Supporting Information

Synthetic Small Molecules that Induce Neurogenesis in Skeletal Muscle

Darren R. Williams, Myung-Ryul Lee, Young-Ah Song, Sung-Kyun Ko, Gun-Hee Kim and Injae Shin*

Department of Chemistry, Yonsei University, Seoul 120-749, Korea

* To whom correspondence should be addressed. E-mail: injae@yonsei.ac.kr

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EXPERIMENTAL SECTION

Construction of the imidazole library: The imidazole library was prepared by following a modified procedure developed by Sarshar et al (*Tetrahedron Lett*. **1996**, *37*, 835) (Scheme S1). A solution of 4-nitrophenyl chloroformate (4 mmol) in CH_2Cl_2 was added to a Wang resin (1 mmol) in CH_2Cl_2 (9 mL) and pyridine (3 mL). After shaking for 12 h, the resin was washed with 10% DMF in CH_2Cl_2. A solution of 2,2'-(ethylenedioxy)bisethylenediamine (10 mmol) and diisopropylethylamine (DIEA, 5 mmol) in DMF was added to the resin. After shaking for 12 h, the resin was washed with DMF.

Pathway A: The amine-containing resin (7 μmol), aldehyde (10 equiv.), ammonium acetate (40 equiv.) and diketone (10 equiv.) was placed in a reaction vial and suspended in acetic acid (300 μL). The reaction vial was placed in a heat block on a shaker at 100 ^oC. After shaking for 4 h at the same temperature, the resin was filtered and washed with DMF, MeOH and CH_2Cl_2 several times. The imidazole derivative was cleaved from a solid support by treatment with TFA for 1.5 h.

Pathway B: A solution of Fmoc-protected *p*-aminomethyl benzoic acid (3 equiv.), BOP (3 equiv.), HOBt (3 equiv.) and DIEA (4 equiv.) in DMF was added to the amine-containing resin (7 μmol). After shaking for 6 h, the resin was washed with 10% DMF in CH_2Cl_2. After removal of Fmoc with 20% piperidine in DMF, the amine resin was reacted with aldehyde (10 equiv.), ammonium acetate (40 equiv.) and diketone (10 equiv.) in acetic acid (300 μL) at 100 ^oC for 4 h in a heat block on a shaker. The resin was filtered and washed with DMF, MeOH and CH_2Cl_2 several times. The imidazole derivative was cleaved from a solid support by treatment with TFA for 1.5 h.

Pathway C: A solution of *p*-formyl benzoic acid (3 equiv.), BOP (3 equiv.), HOBt (3 equiv.) and DIEA (4 equiv.) in DMF was added to the amine-containing resin (7 μmol). After shaking for 6 h, the resin was washed with 10% DMF in CH_2Cl_2. The aldehyde resin was reacted with amine (20 equiv.), ammonium acetate (1.4 equiv.) and diketone (20 equiv.) in acetic acid (300 μL) at 100 ^oC for 4 h in a heat block on a shaker. The resin was filtered and washed with DMF, MeOH and CH_2Cl_2 several times. The imidazole derivative was cleaved from a solid support by treatment with TFA for 1.5 h.

The purity of synthesized imidazole derivatives in the library was determined by using analytical reversed-phase HPLC (Figure S2). About 300 imidazole derivatives with more than 70% purity were obtained from these reactions. All these compounds were characterized by using MALDI-TOF MS.

Preparation of 2-{2-[5-(3-chlorophenyl)]furanyl}-4,5-bis(4-methoxyphenyl)- imidazole (Neurodazine): 5-(3-Chlorophenyl)furfural (0.048 mmol), ammonium acetate (0.57 mmol) and 4,4'-dimethoxybenzil (0.048 mmol) was suspended in acetic acid (500 μL), and then the suspension was heated to 100 ^oC. After stirring for 6 h, the reaction mixture was diluted with ethyl acetate and washed with saturated $NaHCO_3$ and brine. The organic layer was concentrated *in vacuo*. The crude product was purified by flash column chromatography: ^1H NMR (500 MHz, DMSO) δ 12.83 (s, 1 H), 8.0 (s, 1 H), 7.84 (d, 1 H, J = 7.5 Hz), 7.52-7.41 (m, 5 H), 7.40-7.34 (m, 1 H), 7.24 (d, 1 H, J = 3.5 Hz), 7.08-7.0 (m, 3 H), 6.92-6.85 (m, 2 H), 3.81 (s, 3 H), 3.75 (s, 3 H); ^{13}C NMR (125 MHz, DMSO) δ 159.7, 158.8, 151.7, 146.8, 138.3, 137.6, 134.7, 132.7, 131.5, 130.7, 129.0, 128.2, 128.0, 127.7, 124.0, 123.9, 123.0, 115.0, 114.5, 110.2, 109.7, 56.0, 55.8; HR MS calcd for $C_{27}H_{21}ClN_2O_3$ $[M+H]^+$ 457.1241, found 457.1251.

Small-molecule screening: Murine C2C12 myoblasts were seeded in a 96-well plate at a density of 10^4 cells/mL in culture media [Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 μg/mL streptomycin]. After incubation for 24 h, the culture media were replaced with differentiation media (DMEM supplemented with 2% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin) to suppress cell proliferation. Test compounds (1 mM) dissolved in DMSO were added to cell culture (200 μL) at a final concentration of 3 μM (0.3% DMSO in cell culture). For a negative

control, one well containing 200 μL of cell culture was treated with 0.6 μL DMSO without a compound. After incubation for 3 days, culture media containing compound were replenished. After two additional days (total of 5 day incubation with compound), the cells were washed with PBS and loaded with 2 μM FM1-43 (Molecular Probes) dissolved in Ringer buffer containing 100 mM KCl (the amount of Na^+ in buffer was reduced by an equivalent amount of K^+). After 5 min incubation at room temperature, the cells were washed with Ringer buffer three times to remove excess FM1-43. Fluorescent intensity of treated cells (excitation wavelength: 470 nm, emission wavelength: 540 nm) was measured by a fluorescent microplate reader (SpectraMax GeminiEM, Molecular Devices). Cells that exhibited relatively high fluorescence signal after treatment with compounds were observed by light microscopy (Nikon Eclipse TE2000) to detect neurite outgrowth characteristic of neural differentiation.

Four compounds with neurogenesis-inducing activity were identified based on this screening method (Figure 3).

Neurogenesis of murine C2C12 myoblasts by Nz: The cultured C2C12 myoblasts at a density of 10^4 cells/mL in differentiation media (DMEM supplemented with 2% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin) were treated with 2 μM Nz and incubated for 7–10 days to induce neurogenesis.

Neurogenesis of differentiated C2C12 myotubes by a two-step procedure: To differentiate C2C12 myoblasts into myotubes, C2C12 myoblasts were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 μg/mL streptomycin until they reached confluence, after which the media were changed to differentiation media. After 5–7 day incubation, the generated myotubes were treated with 10 μM myoseverin (Sigma) for 20 h. The treated cell cultures were gently washed with differentiation media to harvest the fragmented myotubes and mononucleate cells. The cells were transferred to 6-well plates at a density of 10^4 cells /mL in differentiation media. After 24 h, cells were incubated with 2 μM Nz for 7–10 days to undergo neurogenesis.

Neurogenesis of human skeletal muscle by Nz: Human single muscle fibers were prepared as previously described (Bonavaud, S. *et al. In Vitro Cell Dev. Biol. Anim.* **2002**, *38*, 66-72). Skeletal muscle tissue was taken from the abductor hallucis muscle of three males, aged seven months, three years and five years (provided by Dr. H. W. Kim, department of pathology, Yonsei University College of Medicine). The muscle sample was digested in a Petri dish containing 0.2% collagenase (Sigma) in Ham's F10 media (Gibco) at 37 ˚C for 90 min. Most of the human muscle fibers were released from the tissue after enzymatic digestion. The single muscle fibers were isolated by repeatedly triturating the muscle fragments with a wide-mouthed Pasteur pipette. The best twenty fibers from each patient, as judged by their attachment to the culture plate and the absence of fiber damage and hypercontraction, were selected for further studies.

To obtain mononucleates, single muscle fibers were treated with 15 μM myoseverin for 20 h. Fiber membrane folds, buds, fragments and potential discrete mononucleates were collected by disrupting the fiber with repeated trituration using a wide-mouth pipette. The mononucleates and fiber fragments were harvested by centrifugation and transferred to a cell culture plate. After 6 h, the mononucleates were attached to the plate, allowing the fiber fragments to be washed away. After 12 h, the cells were collected by trypsinization and the number of cells was determined by a hemocytometer. The mononucleates were transferred to matrigel (Collaborative Biomedical Products, 1 mg/mL) coated 6-well plates (BioCoat) in DMEM supplemented with 10% FBS, 5% chick embryo extract (CEE, Sera Laboratories International), 50 units/mL penicillin and 50 μg/mL streptomycin. After 24 h, cells were incubated with 2 μM Nz for 7–10 days to undergo neurogenesis.

To obtain satellite cells, single muscle fibers were plated in one drop of Ham's F10 media (Gibco) into matrigel coated 6-well plates, and were allowed to attach for at least 4 h before adding plating media (Ham's F10 media supplemented with 10% FBS and 1% CEE). After 7

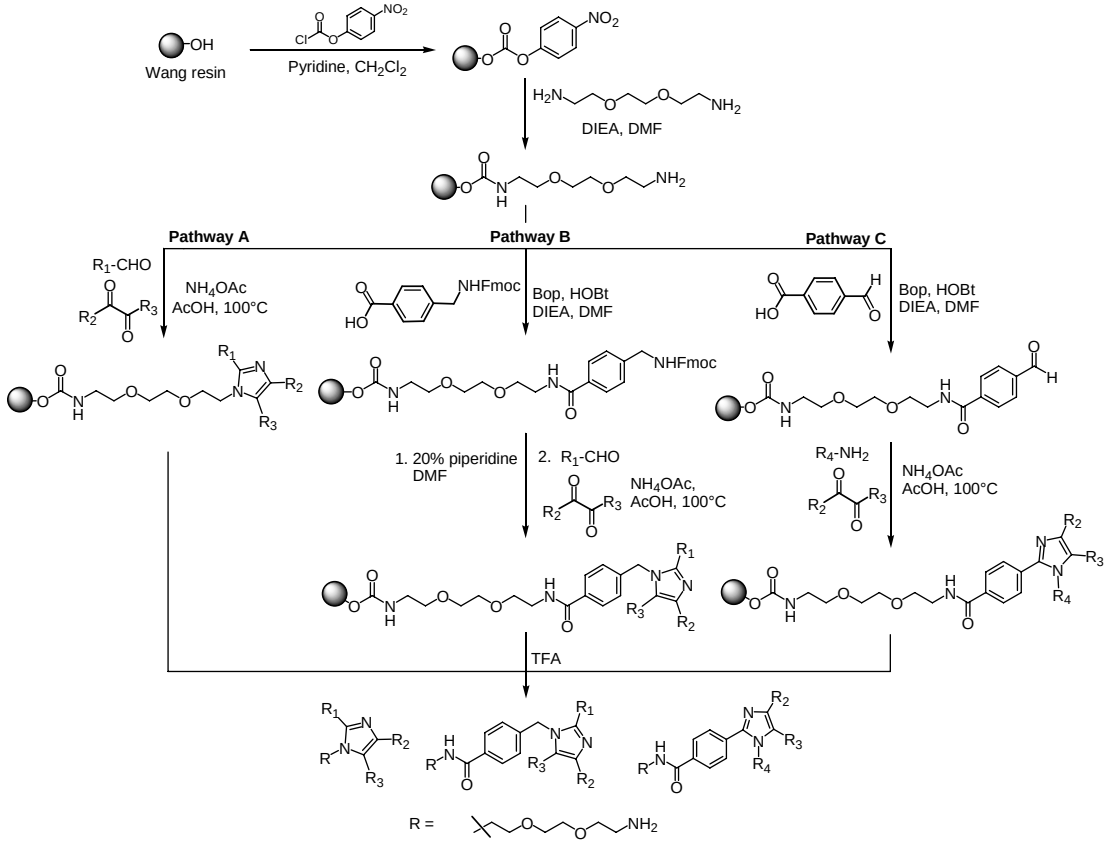
days, the satellite cells were collected by trypsinization (the satellite cells began to migrate from the fiber after 4–5 days) and the number of cells determined by a hemocytometer. These cells were plated at a density to ensure limited cell contact to reduce the onset of terminal myogenesis (e.g. 10^5 cells/well of a 6-well plate) in differentiation media (DMEM supplemented with 2%FBS, 1%CEE, 50 unit/mL penicillin and 50 μg/mL streptomycin). After 24 h, cells were incubated with 2 μM Nz for 7–10 days to undergo neurogenesis.

Western blot analysis: Proteins were separated by 7.5% or 10% SDS-PAGE and transferred onto membranes (Hybond™, Amersham Biosciences, NJ). The membranes were incubated in a blocking solution (PBS buffer containing 5% non-fat skim milk and 0.5% Tween-20) for 1–2 h at room temperature to reduce nonspecific adsorption of antibodies. After the membranes were briefly washed with PBS-Tween, they were incubated with diluted primary antibodies in PBS-Tween for 1 h at room temperature or overnight at 4 ^{o}C. After the membranes were washed with PBS-Tween, they were treated with diluted secondary antibodies in PBS-Tween for 0.5–1 h at room temperature. The treated membranes were visualized by using the ECL kit from Amersham–Pharmacia Biotech. Primary antibodies were used at the following dilutions: mouse monoclonal to neuron-specific βIII-tubulin (TuJ1) – 1:1000 (Abcam, Cambridge, MA); chicken polyclonal to neuron specific enolase – 1:1000 (Abcam, Cambridge, MA); mouse monoclonal to neurofilament 200 (phosphorylated and non-phosphorylated –clone C52) – 1:1000 (Sigma); mouse monoclonal to MyoD1 (5.8A) – 1:1000 (Abcam, Cambridge, MA); mouse monoclonal to s-100 (B32.1) – 1:1000 (Abcam, Cambridge, MA); sheep polyclonal to choline acetyltransferase – 1:1000 (Abcam, Cambridge, MA). Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG (Sigma, 1:2000), rabbit anti-chicken IgY (Sigma, 1:2000) and rabbit anti-sheep IgG (Abcam, Cambridge, MA, 1:2000).

Immunocytochemistry: Cells were fixed for 10 - 30 min with 4% paraformaldehyde in PBS buffer and then washed with PBST buffer (PBS buffer containing 0.1–0.3% Triton X-100). The fixed cells were incubated in a blocking solution (PBS containing 0.1–0.3% Triton X-100 and 0.1% FBS) for 1 h at room temperature to reduce nonspecific adsorption of antibodies. Cells were treated with diluted primary antibodies in PBST buffer for 1 h at room temperature or overnight at 4 ^{o}C and then washed with PBST buffer. Cells were treated with diluted secondary antibodies in PBST buffer for 0.5–1 h at room temperature. Primary antibodies were used at the following dilutions: mouse monoclonal to neuron-specific βIII-tubulin (TuJ1) – 1:500; chicken polyclonal to neuron specific enolase – 1:500; mouse monoclonal to neurofilament 200 (phosphorylated and non-phosphorylated – clone C52) – 1:500; rabbit polyclonal to GAP-43 (Abcam, Cambridge, MA) – 1:200; rabbit polyclonal to MAP-2 (Santa Cruz) – 1:200; rabbit polyclonal to synapsin (Sigma) – 1:200; sheep polyclonal to choline acetyltransferase (Abcam, Cambridge, MA) – 1:500. Secondary antibodies were biotinylated goat anti-mouse IgG (Sigma, 1:200), rabbit anti-chicken IgY H and L (Abcam, Cambridge, MA, 1:500) and rabbit anti-sheep IgG (Abcam, Cambridge, MA, 1:500). Cells were imaged by fluorescence microscopy (Nikon Eclipse TE2000 microscope) or confocal fluorescence microscopy (Bio-Rad Radiance 2100).

Synaptic vesicle recycling studies using FM1-43: Cells were cultured in T25 cm^2 flasks (Nunc). After 5 day incubation, cells were harvested with trypsin/EDTA (Sigma) for 5 min with gentle shaking followed by centrifugation at 600 rpm for 5 min. This relatively gentle approach was employed to preserve the integrity of neurites and axons. Harvested cells were re-suspended gently and seeded at 10^4 cells/well of a 96-well plate. After 8 h, cells were treated with 2 μM FM1-43 dye (Molecular Probes) dissolved in Ringer buffer (external $[Na^+]$ was reduced by an equivalent amount of K^+) for 5 min at room temperature. Cells were then washed with Ringer buffer for 15 min to remove excess FM1-43. Fluorescent intensity (excitation wavelength: 470 nm, emission wavelength: 540 nm) was measured by a fluorescent microplate reader (SpectraMax GeminiEM, Molecular Devices). Cells were imaged by fluorescence microscopy.

cDNA microarray analysis: Microarray experiments were performed using Agilent mouse 20K cDNA chips. To identify genes whose expression was changed substantially in response to Nz treatment, Cy5-labeled RNA from C2C12 cells incubated with 2 μM Nz for 12 h was hybridized together with Cy3-labeled RNA from untreated C2C12 cells. Microarray data were analyzed with significance analysis of microarrays (SAM). Genes exhibiting significant expression changes (> 2-fold) after Nz treatment were selected to investigate affected biological pathways.



Scheme S1. Synthetic pathways to prepare an imidazole library.

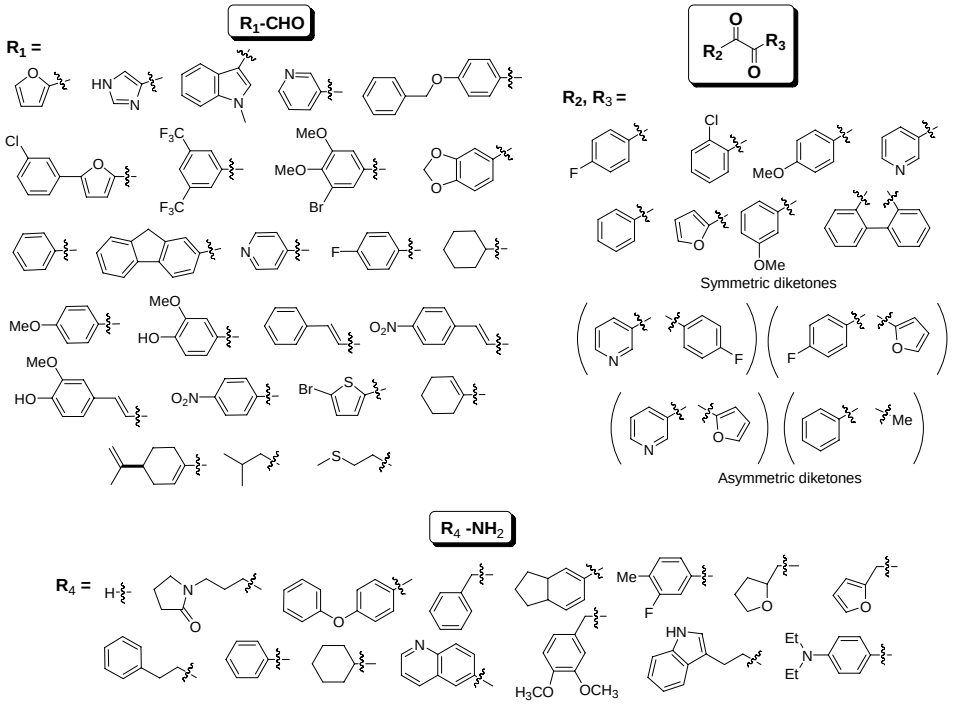
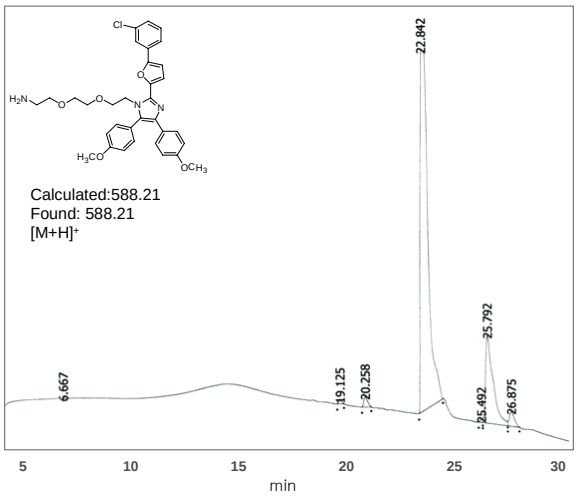


Figure S1. Structures of substituents R_1–R_4.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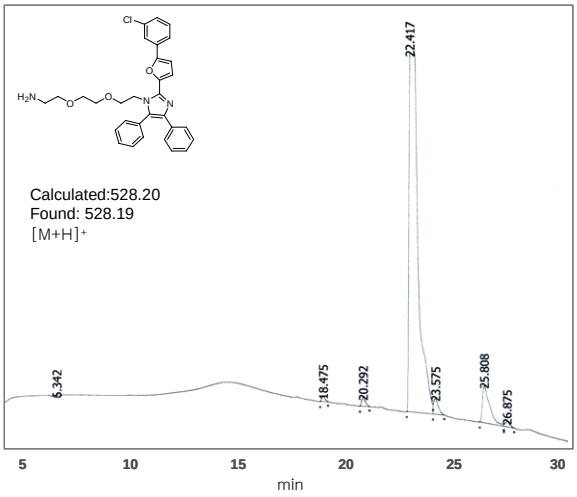
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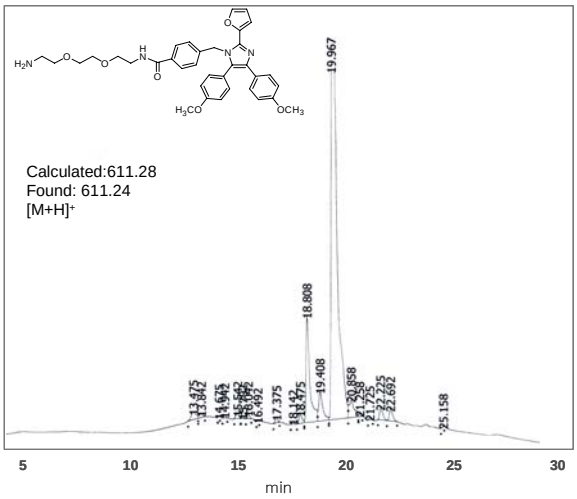
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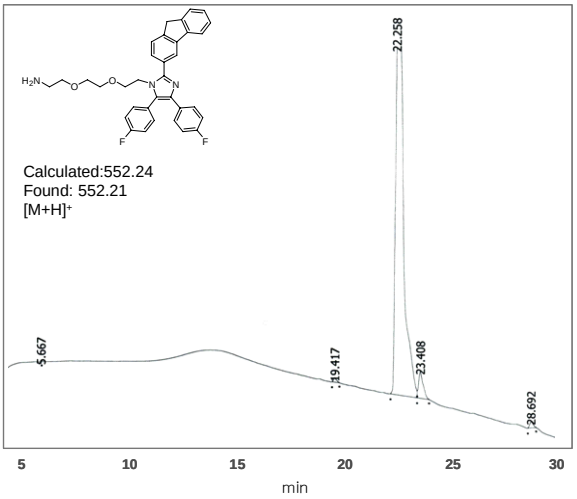
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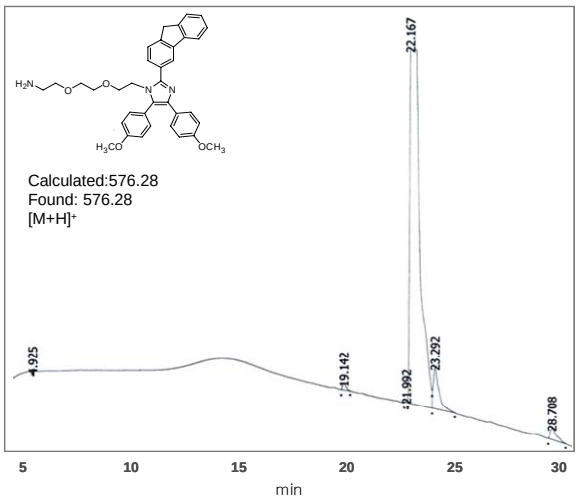
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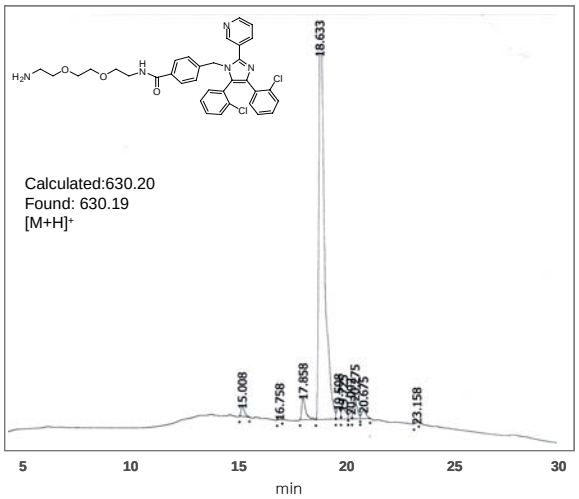
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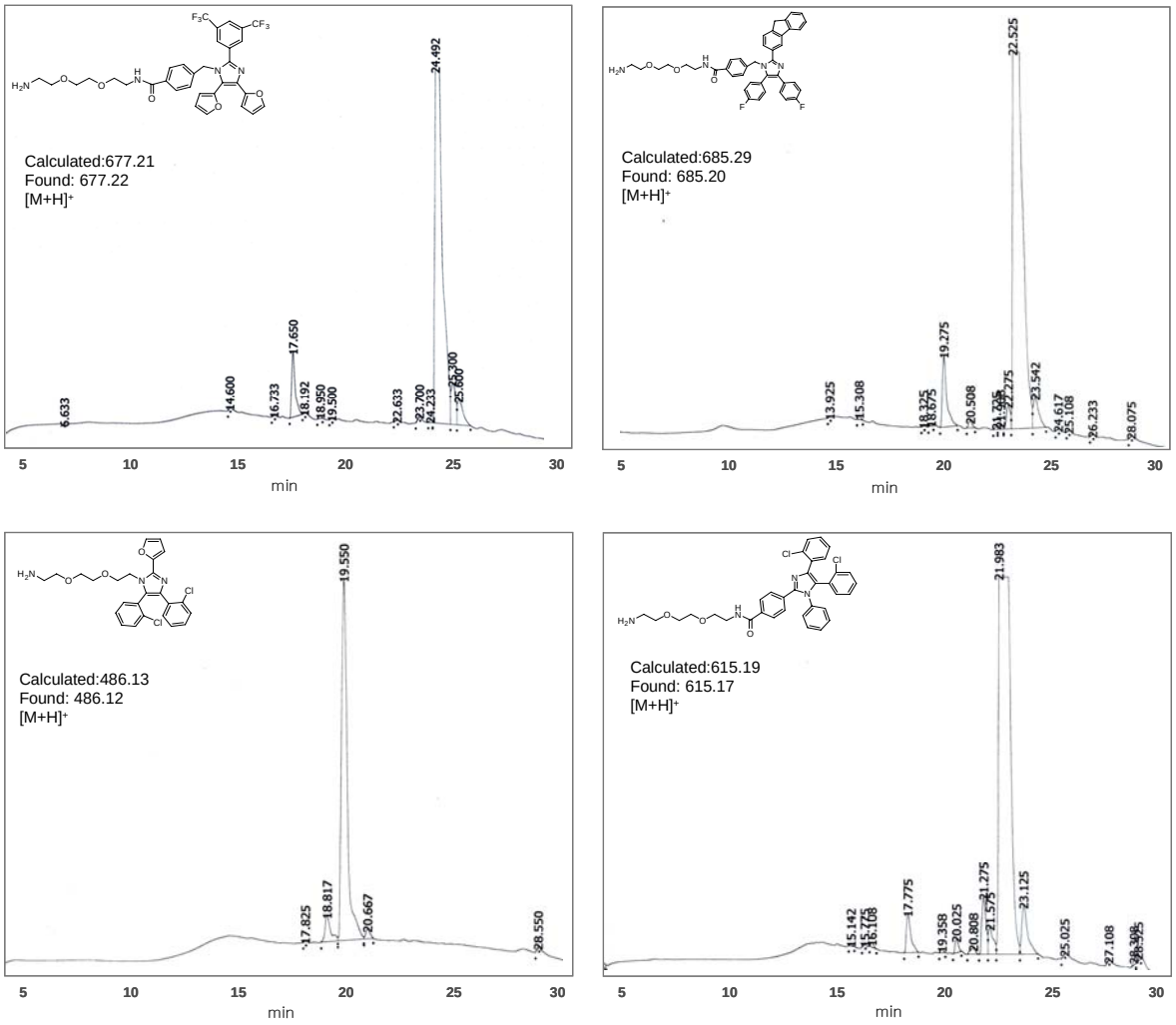

Figure S2. HPLC profiles of imidazole derivatives. A major peak in each HPLC chromatogram is the desired product.

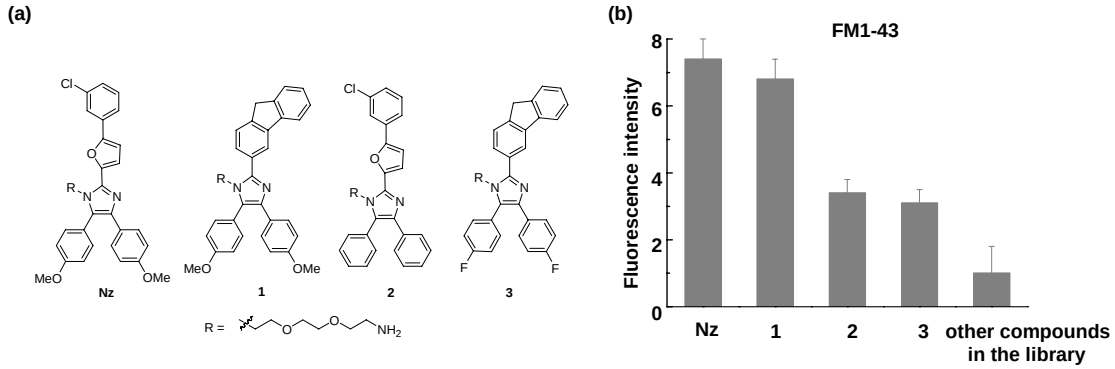

Figure S3. Screening of small molecules that induce neurogenesis in C2C12 myoblasts using FM1-43. (a) Structure of four compounds with neurogenesis-inducing activity based on FM1-43 assay. (b) Fluorescence intensity of imidazole derivatives after incubation of compound-treated C2C12 cells with FM1-43. Four compounds (Nz, **1**, **2** and **3**) exhibit at least three times higher fluorescence intensity than other compounds in the library or control without a compound (error = SD). Among the compounds, neurodazine (Nz) shows the strongest fluorescence intensity.

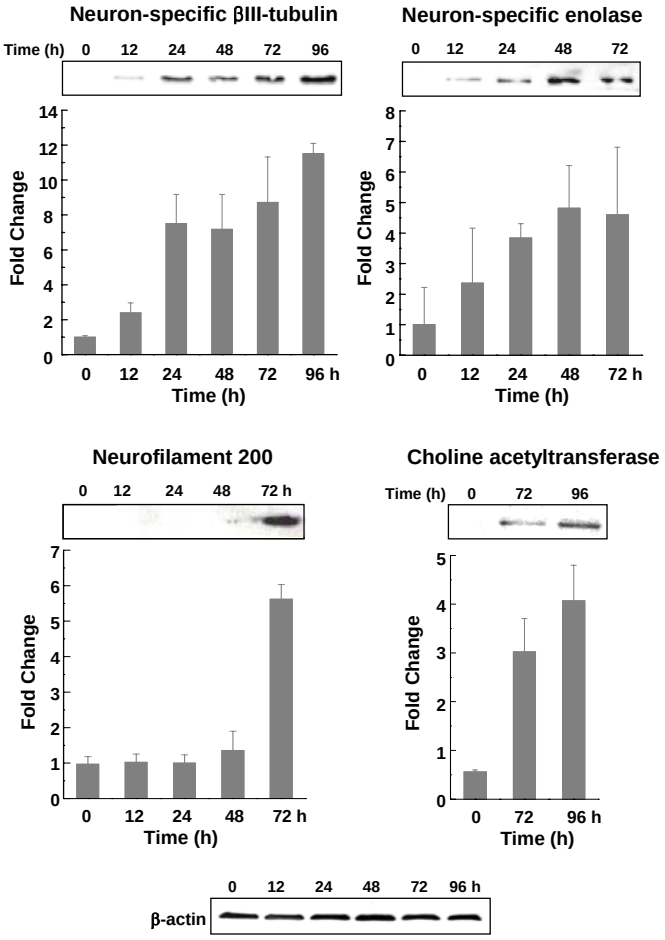

Figure S4. Western blot analysis of the C2C12 cells treated with 2 μM Nz for 7 days (the amount of loaded proteins; 10 μg/lane). All the experiments were performed at least three times (error = SD).

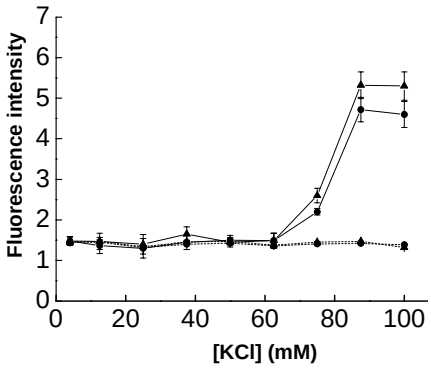

Figure S5. Concentration-dependence of KCl on synaptic vesicle recycling of C2C12 and PC12 cells treated with 2 μM Nz and 100 ng/mL NGF, respectively (–●– : Nz-treated C2C12 cells, ···●··· : untreated C2C12 cells, –▲– : NGF-treated PC12 cells, ···▲··· : untreated PC12 cells).

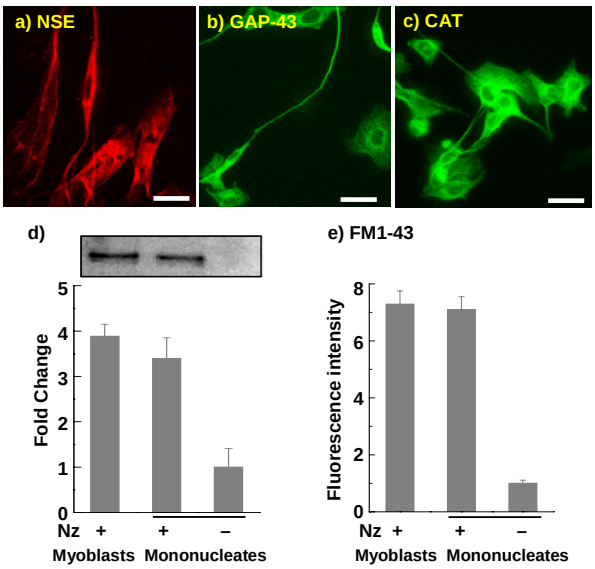

Figure S6. Neurogenesis of differentiated C2C12 myotubes by a two-step procedure. a-c) The upregulation of neuron-specific markers (NSE, GAP-43 and CAT) in mononucleates treated with 2 μM Nz for 7 days is visualized by immunocytochemistry (bar = 50 μm). d) Western blot analysis of C2C12 myoblasts and mononucleates treated with 2 μM Nz for 7 days using anti-NST antibody. e) Synaptic vesicle recycling of myoblasts and mononucleates after treatment with 2 μM Nz for 7 days using FM1-43. All the experiments were performed at least three times (error = SD).

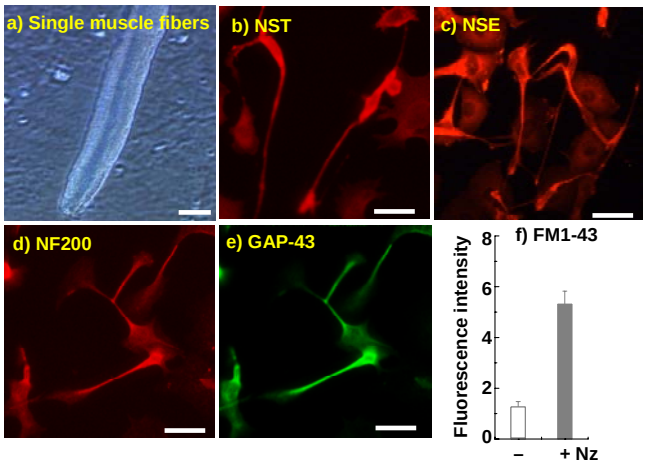

Figure S7. Neurogenesis of human skeletal muscle fibers by Nz. a) Single muscle fibers isolated from the abductor hallucis muscle, b-e) immunocytochemical analysis of mononucleates and satellite cells after treatment with 2 μM Nz for 7 days (bar = 50 μm) and f) synaptic vesicle recycling of mononucleates after treatment with 2 μM Nz for 7 days using FM1-43 (error = SD).

Table S1. Transcriptional changes after treatment of C2C12 myoblasts with Nz

Genes linked to neurogenesis that are upregulated by Nz

Gene	M score[a]
Leucine-rich repeat kinase 1	1.770995
B-cell translocation gene 2, anti-proliferative	1.600475
Nuclear receptor subfamily 2, group F, member 2 regulation of transcription, DNA-dependent	1.685783
ATP-binding cassette, sub-family C (CFTR/MRP), member 4	1.00966
TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor	1.113373
Regulatory factor X domain containing 2 homolog (human)	1.613846
Epoxide hydrolase 1, microsomal	1.630225
Nuclear transcription factor-Y gamma	2.746852
Dystonin	2.181571
Eph receptor B2	1.00211
Tumor necrosis factor receptor superfamily, member 12a	1.223877
Vesicle-associated membrane protein 3	1.274153
Synaptotagmin VI	1.734244
Amyloid beta (A4) precursor protein-binding, family B, member 1	1.473769
Internexin neuronal intermediate filament protein, alpha	1.0148293
Mitogen activated protein kinase kinase 1	1.297401
Bcl2-associated X protein	1.046502
Gamma-aminobutyric acid (GABA-A) receptor, subunit delta	1.103018
Nucleoporin 50	1.203522
Ephrin B2	1.095674
Syntaxin 4A (placental)	1.065573
N-ethylmaleimide sensitive fusion protein attachment protein alpha	1.526439
Proprotein convertase subtilisin/kexin type 9	1.034653
Eph receptor A2	1.038586
Ceroid-lipofuscinosis,	1.004651
Peroxisomal membrane protein 3	1.168817
Neuron navigator 1	2.145832
Enolase 2, gamma neuronal	1.326852
Glial cell line derived neurotrophic factor	1.119861

Genes linked to myogenesis that are affected by Nz

MAD homolog 3	0.238957
Myogenin	0.193628
Plasminogen	0.318897
Cysteine and glycine-rich protein 3	0.05591
Biregional cell adhesion molecule-related/down-regulated by oncogenes (Cdon) binding protein	0.246415
Centromere autoantigen F	-0.75609
Myogenic factor 6	0.273523
Ras homolog gene family, member A	0.501602
E1A binding protein p300	0.056421

Transforming growth factor, beta 1	-0.7101
Myogenic differentiation 1	-0.29099
Vestigial like 2 homolog	-0.29705
Vesicle-associated membrane protein 5	0.790137
Procollagen, type XIX, alpha 1	-0.01803
Cell adhesion molecule-related/down-regulated by oncogenes	-0.0309
Myogenic factor 5	0.708617
Luc7 homolog	-0.53109
Filamin, beta	0.516043
Sirtuin 1	0.146131

[a]M score = $\log_2$ (Fluorescent intensity of labeled Cy5 in mRNA obtained from C2C12 cells treated with Nz)/(Fluorescent intensity of labeled Cy3 in mRNA obtained from untreated C2C12 cells). M scores above 1 are deemed as significant upregulation of expression.