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Multivalency in Recognition and Antagonism of HIV TAR RNA –TAT Assembly using an Aminoglycoside Benzimidazole Scaffold

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Material and Methods

Chemicals

All chemicals were purchased from commercial suppliers and used without further purification. Neomycin B tri-sulfate was purchased from MP Biomedicals (Solon, OH), ditert-butyl dicarbonate (Boc anhydride) was purchased from Advanced ChemTech (Louisville, KY), SC (Sodium cacodylate), EDTA (Ethylenediaminetetraacetic acid), KCl, and sodium phosphate (mono- and di-) salts were purchased from Fisher Scientific (Hampton, NH). DMAP (4-dimethyl-aminopyridine) was purchased from Acros organics (Fair Lawn, NJ). TCDP (1,1'-thiocarbonyldi-2(1*H*)-pyridone), TPS-Cl (2,4,6-triisopropylbenzenesulfonyl chloride), bis(chloro ethyl) amine hydrochloride, sodium azide, 6-bromohexanoyl chloride, propargyl ether, DIPEA (N,N-diisopropylethylamine) and 4M HCl in dioxane were purchased from Sigma Aldrich (St. Louis, MO). Silica plates (w/UV254, aluminum backed) and silica gel (particle size = 40-63 micron, 230×400 mesh) for flash column chromatography were purchased from Sorbent Technologies (Atlanta, GA). All solvents were purchased from VWR. Reaction solvents were distilled over calcium hydride [pyridine, DCM (dichloromethane)]. EtOH (Ethanol) was first distilled with sodium metal and then redistilled over magnesium turnings. Reactions were conducted under N2 using a dry solvent, unless otherwise noted.

Instrumentation

¹H NMR spectra were collected on a Bruker 500 MHz FT-NMR spectrometer, and the MS (MALDI-TOF) spectra were collected using a Bruker Omniflex MALDI-TOF mass spectrometer. FID assays were performed in 96-well plates (Black w/flat bottom, Greiner

Bio-one, Monroe, NC) on a Genios Multi-Detection Microplate Reader, TECAN (McLean, VA) with Magellan software.

Competition FRET Assay. To a solution of 100 nM HIV-1 TAR RNA and 100 nM fluorescein-labeled HIV-1 Tat peptide were added appropriate concentrations (0 nM to 100 μ M) of the small molecules at 25°C; the total volume of the incubation solution was 80 μ L. After 60 min, the change in fluorescence intensity of the sample solution were determined with the Spectra Max fluorimeter detector. The experimental dose response data for the ligands were fit to a sigmoidal dose-response non-linear regression model on GraphPad Prism 4.0 to afford the IC₅₀ values for each ligand.

Ethidium Bromide Displacement Titration. A solution of ethidium bromide (1.25μ M, 1800 μ L) was excited at 545 nm and the fluorescence emission was monitored from 560 to 620 nm in the absence and presence of HIV TAR RNA. The concentration of HIV TAR RNA was 50 nM/strand. A small fraction of ethidium bromide is bound (<20%) under these conditions. Buffer conditions were 10 mM SC, 0.5 mM EDTA, 100 mMKCl at pH 6.8.

Ethidium Bromide Displacement Titration to Determine the Binding Constant via Scatchard Analysis. A solution of ethidium bromide (5.0μ M, 1800μ L) was excited at 545 nm and its fluorescence emission was monitored from 560 to 620 nm before and after the addition of HIV TAR RNA. The concentration of HIV TAR RNA was 200 nM/strand. Buffer conditions were 10 mM SC, 0.5 mM EDTA, 100 mM KCl at pH 6.8.

HPLC Purification. A measurable quantity (~5 mg) of compound **5** was purified on Thermofisher Scientific HPLC instrument equipped with LiCHrospher 100, RP-18 (5 μ) column. Purification was done in the following elution sequence— Solvents: A, Acetonitrile (with 0.1% TFA); B, H₂O (with 0.1% TFA). The solvent gradient used for compound purification was: 0-6 min, 10% A in B; 6-25 min, 40% A in B; 25-30 min, 0% A in B at a flow rate of 1.0 mL min⁻¹.

Synthesis and characterization

Synthesis of N, N-bis(2-azidoethyl)-6-bromohexanamide (6).¹



Scheme S1. Reagents and conditions: (a) NaN_3 , water, 100°C, 48h, 90% (b) Dichloromethane, aq. NaOH (3M), 0°C, 1h, 86%.

Synthesis of bis(2-azidoethyl)amine (10).

To a solution of bis(chloro ethyl) amine hydrochloride (1.4 g, 1.0 mmol) in water (10.0 mL), sodium azide (3.2 g, 5.0 mmol) was added and the reaction mixture was stirred at 100°C for 48h. The reaction mixture was allowed to come at room temperature followed by the addition of 3M NaOH to increase the pH to ~10. The compound was extracted with diethyl ether (3×20.0 mL). The organic layer was dried over Na₂SO₄ and the evaporation of solvents resulted in greenish oil. (1.4 g, 90%): R_f = 0.30 [15% CH₃OH in CH₂Cl₂ (*v/v*)]; IR (neat, cm⁻¹) 3411 (broad), 2976, 2104 (azide), 1618, 1541; ¹H NMR (500 MHz, CDCl₃) δ 3.35 (t, *J* = 6.62 Hz, 4H, N₃-<u>CH₂-CH₂-N-), 2.85 (t, *J* = 6.52 Hz, 4 H, N₃-CH₂-CH₂-N-); ¹³C NMR (125 MHz, CDCl₃) δ 51.27 (N₃-<u>CH₂-CH₂-N-), 48.04 (N₃-CH₂-<u>CH₂-N-).</u></u></u>

Synthesis of N, N-bis(2-azidoethyl)-6-bromohexanamide(6).

To a solution of bis (azido diethyl) amine (10) (0.5 g, 0.3 mmol) in dichloromethane (2.0 mL), an aqueous solution of 3M NaOH (2.6 mL) was added and the reaction mixture was stirred at0°C for 15 min followed by dropwise addition of a solution of 6-bromohexanoyl chloride (1.3 g, 0.6 mmol in 1.0 mL dichloromethane). The reaction mixture was then stirred for 1h at 0°C. The organic layer was washed with 3N HCl (3×3.0 mL), dried over Na₂SO₄

and evaporation of solvent results in greenish oil (0.9 g, 86%). [R_f= 0.5, 50% ethylacetate in hexane (ν/ν)]; IR (neat, cm⁻¹): 3340 (broad), 2914, 2104 (broad, azide), 1701, 1465; ¹H NMR (500 MHz, CDCl₃): δ 3.42-3.50 (m, 6H), 3.37-3.41 (m, 2H), 3.34 (t, J = 6.62 Hz, 2H), 2.30 (t, J = 7.41 Hz, 2H), 1.80 (m, J = 6.93 Hz, 2H), 1.59 (m, J = 7.72 Hz, 2H), 1.40 (m, J = 7.41 Hz, 2H); ¹³C NMR (500 MHz, CDCl₃): δ 173.2, 49.9, 49.4, 48.1, 46.2, 33.7, 32.7, 32.5, 27.7, 24.1; MS (ESI-HRMS) *m*/zcalcd. for C₁₀H₁₈BrN₇O 332.0831, found 332.0834.

Synthesis of Hexa-N-Boc deoxy-neomycin-5"-propargyl ether (7).²



Scheme S2. Reagent and conditions: (a) Toluene (anhydrous), CuI, DIPEA (anhydrous), 90 °C, 18 h, 89%.

Synthesis of Hexa-N-Boc deoxy-neomycin-5''-propargyl ether (7)

To a solution of Hexa-*N*-Boc deoxy-neomycin-5"-azide (**11**) (135.0 mg, 105.0 μ mol) in EtOH (2.5 mL), a solution of propargyl ether (1.3 g, 14.0 mmol in1.0 mL EtOH) was added followed by addition of CuSO₄ (3.2 mg, 20.0 μ mol in 0.5 mL water) and sodium ascorbate (7.9 mg, 4.0 μ mol in 0.5 mL water). The reaction mixture was stirred at room temperature for 48 h. The progress of the reaction was monitored by TLC. The reaction was stopped by adding DCM, followed by filtration. The volatiles were evaporated under reduced pressure. Column chromatography on silica gel [0 to 10 % EtOH in DCM (ν/ν] was used to purify the crude mixture which gave the desired product as a yellowish solid (129.2 mg, 89%):[R_f 0.58, 10 % EtOH in DCM (ν/ν)]; ¹H NMR (500 MHz, Acetone- d_6) δ 8.21 (s, 1H, triazole), 6.52 (t, *J* = 5.83 Hz, 1H, NH_{6IV}), 6.33-6.25 (m, 1H), 6.18-5.98 (m, 5H, NH_{6II}, NH₁₁, NH₃₁, NH_{21V}, and

NH_{2II}), 5.98-5.91 (br, s, 1H), 5.30-5.19 (m, 2H), 5.03-4.93 (m, 3H), 4.90 (dd, J_I = 3.79 Hz, J_2 = 3.94 Hz, 1H), 4.82-4.74 (m, 3H), 4.73-4.66 (m, 3H), 4.47-4.39 (br, s, 2H), 4.32 (m, 2H), 4.30-4.23 (m, 5H), 4.22-4.15 (m, 2H), 4.15-4.08 (m, 1H), 4.08-4.01 (m, 2H), 3.91 (t, J = 7.09 Hz, 1H), 3.88-3.76 (m, 4H), 3.74-3.65 (m, 4H), 3.64-3.52 (m, 7H), 3.52-3.44 (m, 4H), 3.44-3.36 (m, 2H), 3.33-3.17 (m, 4H), 3.05 (m, 1H), 2.87 (m, 1H), 1.67-1.32 (m, 55H, H_{2Ieq}, 6 × boc);¹³C NMR (125 MHz, Acetone- d_6) δ 157.4, 156.6, 156.0, 155.3, 147.5, 122.9, 110.1, 100.1, 98.6, 85.2, 80.3, 79.8, 78.8, 78.4, 78.0, 74.5, 73.7, 73.1, 72.5, 72.2, 71.6, 70.1, 67.3, 56.0, 55.0, 54.0, 52.3, 51.7, 50.7, 41.9, 40.2, 35.0, 25.0, 22.4, 13.4; MS (ESI-HRMS) *m*/zcalcd. for C₅₉H₉₉N₉O₂₅ 1334.6777, found 1334.6830.

Synthesis of bromo ended neomycin dimer (8).

To a solution of compound **7**(43.0 mg, 14.0 µmol) in ethanol (2.0 mL), CuSO₄ (0.5 mg, 3.5 µmol, in 0.2 mL water) and sodium ascorbate (1.4 mg, 7.0 µmol, in 0.2 mL water) were added and the reaction mixture was stirred at room temperature for 15 min. N, N-bis(2-azidoethyl)-6-bromohexanamide (**6**) (2.3 mg, 7.0 µmol) in EtOH (0.2 mL) was added dropwise and the reaction was started at room temperature with vigorous stirring. The progress of the reaction was monitored by TLC. The volatiles were evaporated under reduced pressure and the crude product was purified by column chromatography on silica gel[0 to 15 % EtOH in DCM (v/v)] to yield the desired compound as a yellowish solid (36.2 mg, 80%). [R_j0.38, 0 to 10 % EtOH in DCM (v/v)]; ¹H NMR (500 MHz, CD₃COCD₃): δ 8.34-8.21 (m, 2H, triazole), 8.15-8.04 (m, 2H,triazole), 6.67-6.52 (br, s, 2H, NH_{6IV}), 6.41-6.30 (br, s, 2H), 6.26-5.96 (m, 10H, NH_{6II}, NH₁₁, NH₃₁, NH_{2IV}, and NH_{2II}), 5.31-5.20 (m, 4H), 5.08-5.03(m, 2H), 5.02-4.89 (m, 4H), 4.85 (d, *J* = 8.35 Hz, 2H), 4.81-4.67 (m, 14H), 4.57-4.50(br, s, 2H), 4.41-4.23 (m, 10H), 4.05 (s, 4H), 3.96-3.78(m, 8H), 3.74-3.53 (m, 18H), 3.53-3.38 (m, 8H), 3.36-3.14 (m, 6H), 1.82 (m, *J* = 6.94 Hz, 2H), 1.65-1.33 (m, 116H, H_{2leq}, 12 × boc, linker

protons); ¹³C NMR (75 MHz, Acetone-d₆) δ 157.4, 156.5, 155.9, 155.3, 144.1, 129.6, 125.3, 110.1, 100.2, 98.7, 80.2, 78.8, 78.7, 78.3, 78.1, 74.4, 73.0, 72.4, 72.1, 71.9, 70.1, 67.3, 62.8, 55.7, 52.3, 50.7, 41.9, 40.1, 35.2, 31.7, 26.8, 25.4, 22.4, 13.4; MS MALDI-TOF calcd for C₁₂₈H₂₁₆BrN₂₅O₅₁ (M+H₂O⁺), 3016.40, obsd: 3016.40.

Synthesis of azido ended neomycin dimer (9).



To a solution of **8**(100.0 mg, 3.3 µmol) in DMF (2.0 mL), NaN₃ (100.0 mg, 1.5 mmol) was added and the reaction mixture was stirred at 100 °C for 14 h. The volatiles were removed using rotary evaporator. The reaction mixture was dissolved in ethyl acetate (5.0 mL) and washed with water (3 × 5.0 mL) followed by drying over Na₂SO₄. The evaporation of solvents resulted in a brownish solid (93.8 mg, 95%) in almost quantitative yield. The product was used in the next step without further purification:IR (neat, cm⁻¹): 3300-3500 (broad), 2110 (azide), 1610-1650, 1505; ¹H NMR (500 MHz, CD₃COCD₃) δ 8.31-8.20 (m, 2H, triazole), 8.16-8.06 (m, 2H, triazole), 6.69-6.55 (br, s, 2H, NH_{6IV}), 6.42-6.28 (br, s, 2H), 6.27-5.27 (m, 10H, NH_{6II}, NH₁₁, NH₃₁, NH_{2IV}, and NH_{2II}), 5.27-5.20 (m, 4H), 5.11-5.07 (m, 2H), 5.05-4.85 (m, 6H), 4.83-4.53 (m, 16H), 4.50-4.30 (m, 10H), 4.05 (s, 4H), 3.95-3.80 (m, 8H), 3.77-3.53 (m, 14H), 3.53-3.35 (m, 10H), 3.35-3.15 (m, 8H), 1.65-1.33 (m, 116H, H_{2leq}, 12 × boc, linker protons); ¹³C NMR (125 MHz, Acetone-*d*₆) δ 157.4, 156.9, 156.5, 155.9,

155.3, 144.2, 129.6, 125.3, 110.2, 100.2, 98.7, 85.1, 80.2, 78.8, 78.7, 78.4, 78.3, 78.1, 76.3, 74.4, 73.7, 73.0, 72.6, 72.1, 71.7, 70.1, 67.3, 62.8, 55.8, 55.0, 52.3, 51.0, 50.7, 41.9, 40.1, 35.2, 22.4, 13.4; MS MALDI-TOF calcd. for C₁₂₈H₂₁₆N₂₈O₅₁ (M+H₂O⁺), 2979.50, obsd: 2980.10.

Synthesis of compound 5a.



To a solution of compound**9**(10.0 mg, 3.7 μ mol) in a mixture of ethanol and water [3.0 mL, 3:1, (*v*/*v*)], sodium ascorbate (0.3 mg, 1.7 μ mol, 0.5 eq) and CuSO₄ (140.0 mg, 89.0 μ mol, 0.2 eq) were added followed by the addition of benzimidazole alkyne**1** (1.7 mg, 4.7 μ mol). The reaction mixture wasstirred at room temperaturefor 21 h vigorously and the progress of the reaction was monitored by TLC. The reaction mixture was dried over rotary evaporator. The crude reaction mixture was purified by column chromatography on silica gel [0 to 30 % MeOH in DCM (v/v)] to yield the desired compoundas white powder (7.9 mg, 73.6%): R_f 0.45 [15% MeOH in DCM (*v*/*v*)];¹H NMR (500 MHz, CD₃COCD₃) δ 8.34-8.18 (m, 5H), 8.10 (m, 1H, aromatic proton from benzimidazole), 7.05 (m, 1H), 6.70 (br, s, 2H, NH_{6IV}), 6.38 (s, 2H, NH_{6II}), 6.45-6.02 (m, 8H, NH_{1I}, NH_{3I}, NH_{2IV}, and NH_{2II}), 5.45-5.30 (m, 4H), 5.30-5.20 (m, 4H), 5.14 (s,

4H), 5.04-4.82 (m, 8H), 4.81-4.50 (m, 16H), 4.45 (s, 4 H), 4.41-4.20 (m, 8H), 4.05 (s, 4H), 3.85 (m, 4H), 3.84-3.78 (m, 8H), 3.71-3.55 (m, 12H), 3.52-3.32 (m, 8H), 3.32-3.05 (m, 8H), 2.30-2.20 (m, 4H), 2.20-2.10 (m, 2H), 1.92 (m, 2H), 1.82 (m, 2H), 1.65-1.50 (m, 4H), 1.50-1.15 (m, 116 H, H_{2Ieq}, 12 × boc, linker protons); MS MALDI-TOF calcd. for $C_{149}H_{238}N_{32}O_{52}$ (M+H₂O⁺), 3326.40, obsd: 3325.04.

Synthesis of 5 (DPA 83).



To a solution of **5a**(5.0 mg, 1.5 µmol) in dioxane (1.5 mL), 4N HCl in dioxane (0.4 mL) was added with constant stirring. White precipitate was formed after 10-15 minutes of stirring. The precipitation was enhanced by adding a mixture of hexane and ethyl ether [2.0 mL, 1:1 (v/v)]. The reaction mixture was centrifuged and dissolved in water. The aqueous solution was washed with ethyl acetate (3 × 1 mL). The aqueous layer was lyophilyzdto give the desired compound **5** as pale white solid (2.8 mg, 73%):¹H NMR (500 MHz, D₂O) δ 8.09 (s, 1H, triazole), 8.05 (m, 1H, triazole), 7.98-7.85 (m, 4H), 7.65-7.60 (m, 1H), 7.31-7.18 (m, 4H), 6.02-5.96 (m, 1H), 5.35 (d, *J* = 2.34 Hz, 2H), 5.29 (s, 2H), 5.25 (s, 2H), 4.71-4.62 (m, 14H), 4.61-4.55 (m, 6H), 4.54-4.43 (m, 4H), 4.32-4.22 (m, 4H), 4.17-4.05 (m, 4H), 3.96 (t, *J* = 2.34 Hz, 2H), 3.95-3.92 (m, 4H), 3.81 (d, *J* = 2.34 Hz, 2H), 3.75 (s, 2H), 3.71-3.56 (m, 10H), 3.56-3.42 (m, 8H), 3.42-3.20 (m, 12H), 3.19-3.10 (m, 2H), 2.92-2.86 (m, 4H), 2.45-

2.38 (m, 2H), 2.14-2.08 (m, 1H), 1.95 (s, 1H), 1.83 (q, J = 12.45 Hz, 2H, H_{2Iax}), 1.65 (m, J = 6.73 Hz, 2H, linker protons), 1.55-1.40 (m, 2H, linker protons); MS (MALDI-TOF) m/z calcd. for C₈₉H₁₄₂N₃₂O₂₈2108.28, found 2132.99 [M+Na]⁺;UV (water) $\lambda_{max} = 323$ nm ($\epsilon = 45896$ M⁻¹ cm⁻¹); RP-HPLC (t_R = 15.9 min, Purity 98.9%).

Synthesis of 4.



Scheme S3. Reagents and conditions: (a) Dichloromethane, 4M HCl, r.t., 4h, 92%.

Synthesis of compound 4.

To a solution of Compound **9** (11 mg, 3.71 µmol) in dichloromethane (1.0 mL), 4M HCl in 1,4dioxane (1.0 mL) was added and the reaction mixture was stirred at room temperature for 4h. Precipitation of product was induced by adding diethyl ether (3.0 mL). The precipitated solid was centrifuged and the obtained solid was dissolved in 1.0 mL water. The aqueous solution was then washed with dichloromethane (3×1 mL). The aqueous layer was then lyophilized to afford the desired compound DPA83 (**5**) as a yellowish solid (7.5 mg, 92%): ¹H NMR (500 MHz, D₂O) δ 8.24 – 7.81 (m, 4H), 6.13 – 5.82 (m, 2H), 5.41 – 5.08 (m, 4H), 4.68-4.55 (m, 16H) 4.55 (m, 10H), 4.18 (m, 4H), 4.08 – 3.80 (m, 6H), 3.81–2.91 (m, 22H), 2.37 (d, *J* = 12.4 Hz, 2H), 1.88 (m, 2H), 1.50 (s, 2H), 1.37 – 0.78 (m, 6H); ¹³C NMR (75 MHz, D₂O) δ 163.5, 163.0, 162.5, 162.1, 143.9, 125.6, 122.0, 118.2, 114.3, 110.4, 110.0,

95.4, 94.9, 84.9, 79.7, 76.7, 74.9, 72.8, 72.3, 70.3, 69.6, 67.9, 67.4, 62.5, 53.2, 52.0, 50.7, 49.6, 48.3, 40.4, 40.0, 27.8; MS MALDI-TOF calcd. for C₆₈H₁₂₀N₂₈O₂₇ (M+H₂O⁺), 1778.90, obsd: 1779.19.



































Figure 17. MALDI-TOF spectrum of compound 5a.









concentration plot of compound 5to determine extinction coefficient (right).





Biophysical assays

Thermal Denaturation profileof HIV-1 TAR RNA in the presence of various ligands.



Figure 23. UV melting profile of 1 μ MHIV TAR RNA in the presence of indicated ligands at a stoichiometric ratio of 1:1 (HIV TAR RNA : ligand). Buffer conditions: 100 mMKCl, 10 mM SC, 0.5 mM EDTA, pH 6.8.

FID titration of various ligands with HIV TAR RNA pre-incubated with EtBr.



Figure 24. The raw data from FID titrations (top panel) and their representative sigmoidal fit (bottom panel) to extract the IC₅₀ values. Buffer conditions: 100 mMKCl, 10 mM SC, 0.5 mM EDTA, pH 6.8. [HIV TAR RNA] = 200 nM/strand. [EtBr] = 5 μ M.ND = The binding was too weak to extract the IC₅₀ value.

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