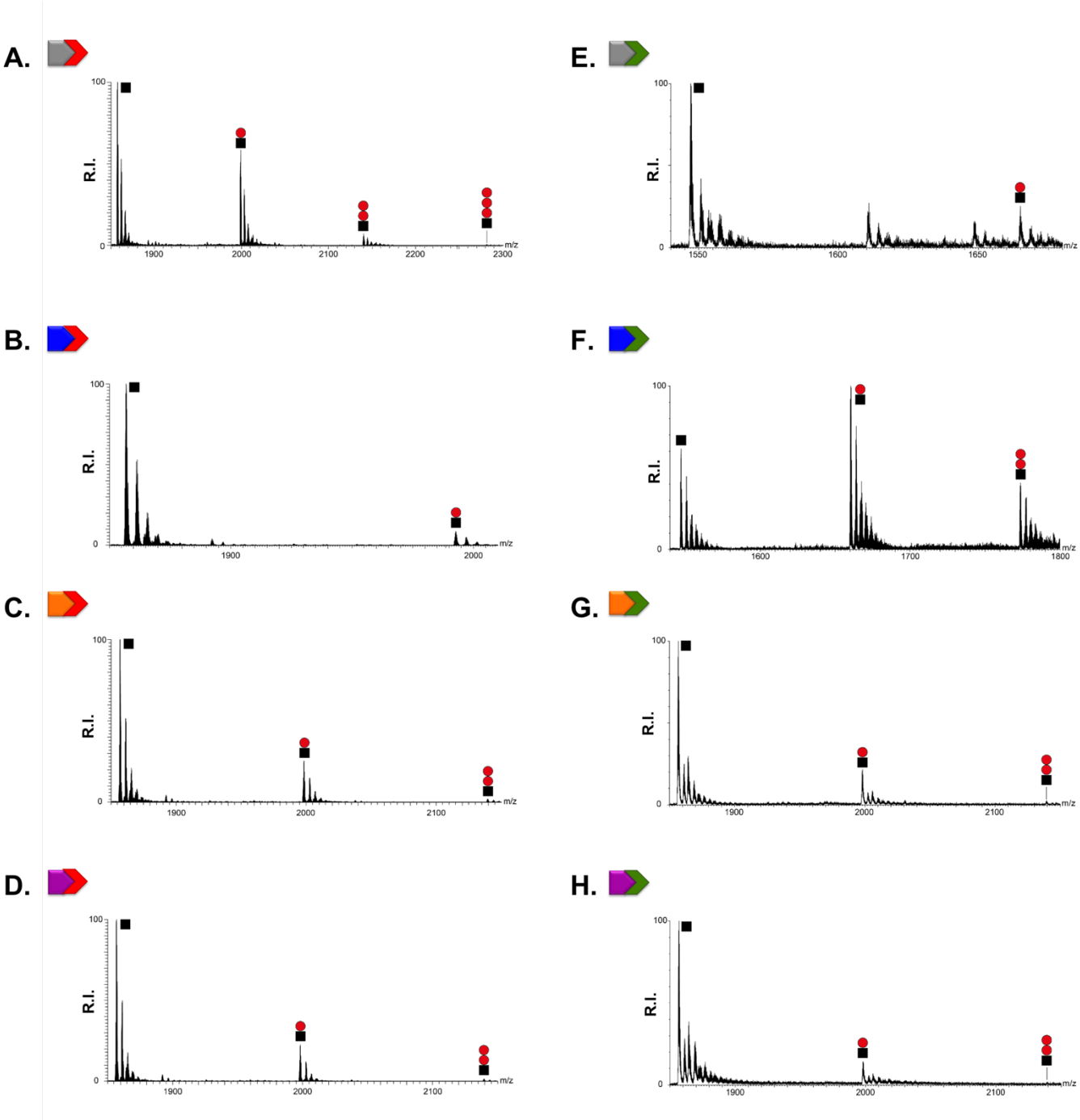


**Table S2. Binding stoichiometries of compound-nucleic acid complexes detected in the ESI-MS spectra.** The interactions established by AQ-L-Tyr and AQ-D-Tyr compounds with TAR constructs employed as target produced different binding stoichiometries. Data are elaborated from the negative ion mode spectra obtained for 10:1 anthraquinone:TAR molar ratio in 150 mM ammonium acetate.

| Compound | 1:1 AQ:TAR | 2:1 AQ:TAR | 3:1 AQ:TAR |
|---|---|---|---|
| **ß-Ala-Y** | X | X | X |
| **G-Y** | X | | |
| **A-Y** | X | X | |
| **DA-Y** | X | X | |
| **ß-Ala-DY** | X | | |
| **G-DY** | X | X | |
| **A-DY** | X | X | |
| **DA-DY** | X | X | |

**Table S3. Variations of TAR melting temperature ($\Delta T_m$) in the presence of an increasing concentration of the compounds of the L-Tyr and D-Tyr series.** Reported values are the mean ± standard error of the mean (SEM) of triplicate experiments performed on samples containing 1 μM of nucleic acid substrate and 10 μM of compound. The reference $T_m$ value for TAR, cTAR and the TAR/cTAR hybrid in the absence of compound are 69.3, 53.8 and 69.4 °C, respectively.

| L-Tyr | $\Delta T_m$ TAR (°C) | $\Delta T_m$ cTAR (°C) | $\Delta T_m$ TAR/cTAR (°C) | D-Tyr | $\Delta T_m$ TAR (°C) | $\Delta T_m$ cTAR (°C) | $\Delta T_m$ TAR/cTAR (°C) |
|---|---|---|---|---|---|---|---|
| **ß-Ala-Y** | 0.03 ± 0.05 | 0.23 ± 0.27 | 0.60 ± 0.05 | **ß-Ala-DY** | 2.62 ± 0.11 | 5.81 ± 0.18 | 1.06 ± 0.14 |
| **G-Y** | 1.29 ± 0.04 | 2.38 ± 0.24 | 1.19 ± 0.28 | **G-DY** | 2.05 ± 0.20 | 3.34 ± 1.31 | 0.94 ± 0.04 |
| **A-Y** | 2.88 ± 0.04 | 4.48 ± 0.14 | 1.10 ± 0.24 | **A-DY** | 0.40 ± 0.74 | 2.50 ± 1.44 | 0.41 ± 0.06 |
| **DA-Y** | 0.52 ± 0.05 | 2.32 ± 0.03 | 1.10 ± 0.01 | **DA-DY** | 0.34 ± 0.09 | 2.37 ± 0.53 | 0.41 ± 0.11 |

**Figure S3. Representative ESI-MS spectra of samples obtained by mixing NC with compounds of the L-Tyr and D-Tyr series.** In the figure are reported the ESI-MS spectra acquired in positive ion mode of samples obtained by mixing 10 μM of NC with 100 μM of (**A**) **ß-Ala-Y**, (**B**) **G-Y**, (**C**) **A-Y**, (**D**) **DA-Y**, (**E**) **ß-Ala-DY**, (**F**) **G-DY**, (**G**) **A-DY**, (**H**) **DA-DY** in 150 mM ammonium acetate. The grey ▲ symbol corresponds to NC protein and the red ● to the ligand. The stoichiometry of bound species is graphically represented in each spectrum. Lower intensity signals near free/bound species consist of typical sodium and ammonium adducts.

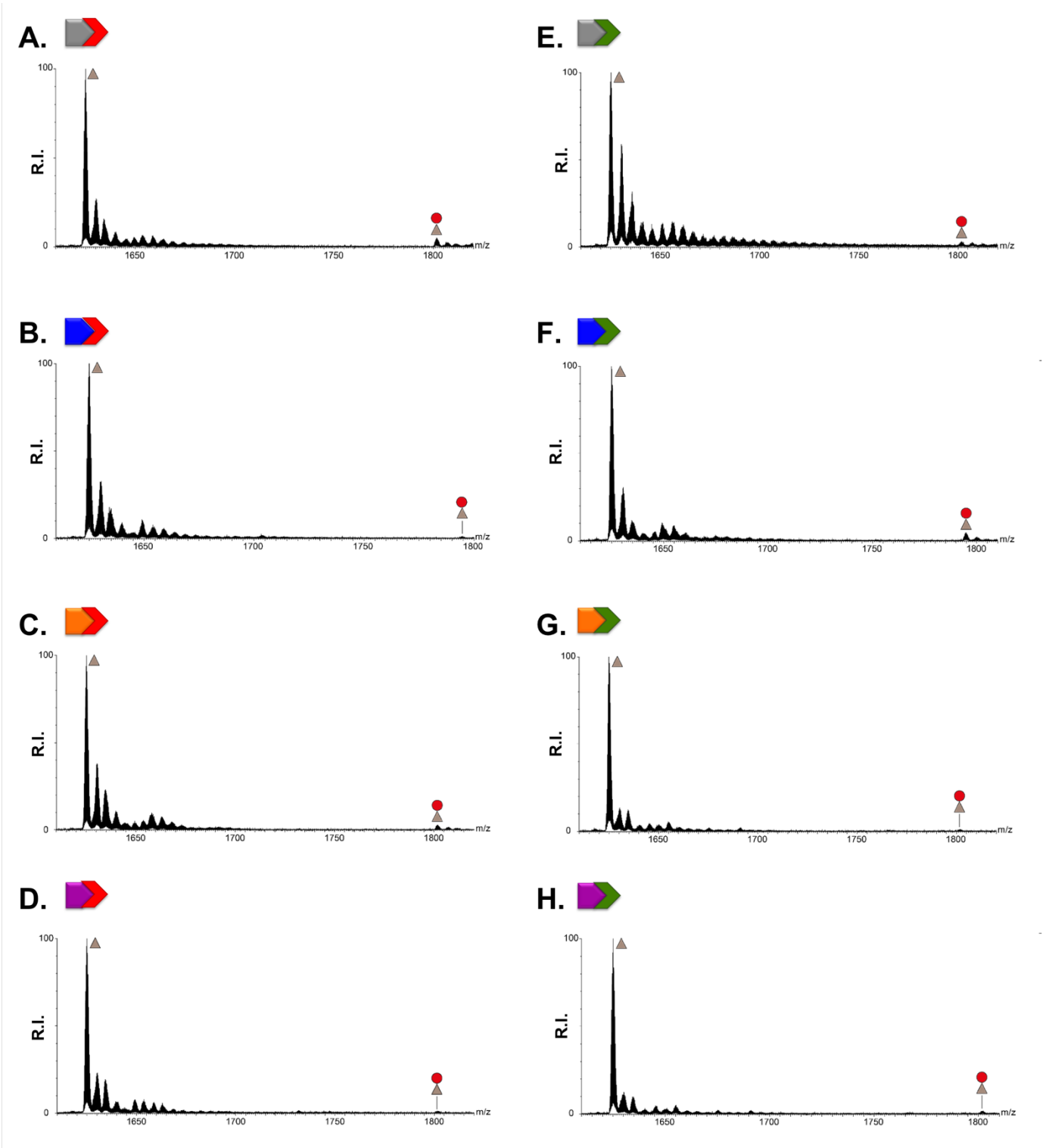


**Figure S4. Representative ESI-MS spectra of samples obtained by mixing Tat with compounds of the L-Tyr and D-Tyr series.** In the figure are reported the ESI-MS spectra acquired in positive ion mode of samples containing 10 μM of Tat (**A**), and obtained mixing 10 μM of Tat with 100 μM of **ß-Ala-Y** (**B**) in 150 mM ammonium acetate. In both spectra A and B, the peak detected at m/z 698.98 corresponds to the Tat protein. In spectrum B, compound **ß-Ala-Y** was identified at m/z 707.27. Lower intensity signals consist of typical sodium and ammonium adducts.

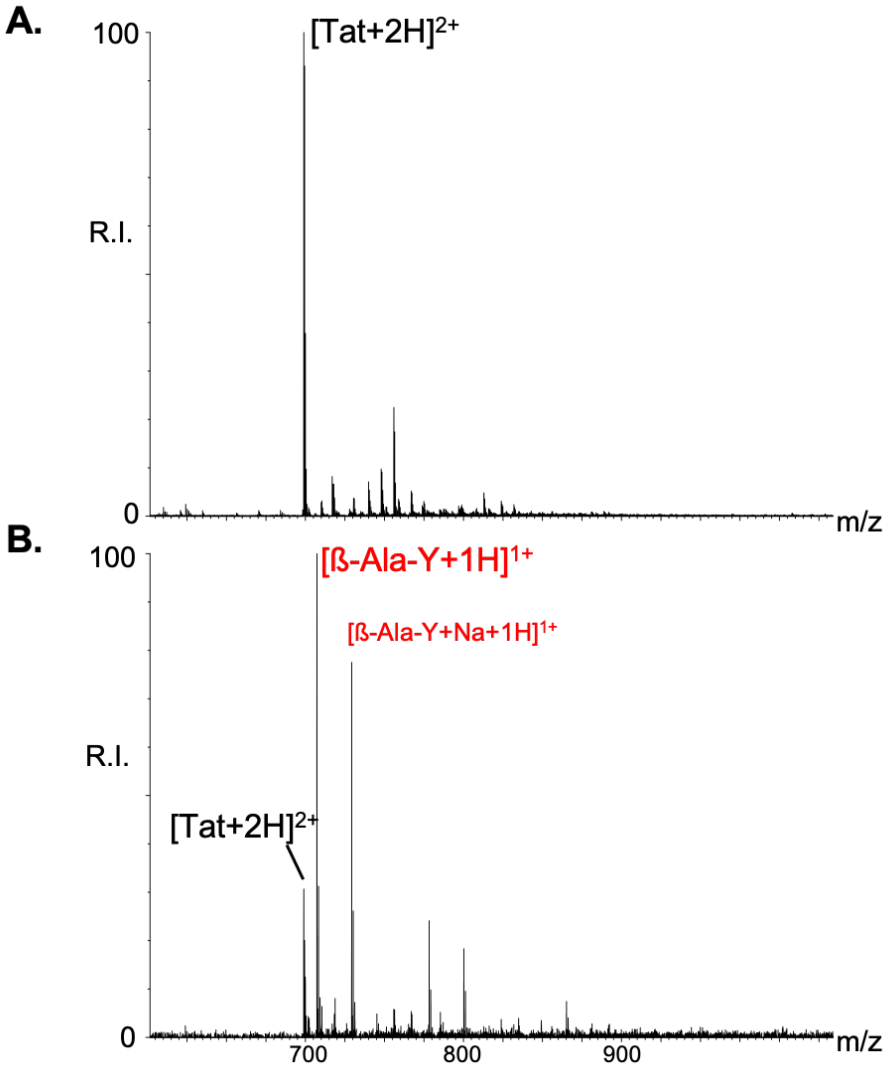


**Table S4. Experimental and calculated monoisotopic masses (Da) of 2,6-AQ:Tat complexes.** Expected masses of 1:1 complexes were calculated from the sequence of Tat protein (GRKKRRQRRR) and the elemental composition of each compound. No complexes were detected in the ESI-MS spectra.

| Compound | 1:1 AQ:Tat complex | |
|---|---|---|
| | Experimental Mass (Da) | Calculated Mass (Da) |
| **ß-Ala-Y** | Not detected | 2102.19 |
| **G-Y** | Not detected | 2074.17 |
| **A-Y** | Not detected | 2102.19 |
| **DA-Y** | Not detected | 2102.19 |
| **ß-Ala-DY** | Not detected | 2102.19 |
| **G-DY** | Not detected | 2074.17 |
| **A-DY** | Not detected | 2102.19 |
| **DA-DY** | Not detected | 2102.19 |

**Figure S5. ESI-MS spectra of samples obtained by adding NC to the preformed ligand•TAR complexes.** The tested compounds are (**A**) **A-Y**, (**B**) **A-DY**, (**C**) **DA-Y**, (**D**) **DA-DY**. Lower intensity signals near free/bound species consist of typical sodium and ammonium adducts. Gray ▲ corresponds to NC protein; ■ to the TAR RNA substrate; and red ● to the ligand. For the sake of clarity only the 5+ and 6+ charge states of the binary (RNA-ligand) and ternary (RNA-ligand- protein) complexes are labeled in the spectra.

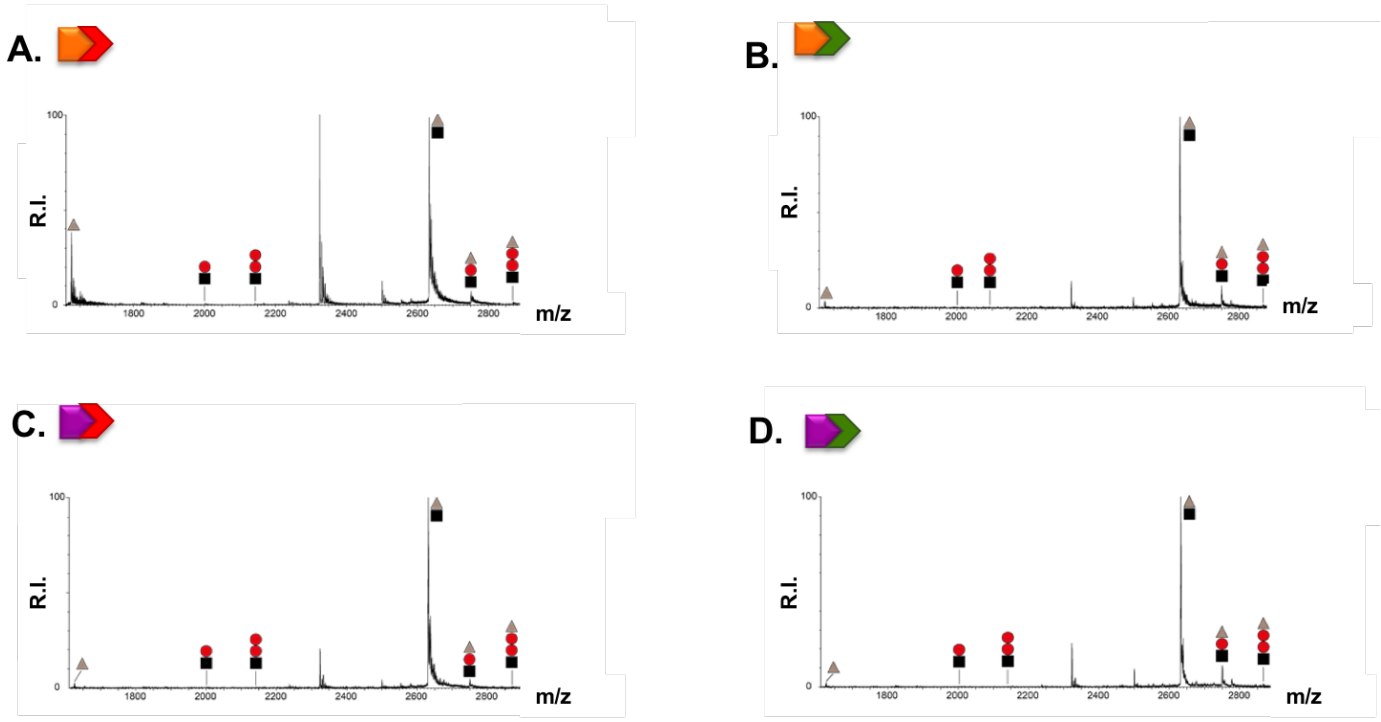


**Figure S6. Parallel Artificial Membrane Permeation Assays (PAMPA).** The graph reports the percentage of each compound detected in each compartment after 24 hours of incubation. In these assays, we included **ß-Ala-DK** and **ß-Ala-DOrn** as representative derivatives of the ß-Ala series of aliphatic charged compounds bearing D-Lysine and D-Ornithine as terminal residue, respectively.