SUPPORTING INFORMATION

Preparation of a 3′-azido-3′-deoxythymidine (AZT) derivative, which is blood-brain barrier permeable

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1. General Methods

Column chromatography was performed on Merck 60 silica gel [70-230 or 230-400 mesh (flash)], and MPLC on Fluka 100 C8-reversed phase silica gel. All NMR spectra were recorded on a Bruker DPX 300 instrument operating at 300MHz for ^{1}H, and 75MHz for ^{13}C, unless otherwise indicated. The chemical shifts are reported in δ ppm with TMS as reference standard. IR spectra were recorded on a BOMEM FT-IR M100 C15 spectrometer and values are reported in cm^{-1}. For IR sample preparations, compounds were dissolved in appropriate solvents such as dichloroform or chloroform and a thin film of the solution was spread between polished NaCl salt plates. Attenuated total reflection (ATR), an accessory for IR spectroscopy, was used for compounds which are soluble only in MeOH or H_2O. Low resolution mass spectra were recorded on a Micromass PLATFORM II spectrometer in the electron impact (EI) or fast atom bombardment (FAB) modes. HR-FABMS were obtained on a Jeol JMS 700 high resolution mass spectrometer at the Korea Basic Science Institute (Daegu), and MALDI-TOF-MS on a Micromass M@DI at the Biomolecular Diversity Core Facility (POSTECH). Analytical HPLC was performed on Agilent 1100-HPLC Chemstation with an analytical column ZORBAX SB-C8 (5 μm, 300Å, 4.6 X 250 mm), and preparative HPLC on Agilent 1100-HPLC Chemstation with a semi-preparative column GRACEVYDAC C18 (5 μm, 300Å, 10 X 250 mm). Optical rotations were measured with a JASCO DIP-360 digital polarimeter.

2. Synthetic Chemistry

3′-Azido-3′-deoxythymidine-succinate (1).

To a solution of 3′-Azido-3′-deoxythymidine (AZT) (133 mg, 0.49 mmol) in pyridine (2 ml) at rt, were added succinic anhydride (147 mg, 1.47 mmol) and DMAP (12 mg, 0.10 mmol). After stirring for 4 hrs, the solution was repeatedly concentrated at 50℃ under reduced pressure by azeotropic distillation with toluene. The residue was dissolved in EtOAc and then washed with

H_2O and brine. The organic layer was dried over Na_2SO_4, filtered and condensed in vacuo to give the crude product, which was purified by column chromatography on flash silica gel to yield **1** (169 mg, 94%) as a colorless foamy solid.

R_f: 0.25 (CH_2Cl_2 : MeOH = 20:1); $[\alpha]_D^{27}$ 79.52 (*c* 1.24, $CHCl_3$) [lit.[1] $[\alpha]_D^{24}$ 70.6 (*c* 1.5, $CHCl_3$)]; IR (film, cm^{-1}) 2850-3400 (broad, O-H), 3179 (Csp^2-H), 3081 (N-H), 2108 (N_3), 1734 (C=O, ester), 1699 (C=O, carboxylic acid), 1685 (C=O, amide), 1653 (C=C); ^1H NMR ($CDCl_3$) 1.80 (s, 3H, CH_3), 2.25-2.98 (m, 6H, CH_2CH_2 and H_2'), 4.03 (s, 1H, H_4'), 4.21-4.39 (m, 2H, H_5'), 4.83 (dd, 1H, J=6.36 Hz, H_3'), 5.90 (t, J= 5.61 Hz, 1H, H_1'), 7.43 (s, 1H, Csp^2-H), 10.49 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) 12.55, 28.81, 29.13, 38.18, 60.36, 63.26, 83.04, 85.99, 109.79, 137.71, 149.49, 166.53, 172.90, 177.68; HR-FABMS $[M+H]^+$ calcd for $C_{15}H_{17}N_5O_7$ m/z 368.1128, found 368.1205.

1-*O*-trityl-2,3,4,5-tetra-*O*-(*N*-{bis-[3-(*N´*,*N´´*-bis-Boc-guanidino)-propyl]}-6-aminohexanoyl)-D-sorbitol (2) was prepared according to a literature procedure.[2]

1-*O*-(3´-azido-3´-deoxythymidine-succinoyl)-2,3,4,5-tetra-*O*-(*N*-{bis-[3-(*N´*,*N´´*-bis-Boc-guanidino)-propyl]}-6-aminohexanoyl)-6-*O*-trityl-D-sorbitol (3).

To a solution of **2** (802 mg, 0.25 mmol) in CH_2Cl_2 (7 ml) at rt were added **1** (176 mg, 0.48 mmol), EDC (94 mg, 0.50 mmol), and DMAP (15 mg, 0.12 mmol). After stirring for 2 days, the solution was concentrated under reduced pressure. The residue was dissolved in EtOAc and washed with aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated to give the crude product, which was purified by column chromatography on silica gel to give **3** (851 mg, 96%) as a colorless foamy solid.

R_f: 0.40 (CH_2Cl_2 : MeOH = 15:1); IR (film, in cm^{-1}) 3145 (Csp^2-H), 2106 (N_3), 1720, 1639, 1615, 1154; ^1H NMR ($CDCl_3$) 0.98-2.15 (m, 187H), 2.18-2.90 (m, 36H), 3.20-3.52 (m, 18H), 3.68-3.89 (m, 2H), 4.18-5.53 (m, 10H), 6.04 (s, 1H), 7.12-7.49 (m, 16H), 8.45 (br s, 8H), 11.49 (br s, 8H); ^{13}C NMR ($CDCl_3$, 125MHz) 12.54, 27.97, 28.06, 28.22, 28.32, 28.88, 29.34, 33.97, 37.28, 39.61, 51.55, 52.23, 53.43, 53.78, 60.25, 63.22, 69.11, 70.09, 70.26, 72.37, 79.12, 81.88, 82.87, 111.16, 127.22, 127.50, 127.88, 127.96, 128.16, 128.41, 128.52, 128.66, 136.07, 143.41, 150.01, 153.10, 156.08, 163.62, 171.68, 171.89.

1-*O*-(3´-azido-3´-deoxythymidine-succinoyl)-2,3,4,5-tetra-*O*-(*N*-{bis-[3-(*N´*,*N´´*-bis-Boc-guanidino)-propyl]}-6-aminohexanoyl)-D-sorbitol (4).

A column of flash silica gel was packed first in hexane with 1% Et_3N and then hexane with 1% TFA; a sea sand layer was placed in-between. Compound **3** (471 mg, 130 μmol) was dissolved in CH_2Cl_2 containing 1% TFA and was sonicated for several seconds. The solution was loaded

on the column and eluted with increasing MeOH in CH_2Cl_2 to give **4** (246 mg, 56%) as a colorless foamy solid.

R_f: 0.45 (CH_2Cl_2 : MeOH = 10:1); IR (film, in cm^{-1}) 2850-3400 (O-H), 2109 (N_3), 1738, 1682, 1615, 1137; ^1H NMR ($CDCl_3$) 0.88-2.15 (m, 187H), 2.18-2.90 (m, 36H), 3.20-3.52 (m, 18H), 3.68-3.89 (m, 2H), 4.18-5.53 (m, 10H), 6.06 (s, 1H), 7.28 (s, 1H), 8.82 (br s, 8H), 11.40 (br s, 8H); ^{13}C NMR ($CDCl_3$) 12.59, 14.33, 21.18, 22.82, 23.09, 23.84, 24.10, 27.73, 27.95, 28.03, 28.13, 29.06, 29.83, 33.75, 37.31, 38.64, 45.94, 49.67, 50.63, 52.76, 53.03, 53.61, 60.54, 63.50, 68.29, 69.11, 69.48, 69.82, 70.44, 71.17, 71.76, 72.64, 77.43, 81.95, 82.63, 84.58, 85.16, 111.24, 114.68, 136.26, 150.44, 152.52, 153.10, 154.99, 155.89, 159.45, 161.56, 162.03, 164.28, 170.49, 170.80, 171.33, 171.64, 172.13, 172.87, 173.27.

1-*O*-(3´-azido-3´-deoxythymidine-succinoyl)-2,3,4,5-tetra-*O*-(*N*-{bis-[3-(*N´*,*N´´*-bis-Boc-guanidino)-propyl]}-6-aminohexanoyl)-6-*O*-(*N*-Boc-6-aminohexanoyl)-D-sorbitol (5).

To a solution of **4** (241 mg, 71 μmol) in CH_2Cl_2 (4 ml) at rt were added HOOC-$(CH_2)_5$-NHBoc (28 mg, 121 μmol), EDC (27 mg, 142 μmol), and DMAP (4 mg, 35 μmol). After stirring for 2 days, the solution was concentrated under reduced pressure. The residue was dissolved in EtOAc and washed with aq. $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated to give the crude product, which was purified on silica gel to give **5** (135 mg, 53%) as a colorless foamy solid.

R_f: 0.55 (CH_2Cl_2 : MeOH = 10:1); IR (film, in cm^{-1}) 2104 (N_3), 1718, 1696, 1654, 1617, 1135; ^1H NMR ($CDCl_3$) 0.95-2.12 (m, 200H), 2.14-2.97 (m, 38H), 3.05-3.59 (m, 20H), 3.62-3.89 (m, 2H), 4.12-5.53 (m, 12H), 6.08 (s, 1H), 7.30 (s, 1H), 8.52 (br s, 8H), 11.44 (br s, 8H); ^{13}C NMR ($CDCl_3$) 12.61, 22.76, 23.11, 24.70, 24.87, 25.35, 25.69, 26.25, 26.66, 28.06, 28.15, 28.40, 28.53, 29.05, 29.42, 29.76, 29.85, 31.30, 31.99, 34.10, 35.62, 36.04, 37.36, 37.80, 38.83, 39.30, 40.5749.87, 51.16, 52.53, 53.57, 60.28, 62.88, 69.17, 72.51, 77.43, 79.03, 79.33, 81.95, 83.16, 83.53, 86.02, 111.21, 135.67, 136.26, 150.36, 153.18, 153.32, 153.94, 156.44, 163.09, 163.55, 164.12, 172.01, 173.05, 173.56, 177.91, 179.59.

1-*O*-(3´-azido-3´-deoxythymidine-succinoyl)-2,3,4,5-tetra-*O*-[*N*-{bis-(3-guanidinopropyl)}-6-aminohexanoyl]-6-*O*-(6-aminohexanoyl)-D-sorbitol·9HCl (6).

Compound **5** (134 mg, 37 μmol) was added into a solution of EtOAc saturated with gaseous HCl (4 ml) at rt., and the solution was stirred for 2 days. After evaporation, the precipitate was dissolved in deionized water and lyophilized to give **6** (83 mg, quant. yield) as a colorless foamy solid, which was used in next step without further purification.

IR (ATR, in cm^{-1}) 2071 (N_3), 1743, 1620; ^1H NMR (MeOD) 1.01-2.00 (m, 46H), 2.01-2.31 (m, 14H), 2.32-3.11 (m, 26H), 3.17-3.25 (m, 16H, partially overlapped with MeOD peak), 3.29-3.99

(m, 5H), 4.12-5.53 (m, 12H), 6.20 (s, 1H), 7.62 (s, 1H); ^{13}C NMR (MeOD) 12.78, 14.67, 24.26, 24.85, 25.49, 26.99, 27.19, 28.34, 30.12, 34.50, 34.81, 37.84, 38.58, 39.92, 40.71, 51.91, 52.20, 54.70, 61.62, 62.11, 62.72, 63.90, 64.09, 65.05, 65.49, 70.20, 71.78, 83.21, 84.58, 86.21, 86.64, 87.98, 111.49, 112.10, 138.13, 152.30, 158.81, 158.86, 166.40, 166.56, 173.17, 173.84, 174.72, 174.95, 175.17, 175.35, 175.77, 177.37; MALDI-TOF-MS $[M+H]^+$ calcd for $C_{82}H_{157}N_{34}O_{17}$ m/z 1890.2466, found 1890.2445.

1-*O*-(3´-azido-3´-deoxythymidine-succinoyl)-2,3,4,5-tetra-*O*-[*N*-{bis-(3-guanidinopropyl)}-6-aminohexanoyl]-6-*O*-[6-(fluoresceinyl-5-thioureido)-hexanoyl]-D-sorbitol·8HCl (7).

To a solution of **6** (82 mg, 37 μmol) in DMF (3 ml) at rt were added diisopropylethylamine (0.15 ml, 0.74 mmol), and fluorescein-5-isothiocyanate (24 mg, 48 μmol). After stirring for 24 hrs in the dark, the solution was repeatedly concentrated at 45□ under reduced pressure by azeotropic removal with toluene. The residue was purified sequentially by using MPLC and RP-HPLC (GRACEVYDAC, C18), (2.0 ml/min, 10% to 60% CH_3CN in H_2O, 220 nm) to give **7** (59 mg, 63%) as a sticky orange colored solid.

UV (MeOH): $\lambda_{max\ 1}$ = 266 nm, ε = 18603 $cm^{-1}\ M^{-1}$, $\lambda_{max\ 2}$ = 497 nm, ε = 9179 $cm^{-1}\ M^{-1}$; ^{1}H NMR (D_2O) 1.03-1.98 (m, 36H), 2.01-2.26 (m, 21H), 2.29-2.97 (m, 21H), 3.19-3.60 (m, 22H), 3.64-3.99 (m, 6H), 4.12-5.53 (m, 12H), 6.19-6.32 (m, 3H), 6.69-6.81 (m, 2H), 7.31-7.38 (m, 2H), 7.55-7.82 (m, 4H); ^{13}C NMR (MeOD, 125MHz) 11.17, 23.70, 24.01, 25.83, 28.52, 33.18, 36.29, 37.01, 38.51, 47.09, 47.26, 47.43, 47.60, 47.77, 47.89, 47.94, 48.05, 48.11, 48.22, 50.35, 53.35, 60.53, 63.43, 68.88, 75.81, 81.70, 85.13, 110.53, 128.73, 136.55, 141.97, 150.79, 164.92, 172.27, 187.59; MALDI-TOF-MS $[M]^+$ calcd for $C_{103}H_{167}N_{35}O_{22}S$ m/z 2278.2746, found 2278.1489; analytical HPLC (BU-300, 266 nm, 1 ml min^{-1}, 40% to 60% CH_3CN gradient in H_2O during 12 min, t_R=2.31 min), purity 99+ %.

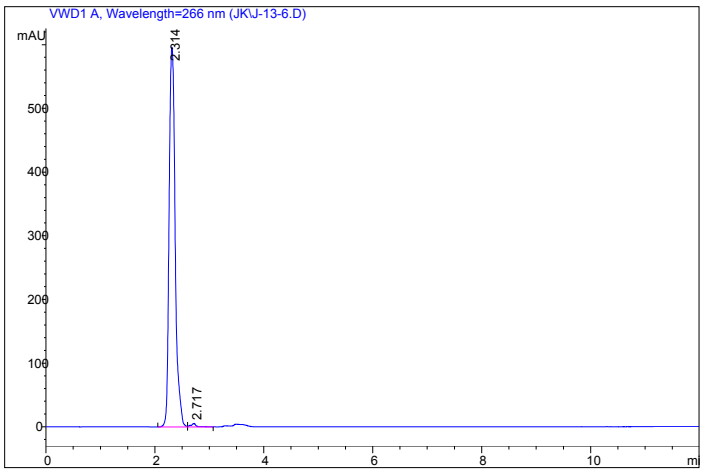

Figure 1. Analytical HPLC chromatogram of **7** under gradient condition (40% to 60% CH_3CN) in H_2O during 12 min, t_R=2.31 min), purity 99+ %.

3. Cellular Uptake and Intracellular Localization Studies

Cell culture: HeLa cells were cultured at 37 □ in a humidified 5% CO_2 containing air environment in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and 10% (v/v) fetal bovine serum (FBS, Sigma) with antibiotics. The subculture was conducted every 2-3 days using the cells grown to subconfluence.

Uptake experiments: For each assay, HeLa cells were seeded into a 35-mm glass bottomed dish (SPL) and cultured for 24 hrs. After removing the medium, HeLa cells were washed with PBS (X1). The cells were incubated for 30 min at 37 □ in 2 ml of DMEM containing 10 μM of **7**. For subcellular staining, HeLa cells were pretreated with **7** as described and then 100 nM mitotracker or 200 nM lysotracker was added and incubated for another 30 min. Rhodamine B-dextran conjugate (1 mg/ml) was incubated together with **7** for 1 hr.

Confocal laser scanning microscopy (CLSM): Each dish of HeLa cells was washed five times with cold PBS, and then CLSM was performed by using an Olympus Fluoview FV1000 (N.A. 1.30, 40X) *without fixing* the cells. Fluorescence was analyzed and collected using the following excitation and emission bands: FITC, 488 nm (ex), 520-550 nm (em); mitotracker, lysotracker, and rhodamine B-dextran, 543 nm (ex), 600-700 nm (em). Merged images and intensity profiles were obtained by the Olympus Fluoview Viewer.

Protocols for the tissue biodistribution study were previously described.[3]

4. Reference

1. H. Tamamura, T. Ishihara, H. Oyake, M. Imai, A. Otaka, T. Ibuka, R. Arakaki, H. Nakashima, T. Murakami, M. Waki, A. Matsumoto, N. Yamamoto and N. Fujii, *J. Chem. Soc., Perkin Trans.*, 1998, **1**, 495.
2. K. K. Maiti, W. S. Lee, T. Takeuchi, C. Watkins, M. Fretz, D. C. Kim, S. Futaki, A. Jones, K. T. Kim and S. K. Chung, *Angew. Chem. Int. Ed.*, 2007, **46**, 5880-5884.
3. K. K. Maiti, O. Y. Jeon, W. S. Lee, D. C. Kim, K. T. Kim, T. Takeuchi, S. Futaki, S. K. Chung, *Angew. Chem. Int. Ed.*, 2006, **56**, 2907-2912.