SUPPLEMENTARY INFORMATION

Polyornithine-based polyplexes to boost effective gene silencing in CNS disorders

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Synthesis of Pyridyl Dithiol Cysteamine (PD)

Pyridyl dithiol ethylamine HCl salt was synthesized essentially as described in van der Vlies *et al.* (*36*). Briefly, in a two-neck round bottom flask fitted with a stirrer bar and a stopper, aldrithiol (15 mmol, 3 eq.) was solubilized in 15.15 mL of MeOH under N_2 atmosphere and vigorous stirring. Afterward, cysteamine·HCl (5.0 mmol, 1 eq.) was dissolved in 7.54mL of MeOH and added to the reaction mixture under N_2 atmosphere. The reaction was allowed to proceed overnight under constant stirring at room temperature under N_2 atmosphere. Then, the product was precipitated in fresh diethyl ether, filtered off and washed 2x with diethyl ether. A white solid was obtained after freeze-drying. Product identity was carried out by ^{1}H-NMR study in d6-DMSO (**Figure S1**).

Calculation of the Hydrodynamic Radius (Rh)

The hydrodynamic radius (Rh) (**Table S1)** has been estimated by means of the Stokes-Einstein equation where *k* is the Boltzman constant, T is the absolute temperature (25°C), η is the medium viscosity (997.13kg/m^3) and D(m^2/s) the diffusion coefficient values obtained by DOSY-NMR analysis (**Figure 1(B)**).

Cell Culture

a) OPCs

Cortical hemispheres were finely chopped with spring bow dissecting scissors and digested for 30 min at 37°C with papain (20 U/mL, Worthington, UK). After adding ovomucoid inhibitor (Worthington, UK), the tissue was gently triturated with fire-polished glass pipettes and subsequently passed through a 70 μm cell strainer. Only for tissue derived from 10-12-day-old mice myelin debris was removed by centrifuging 3.5 mL of 90% isotonic Percoll (GE, Sweden)

added to 11 mL of cell suspension for 20 min at 800 g. The myelin debris layer was removed, and the pellet was washed once with HBSS solution (Invitrogen, UK). OPCs were incubated with A2B5-conjugated microbeads (Miltenyi Biotec, San Francesco, CA, USA) and isolated for positive selection with appropriately sized columns. OPCs were resuspended in OPCs medium (DMEM supplemented with 1% penicillin/streptomycin, 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), 0.1% N-acetyl-L-cysteine (Sigma-Aldrich), 0.1% biotin (Sigma-Aldrich), 0.1% Trace Element B (Cellgro), and 0.1% Forskolin (Sigma-Aldrich) and seeded into multi-well plates coated with PDL at a density of 35000 cells/cm^2. Cells were grown at 37°C with 5% CO_2 in the presence of growth factors 20 ng/mL PDGF-AA, 10ng/mL bFGF and half of the medium was changed every two days maintaining the same final concentration of growth factors.

For the cell expansion experiment, OPCs were grown in T25 flasks for ten days in the presence of growth factors. After detaching with papain (2 U/mL), cells were sorted with A2B5-conjugated microbeads (Miltenyi Biotec) to ensure the use of undifferentiated OPCs. Cells were plated into multi-well plates coated with PDL at a density of 35000 cells/cm^2 in the presence of growth factor-free OPC medium for four days.

b) BO-1

BO-1 cells were kindly provided by Prof. W. Baumgärtner from Department of Pathology of the University of Veterinary Medicine Hannover (Germany). B104 neuroblastoma rat cell line was purchased from IRCCS Azienda Ospedaliera Universitaria San Martino, Interlab Cell Line Collection (ICLC) (Italy).

Materials B104:

DMEM (Cat. 41965) was purchased from Gibco. ITS+Premix was supplied by BD Biosciences. Trypsin/EDTA solution was purchased from Millipore.

Materials BO-1:

DMEM (Cat. 41965) was obtained from Gibco. ITS+Premix was purchased at BD Biosciences. Biotin, poly-L-Lysine, progesterone, and putrescine were purchased from Sigma-Aldrich.

Culture of B104 cells and preparation of conditioned medium

For B104 conditioned medium (CM) production, B104 cells were seeded in T175 flasks at a density of $4.67x10^6$ cells/flask, with 49 mL of culture medium and kept in the incubator (at 37 °C, 5% CO_2/95% air and 95% humidity). After 24h, the medium was exchanged by 35 mL DMEM containing 1% ITS+Premix and 1% Penicillin/Streptomycin. After three days, the media was collected and centrifuged (10 min, 300 g) and the supernatant was filtered first through 0.45 µm filter and second through a 0.22 µm filter. The filtered media (B104 CM) was aliquoted and stored at -20°C until use (*92*).

Culture of BO-1 cells

BO-1 cell media was composed of 33 mL DMEM medium, 15 mL B104 CM, 500 µl penicillin and streptomycin, 500 µl ITS+Premix, 50 µl putrescine, 5 µl biotin, and 5 µl progesterone.

For cell passage, BO-1 cells were detached from the flask mechanically, collected and centrifuged (10 min, 300 g). Cell pellet was re-suspended in BO1 culture medium and cells were seeded at a density of 0.3×10^6 cells/ml. For cytotoxicity and gene silencing studies, cells were seeded at 200,000 cells/well in 6-well plates.

c) CG-4

CG-4 cell line was kindly provided by Mário Grãos from the Unit of Cellular Biology, Biocante CNC, UC Biotech (Portugal). B104 neuroblastoma rat cell line was purchased to IRCCS Azienda Ospedaliera Universitaria San Martino, Interlab Cell Line Collection (ICLC) (Italy).

Materials:

DMEM/F12, Trypsin/EDTA solution, FPS, penicillin, streptomycin and amphotericin B were supplied by Life Technologies. DMEM high glucose 4500 mg/L was obtained from Hyclone. All the rest of the supplements were purchased from Sigma–Aldrich.

Culture of B104 cells and preparation of CM

B104 cells were maintained in B104 proliferation medium supplemented with 10% (v/v) FBS, penicillin (10 U/mL), streptomycin (10 µg/mL) and amphotericin B (2.5 µg/mL)], at 37 °C, 5% CO_2/95% air and 95% humidity. Cells were previously seeded at a density of 15,000 cells/cm^2 in proliferation medium for 24 h to obtain conditioned medium (B104 CM). Then, cells were washed three times with Puck's solution, and the medium was replaced by defined medium, composed by DMEM/F12 supplemented with holo-transferrin (10 µg/mL), sodium selenite (5 ng/mL), putrescine (16 µg/mL), progesterone (6.3 ng/mL), penicillin (10 U/mL),

streptomycin (10 μg/mL) and amphotericin B (2.5 μg/mL). Three days later, the conditioned medium was collected and was added PMSF (1 μg/mL). Next, the medium was centrifuged (10 min, 1000 g at 4°C) and the supernatant was filtered (0.22 μm filter) and stored at − 20 °C.

Culture of CG-4 cells.

The CG-4 cell line was maintained as reported (*90*). Briefly, cells were harvested by washing three times with Puck´s solution, followed by dissociation and detachment using trypsin (500 μg/mL)-EDTA (200 μg/mL) solution. Trypsin was inactivated by adding 10× the volume of recovery medium (DMEM high glucose — 4500 mg/L, with 5% (v/v) FBS sodium pyruvate (2 mM), human insulin (5 μg/mL), penicillin (10 U/mL), streptomycin (10 μg/mL) and amphotericin B (2.5 μg/mL)) and cells were centrifuged (5 min, 200 g), re-suspended, counted and seeded on flasks previously coated with PDL (100 μg/mL, overnight at 37 °C) at a density of 2,500 cells/cm^2 in *recovery medium.* CG-4 cells were allowed to adhere for 30 minutes inside a CO^2 incubator at 37 °C, 5% CO_2/95% air and 95% humidity. After cell attachment, the recovery medium was replaced by CG-4 *proliferation medium* composed by DMEM high glucose supplemented with apo-transferrin (50 μg/mL), biotin (9.8 ng/mL), sodium selenite (40 ng/mL), 30% of B104 cell line – conditioned medium, penicillin (10 U/mL), streptomycin (10 μg/mL) and amphotericin B (2.5 μ g/mL). Cells were maintained in the CO_2 incubator at 37°C, and the medium was changed every other day. For MTS and gene silencing assays, CG-4 cells were seeded at a density of 7,000 cells/cm^2.

d) NSCs

Neural stem cells (NSCs) were isolated from the sub-ventricular zone of adult C57BL/6 mice, as previously described (*93*).

Materials

NeuroCult basal medium, NeuroCult proliferation supplement, and heparin were purchased from STEMCELL Technologies. Human EGF and recombinant human FGF-basic (FGF2) were provided by PeproTech. Penicillin-streptomycin was supplied by Gibco Life Technologies, and laminin was obtained from Sigma Aldrich.

Culture of NSCs:

NSCs were cultured as neurospheres in NeuroCult basal medium containing 10% NeuroCult proliferation supplement, 2 mg/mL heparin, 20 ng/mL recombinant human EGF, 10 ng/mL recombinant human FGF2, and 100 U/mL penicillin-streptomycin, and passaged every 3–5 days. For cytotoxicity studies or gene silencing tests, plates were previously coated with laminin (0.05 mg/mL) to obtain adherent cells instead of neurosphere formation. For both studies, cells were seeded in 6-well plates at a density of 500,000 cells/well.