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Synthetic Small Molecules That Induce Neuronal Differentiation in Neuroblastoma and Fibroblast Cells

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Abstract
An investigation was conducted to demonstrate that neurodazine (Nz) and neurodazole (Nzl), two imidazole-based small molecules, promote neuronal differentiation of both neuroblastoma and fibroblast cells. The results show that differentiated cells generated by treatment with Nz and Nzl express neuron-specific markers. The ability of Nz and Nzl to induce neurogenesis of neuroblastoma and fibroblast cells was found to be comparable to those of the known neurogenic factors, retinoic acid and trichostatin A. In addition, the differentiated cells by Nz and Nzl are observed to express different isoforms of glutamate receptors. The results of signaling pathway studies reveal that two substances enhance neurogenesis in neuroblastoma cells by activating Wnt and Shh signaling pathways and neurogenesis in fibroblast cells by mainly activating the Wnt signaling pathway. Observations made in the present study suggest that Nz and Nzl will serve as chemical tools to generate specific populations of neuronal cells from readily available and simply manageable cells.
Introduction

Neural development is a strictly regulated process that integrates cell proliferation, differentiation, and programmed cell death.¹ Neurogenesis in the nervous system of animals takes place throughout life and is critical for key aspects of memory, learning and thinking. Loss of the structure and function of neurons causes progressive and irreversible deterioration of the nervous system and, consequently, it leads to neurodegenerative disease, including stroke, Parkinson’s, Alzheimer’s and Huntington’s diseases. When they are damaged or destroyed, neurons generally are not regenerated effectively under normal physiological conditions. As a result, the development of approaches that induce neuronal differentiation is greatly relevant to the treatment of neurodegenerative diseases. Potential therapeutic methods for this purpose involve the stimulation of neurogenesis to induce the generation of new neurons within the adult nervous system or the cell therapy in which differentiated neurons are injected into a patient.²⁻⁴ A promising strategy for induction of neurogenesis is termed 'small molecule-based cellular alchemy',⁵ in which synthetic small molecules that accelerate alterations of cell fate are utilized to generate neurons. This approach has several advantages over genetic methods, such as low cost, the ability to modulate functions of the target in a rapid and reversible manner and to attain reproducible activity, and relatively facile development of appropriate pharmaceutical agents.⁶,⁷

Over a last decade, several small molecules have been shown to promote neuronal differentiation in vitro and in vivo.⁵,⁸⁻¹² One neurogenesis inducer of this type is retinoic acid which is known to induce differentiation of neural, mesenchymal and embryonic stem cells into neurogenic cells (Fig. 1).¹³⁻¹⁶ However, this substance displays multiple bioactivities, including the regulation of embryogenesis, development of bone and maintenance of epithelium.¹⁷⁻¹⁹ In addition to retinoic acid, several other small molecules in the family of known protein inhibitors have been identified as being neuronal differentiation inducers. For example, the histone deacetylase inhibitor, trichostatin A, is known to enhance conversion of neural stem cells to physiologically active neurons.²⁰ Also, glycogen synthase kinase-3 (GSK-3) inhibitors, such as kenpaullone and SB-216763, promote neurogenesis of human neural progenitor cells and mouse pluripotent P19 cells.²¹ Moreover, the results of high-throughput screening studies demonstrated the existence of several small molecules, such as TWS119, neuropathiazol and isoxazole 9, which convert embryonic stem cells, hippocampal neural stem cells and neural progenitor cells
into neurogenic cells. Interestingly, a small molecule KHS101, which binds to transforming acidic coiled-coil-containing protein 3, was observed to induce neurogenesis of hippocampal progenitor cells as well as to enhance neuronal differentiation significantly in rats. It is also known that treatment with a combination of inhibitors of SMAD and GSK-3 signaling pathways converts human fibroblasts into functional neurons.

As part of an intense effort aimed at the discovery of neurogenesis inducing small molecules, we recently identified two small molecules, neurodazine (Nz) and neurodazole (Nzl), which promote neurogenesis in pluripotent embryonic carcinoma P19 cells and skeletal muscle cells. This finding stimulated an effort aimed at determining whether Nz and Nzl are able to induce neurogenesis in simply manageable and easily available cells, such as neuroblastoma and fibroblast cells. Neuroblastoma cells, which are transformed neural crest derived cells and are capable of unlimited proliferation in vitro, possess many of the biochemical and functional properties of neurons after differentiation. Human neuroblastoma cells, SH-SY5Y cells, have the ability to be differentiated into neurogenic cells and, as a result, they have been employed as neuronal cell models in studies of Parkinson’s and Alzheimer’s diseases. Also, mouse neuroblastoma cells, Neuro-2a cells, which can be differentiated into neurogenic cells within a few days, have been used to explore neuronal differentiation, axonal growth and signaling pathways. Among many different fibroblast cell lines that are currently available, NIH3T3 cells are often used as models in studies of osteogenesis and cardiogenesis studies. However, the results of only a few investigations have shown that NIH3T3 cells can be differentiated into neurogenic cells by small molecules (e.g. 5-aza-2′-deoxycytidine and trichostatin A). Although the outcomes of the previous studies are promising, additional efforts are required in order to demonstrate that NIH3T3 cells are suitable for study of neuronal differentiation. In the investigation described below, we evaluated the potential of Nz and Nzl to induce neurogenesis in SH-SY5Y, Neuro-2a and NIH3T3 cells. In this exploration, we uncovered evidence supporting the conclusion that Nz and Nzl promote neuronal differentiation of simply manageable and easily available neuroblastoma and fibroblast cells.

Results

Neurogenesis of neuroblastoma cells by Nz or Nzl
To test if Nz and Nzl have neurogenesis inducing activities in neuroblastoma cells, human SH-SY5Y cells were initially incubated with 5 μM Nz or 5 μM Nzl for 10 days and then subjected to immunocytochemical analysis using antibodies against neuron-specific markers, including neuron-specific βIII tubulin (Tuj1), microtubule-associated protein 2 (MAP2), neurofilament 200 (NF200) and neuron-specific enolase (NSE). As a positive control, the cells were treated with the known neurogenic factor, retinoic acid (10 μM). The results of immunocytochemical analysis show that treatment with either Nz or Nzl promotes expression of neuronal proteins in SH-SY5Y cells, a phenomenon which also occurs in retinoic acid treated cells (Fig. 2 and Fig. S1A). The ability of Nz and Nzl to enhance neurogenesis of SH-SY5Y cells was found to be comparable to that of retinoic acid.

The neurogenesis inducing activity of Nz and Nzl in SH-SYS5Y cells was further examined by employing western blot analysis of neuronal proteins. The results indicate that both Nz and Nzl enhance expression of Tuj1, NF200 and NSE in the treated cells with a pattern similar to that observed for retinoic acid treated cells (Fig. 3A and Fig. S1B). In addition, the results of reverse transcription polymerase chain reaction (RT-PCR) analysis reveal that upregulation of neuron-specific genes, such as Tuj1, NF200, NeuroD and MAP2, takes place in cells cultured with Nz and Nzl, which also occurs in retinoic acid treated cells (Fig. 3B and Fig. S1C). Collectively, the results presented above show that Nz and Nzl promote neurogenesis of SH-SYS5Y cells with activities similar to that of retinoic acid.

The effect of Nz and Nzl on induction of neurogenesis of mouse neuroblastoma cells was explored next. For this purpose, mouse Neuro-2a cells were incubated with 5 μM Nz or 5 μM Nzl for 10 days. The cells were also cultured for 10 days in the presence of 10 μM retinoic acid as a positive control. At the end of each culture period, immunocytochemical analysis was conducted with several neuron-specific antibodies. The results show that treatment with Nz or Nzl promotes the expression of neuronal markers, such as Tuj1, MAP2, NF200 and Tau, in the cultured cells (Fig. 4). The neurogenesis inducing activities of Nz and Nzl were also demonstrated by using western blot analysis of neuron-specific proteins, such as Tuj1, NSE, GAP43 (growth associated protein 43) and NF200 (Fig. 5A). The results of RT-PCR analysis also show that neuronal markers are upregulated in Neuro-2a cells cultured cells with Nz and Nzl in the same fashion as those treated with retinoic acid (Fig. 5B and Fig. S2). Taken together, the
observations indicate that Nz or Nzl converts human and mouse neuroblastoma cells into neurogenic cells with activities that are similar to that of the known neurogenic factor, retinoic acid.

**Expression of neurotransmitter receptors in Nz or Nzl treated neuroblastoma cells**

A variety of neurotransmitter receptors are differentially expressed during neuronal differentiation. Glutamate, the principal excitatory neurotransmitter present in most mammalian neurons, acts on glutamate receptors located primarily on the membrane of neuronal cells. Glutamate receptors, which are associated with the glutamate-mediated postsynaptic excitation of neurons, play important roles in neural communication, memory formation and learning.[37,38] These receptors are generally classified into two groups, called ionotropic and metabotropic glutamate receptors, according to the activation mechanism.[39,40] Ionotropic glutamate receptors form ion channel pores and are stimulated upon binding of glutamate. In contrast, metabotropic glutamate receptors (mGluRs) indirectly activate ion channels located on plasma membranes through G protein associated signaling pathways.

To examine whether neuronal cells derived by treatment of neuroblastoma cells with Nz and Nzl express glutamate receptors, SH-SY5Y and Neuro-2a cells were first incubated with 5 μM Nz or 5 μM Nzl. Then, RT-PCR analysis of six ionotropic (NMDAR1, GluK2, GluK4, NMDA2A, NMDA2B, AMPA1(α1)) and four metabotropic (mGluR3, mGluR5, mGluR6 and mGluR7) glutamate receptors was performed. The results show that Nz and Nzl enhance the expression of certain types of glutamate receptors during neuronal differentiation of neuroblastoma cells. Specifically, Nz or Nzl treated SH-SY5Y cells express the ionotropic glutamate receptors NMDAR1 and GluK4 and the metabotropic glutamate receptor mGluR6 (Fig. 6), but they do not express other isoforms of ionotropic (NMDA2A, NMDA2B, GluK2 and AMPA1(α1)) and metabotropic (mGluR3, mGluR5 and mGluR7) glutamate receptors (data not shown). Moreover, Nz or Nzl treated Neuro-2a cells express different but some overlapped glutamate receptors as compared to treated SH-SY5Y cells. Specifically, Neuro-2a cell derived neurogenic cells enhance the expression of GluK4, mGluR3, mGluR5 and mGluR7 (Fig. S3) but not of NMDAR1, NMDA2A, NMDA2B, GluK2, AMPA1(α1) and mGluR6 (data not shown). The combined results show that certain isoforms of glutamate receptors are expressed during Nz
and Nzl promoted neuronal differentiation of neuroblastoma cells. The fact that glutamate receptors are implicated in excitation-coupled neurogenesis suggests that the upregulation of ionotropic and metabotropic glutamate receptors elicited by Nz and Nzl is part of differentiation process leading to functional neurons.

**Effect of Nz or Nzl on signaling pathways associated with neurogenesis of neuroblastoma cells**

To elucidate the underlying mechanism of neurogenesis in neuroblastoma cells, the effect of Nz and Nzl on signaling pathways associated with neurogenesis were explored. Previously, we provided evidence to support the conclusion that Nz and Nzl induced neurogenesis in pluripotent P19 cells took place through their activation of Wnt and Shh signaling pathways. Consequently, the effect of Nz and Nzl on the Wnt signaling pathway during neurogenesis in neuroblastoma cells was determined. For this purpose, SH-SY5Y and Neuro-2a cells were separately incubated for 8 days with 5 µM Nz or 5 µM Nzl in the presence or absence of the two Wnt pathway inhibitors, NSC668036 (20 µM) and PKF118-310 (25 nM). It is known that NSC668036 binds to the PDZ domain of the Disheveled (Dsh) protein, a Wnt pathway signaling molecule, and PKF118-310 suppresses the formation of a complex of Tcf4 and β-catenin in the Wnt pathway. The results of immunocytochemical analysis of the incubation mixtures show that the efficiencies of neurogenesis of SH-SY5Y cells induced by Nz and Nzl are substantially reduced in the presence of each of the inhibitors (Fig. 7 and Fig. S4). Similarly, the neurogenesis inducing activities of Nz and Nzl in Neuro-2a cells are also attenuated in the presence of each of Wnt pathway inhibitors (Fig. 8 and Fig. S5).

In order to determine whether the Shh signaling pathway is involved in neuronal differentiation of neuroblastoma cells induced by Nz and Nzl, SH-SY5Y and Neuro-2a cells were cultured with 5 µM Nz or 5 µM Nzl for 8 days in the presence and absence of Cur61414 (25 µM), an inhibitor of smoothened (SMO) in the Shh signaling pathway. The results of immunocytochemistry analysis show that the neurogenesis inducing activities of Nz or Nzl are markedly attenuated in both of the neuroblastoma cells when Cur61414 is present (Fig. 7 and 8 and Fig. S4 and S5). The effect of Nz and Nzl on the Notch signaling pathway during neurogenesis of neuroblastoma cells was also evaluated. In this experiment, both SH-SY5Y and
Neuro-2a neuroblastoma cells were incubated for 8 days with 5 μM Nz or 5 μM Nzl in the presence and absence of MK0752 (25 μM), an inhibitor of γ-secretase that blocks the neurogenesis associated Notch pathway. The results indicate that MK0752 has little inhibitory effect on neuronal differentiation of both neuroblastoma cells promoted by Nz and Nzl (Fig. 7 and 8 and Fig. S4 and S5). Observations made in these signaling pathway studies provide evidence to support the conclusion that Nz and Nzl enhance neurogenesis in neuroblastoma cells by activating the Wnt and Shh signaling pathways and not by activating the Notch signaling pathway (Fig. S6).

**Neurogenesis of fibroblasts by Nz or Nzl**

We next turned our attention to determining whether Nz and Nzl have the ability to convert fibroblasts into neurogenic cells in a simple culture system. Mouse NIH3T3 fibroblast cells, which have been successfully used to generate neuron-like cells by treatment with small molecules, were utilized in this experiment. To examine neurogenesis inducing activities, NIH3T3 fibroblast cells were cultured with Nz or Nzl for 10 days as well as the known neurogenic agent, trichostatin A, as a positive control. Immunocytochemical studies were then conducted to characterize the phenotype of the neuron-like cells that were generated from fibroblast cells. The results show that treatment with Nz or Nzl induces expression of several neuron-specific markers in NIH3T3 cells that are similar to that elicited by trichostatin A (Fig. 9 and Fig. S7A).

The neurogenesis inducing activities of Nz and Nzl in NIH3T3 cells were also examined by using western blot and RT-PCR analyses of neuron-specific markers. As the images displayed in Fig. 10A show, both substances enhance the expression of neuron-specific proteins such as Tuj1, NSE and NF200 in NIH3T3 cells. In addition, Nz or Nzl treated fibroblast cells also produce neuronal genes such as Tuj1, NeuroD, Mash1 and Ngn1 (Fig. 10B). Notably, the expression levels of neuronal markers in Nz and Nzl treated cells are comparable to those in cells cultured with trichostatin A (Fig. S7B and S7C). When combined, the results demonstrate that Nz and Nzl have the ability to convert fibroblasts into cells possessing both morphological and phenotypic properties of neurons with activities that are similar to that of trichostatin A.
Expression of neurotransmitter receptors in Nz or Nzl treated fibroblasts

Because Nz and Nzl have neurogenesis-inducing activities in NIH3T3 fibroblasts, we evaluated whether the neurogenic cells derived by treatment of fibroblasts with these small molecules expressed glutamate receptors. In this study, NIH3T3 cells were exposed to Nz or Nzl and then RT-PCR analysis of six ionotropic and four metabotropic glutamate receptors was conducted over a 9 day period. The results show that mRNA expression takes place in the AMPA1(α1) and GluK2 isoforms of ionotropic glutamate receptors and mGluR3 and mGluR7 isoforms of metabotropic glutamate receptors (Fig. 11), but not in the NMDAR1, GluK4, NMDA2A, NMDA2B, mGluR5 and mGluR6 isoforms (data not shown).

Effect of Nz or Nzl on signaling pathways associated with neurogenesis of fibroblasts

To understand the cellular basis of the neuronal differentiation promoted by Nz or Nzl, signaling pathways associated with neurogenesis of fibroblasts were evaluated using immunocytochemical analysis. The studies were performed utilizing inhibitors for Wnt, Shh and Notch signaling pathways. For example, NIH3T3 cells were incubated with Nz or Nzl in the presence of the Wnt pathway inhibitors, NSC668036 (15 μM) and PKF118-310 (20 nM). The results show that the neurogenesis inducing efficiency of Nz and Nzl are greatly attenuated when NSC668036 and PKF118-310 are present (Fig. 12 and Fig. S8). In contrast, neither of the Shh (Cur61414) and Notch signaling pathway inhibitors (MK0752) have a significant effect on neuronal differentiation of NIH3T3 fibroblast cells induced by Nz and Nzl (Fig. 12 and Fig. S8). The findings suggest that Nz and Nzl promote neurogenesis in NIH3T3 fibroblast cells mainly through activation of the Wnt pathway but they may not affect Shh and Notch signaling pathways (Fig. S9).

Conclusions

Over the last decade, a number of attempts have been made to design methods to enhance neurogenesis in embryonic and neural stem cells. A highly convenient and attractive strategy for the generation of neurons involves induction of neuronal differentiation of easily available and simply manageable cells or tissues by using synthetically accessible small molecules. In this study described above, we demonstrate that the imidazole derivatives, Nz and Nzl, promote
neuronal differentiation in both neuroblastoma and fibroblast cells. The ability of Nz and Nzl to induce neurogenesis in these cells is comparable to those of the known neurogenic factors, retinoic acid and trichostatin A. Interestingly, neurogenic cells derived from neuroblastoma and fibroblast cells cultured with Nz and Nzl express glutamate neurotransmitter receptors although the expression patterns depend on the cell types. The results of signaling pathway studies indicate that these small molecules enhance neurogenesis in neuroblastoma cells by activating the Wnt and Shh signaling pathways and neurogenesis in fibroblast cells mainly by activating the Wnt signaling pathway. When combined with observations we have made in previous studies,5,8,12 the results show that Nz and Nzl convert skeletal muscle cells, pluripotent embryonic carcinoma cells, neuroblastoma cells and fibroblasts into neurogenic cells. As a consequence, Nz and Nzl will serve as useful chemical tools in methods employed to generate specific populations of neuronal cell types from pluripotent cells as well as easily available and simply manageable cells.

**Experimental**

**Cell culture**

Human neuroblastoma SH-SY5Y cells (ATCC® CRL 2266™) were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, 50 μg/mL streptomycin (Gibco, Paisley, UK) and 2 mM L-glutamine (Gibco, Paisley, UK). Mouse neuroblastoma Neuro-2a cells (ATCC® CCL 131™) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. Mouse NIH3T3 fibroblast cells (ATCC® CRL 1658™) were grown in DMEM supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. Cells were maintained at 37 °C in humidified atmosphere with 5% CO₂.

**Neuronal differentiation of neuroblastoma cells by Nz or Nzl**

SH-SY5Y cells were seeded at a density of 1 x 10⁶ cells per mL in 90 mm petri dishes under non-adherent culture conditions and allowed to aggregate for 3 days with 5 μM Nz or 5 μM Nzl. Aggregated embryonic bodies were dissociated into single cells by treatment with 0.25% trypsin-
EDTA solution (Sigma). Cells were seeded in a tissue culture dish at a density of approximately 1 x 10^5 cells per mL in culture media. After incubation for 24 h, the culture media were replaced with differentiation media (MEM supplemented with 4% FBS, 50 units/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine) containing 5 μM Nz or 5 μM Nzl. Differentiation media were replenished every 2 days. Cells were harvested and analyzed at different time periods during differentiation.

Neuro-2a cells were seeded at a density of 5 x 10^5 cells per mL in 90 mm petri dishes under non-adherent culture conditions and allowed to aggregate for 3 days with 5 μM Nz or 5 μM Nzl. Aggregated embryonic bodies were dissociated into single cells by treatment with 0.25% trypsin-EDTA solution. Cells were seeded in a tissue culture dish at a density of approximately 5 x 10^4 cells per mL in culture media. After incubation for 24 h, the culture media were replaced with differentiation media (DMEM supplemented with 2% FBS, 50 units/mL penicillin, 50 μg/mL streptomycin) containing 5 μM Nz or 5 μM Nzl. Differentiation media were replenished every 2 days. Cells were harvested and analyzed at different time periods during differentiation. As a positive control, SH-SY5Y and Neuro-2a cells were incubated with 10 μM retinoic acid.

Neuronal differentiation of fibroblast cells by Nz or Nzl
NIH3T3 fibroblast cells were seeded at a density of 1 x 10^5 cells per mL in petri dishes under non-adherent culture conditions and allowed to aggregate for 5 days with 2.5 μM Nz or 4 μM Nzl. Aggregated embryonic bodies were dissociated into single cells by treatment with 0.25% trypsin-EDTA solution. Cells were seeded in a tissue culture dish at a density of approximately 1 x 10^4 cells per mL in culture media. After incubation for 24 h, the culture media were replaced with differentiation media (DMEM supplemented with 2% FBS, 50 units/mL penicillin, 50 μg/mL streptomycin) containing 2.5 μM Nz or 4 μM Nzl. Differentiation media were replenished every 2 days. Cells were harvested and analyzed at different time periods during differentiation. As a positive control, NIH3T3 fibroblast cells were incubated with 50 nM trichostatin A.

Immunocytochemistry
Cells were fixed for 20 min with 4% paraformaldehyde and 0.1% Triton X-100 in PBS buffer and then washed with PBS. The fixed cells were incubated in a blocking solution (PBS containing 0.5% FBS) for 1 h at room temperature to reduce nonspecific adsorption of antibodies. The cells were treated with diluted primary antibodies in a blocking solution for 1 h at room temperature or overnight at 4 °C and then washed with PBS. The cells were treated with diluted secondary antibodies in a blocking solution for 1 h at room temperature. Primary antibodies were used in the following dilutions: rabbit monoclonal to Tuj1–1:200; mouse monoclonal to NSE–1:200; mouse monoclonal to NF200–1:200; mouse monoclonal to GAP-43 (Santa Cruz)–1:200; rabbit polyclonal to MAP2 (Santa Cruz)–1:200. Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG, goat anti-mouse IgM. Cells were imaged by fluorescence microscopy (Nikon Eclipse TE2000 microscope).

**Western blot analysis**

Proteins were separated by using 8% or 10% SDS-PAGE and transferred to membranes (Amersham Biotech). The membranes were incubated in a blocking solution (TBS 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 being briefly washed with TBST (TBS buffer containing 0.5% Tween-20), the membranes were incubated with diluted primary antibodies in TBST for 1 h at room temperature or overnight at 4 °C. After the membranes were washed with TBST, they were treated with diluted secondary antibodies in TBST for 1 h at room temperature. The treated membranes were visualized by using the ECL kit (Amersham Biotech). Primary antibodies were used in the following dilutions: rabbit monoclonal to Tuj1–1:1000; mouse monoclonal to NSE–1:1000; mouse monoclonal to NF200–1:1000; mouse monoclonal to GAP-43 (Santa Cruz)–1:1000; rabbit polyclonal to MAP2 (Santa Cruz)–1:1000. Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG and goat anti-mouse IgM.

**Neuronal signaling pathway study**

SH-SY5Y cells were seeded in a tissue culture dish at a density of approximately 1 x 10^5 cells per mL in culture media. In a separate experiment, Neuro-2a cells were seeded in a tissue culture dish at a density of approximately 5 x 10^4 cells per mL in culture media. After incubation for 24
h, the culture media of both SH-SY5Y and Neuro-2a cells were replaced with differentiation media containing 5 µM Nz or 5 µM Nzl in the presence or absence of 20 µM NSC668036, 25 nM PKF118-310, 25 µM Cur61414 or 25 µM MK0752. After every 2 days, differentiation media containing Nz or Nzl without an inhibitor were replenished.

NIH3T3 fibroblast cells were seeded in a tissue culture dish at a density of approximately 1 x 10⁴ cells per mL in culture media. After incubation for 24 h, the culture media was replaced with differentiation media containing 2.5 µM Nz or 4 µM Nzl in the presence or absence of 15 µM NSC668036, 20 nM PKF118-310, 25 µM Cur61414 or 20 µM MK0752. After every 2 days, differentiation media containing Nz or Nzl without an inhibitor were replenished. Immunocytochemical analyses were conducted according to the procedure described above.

Acknowledgements

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References

Fig. 1 Structures of small molecules with neurogenesis-inducing activities.
Fig. 2 Neurogenesis of SH-SY5Y cells induced by (A) Nz and (B) Nzl. Human SH-SY5Y cells were incubated with 5 µM of each compound for 10 days. The cells were fixed and stained with neuron-specific markers. (C) denotes the untreated control followed by the same differentiation protocol (bar: 50 µm).
Fig. 3 Expression of neuron-specific markers in SH-SY5Y cells was examined after treatment with 5 µM Nz or 5 µM Nzl by using (A) western blot and (B) RT-PCR analyses.
Fig. 4 Neurogenesis of Neuro-2a cells induced by (A) Nz and (B) Nzl. Mouse Neuro-2a cells were incubated with 5 µM of each compound for 10 days. The cells were fixed and stained with neuron-specific markers. (C) denotes the untreated control followed by the same differentiation protocol (bar: 50 µm).
Fig. 5 Expression of neuron-specific markers in Neuro-2a cells was examined after treatment with 5 µM Nz or 5 µM Nzl by using (A) western blot and (B) RT-PCR analyses.
Fig. 6 Expression of glutamate receptors in SH-SY5Y cells was examined after treatment with 5 µM Nz or 5 µM Nzl by using RT-PCR.
Fig. 7 Effects of inhibitors of Wnt, Shh and Notch signaling pathways on neurogenesis of SH-SY5Y cells induced by Nz. SH-SY5Y cells were incubated with 5 \( \mu \)M Nz in the presence or absence of 20 \( \mu \)M NSC668036, 25 nM PKF118-310, 25 \( \mu \)M Cur61414, and 25 \( \mu \)M MK0752 for 8 days. The cells were stained with NF200 antibody. Bottom images are of DAPI stained cells (bar: 50 \( \mu \)m).
**Fig. 8** Effects of inhibitors of Wnt, Shh and Notch signaling pathways on neurogenesis of Neuro-2a cells induced by Nz. Neuro-2a cells were incubated with 5 µM Nz in the presence or absence of 20 µM NSC668036, 25 nM PKF118-310, 25 µM Cur61414, and 25 µM MK0752 for 8 days. The cells were stained with NF200 antibody. Bottom images are of DAPI stained cells (bar: 50 µm).
Fig. 9 Neurogenesis of NIH3T3 fibroblast cells induced by (A) Nz and (B) Nzl. Mouse NIH3T3 fibroblast cells were incubated with 2.5 µM Nz or 4 µM Nzl for 10 days. The cells were fixed and stained with neuron-specific markers. (C) denotes the untreated control followed by the same differentiation protocol (bar: 50 µm).
Fig. 10 Expression of neuron-specific markers in NIH3T3 cells was examined after treatment with 2.5 µM Nz or 4 µM Nzl by using (A) western blot and (B) RT-PCR analyses.
Fig. 11 Expression of glutamate receptors in NIH3T3 cells was examined after treatment with 2.5 µM Nz or 4 µM NzI by using RT-PCR.
Fig. 12 Effects of inhibitors of Wnt, Shh and Notch signaling pathways on neurogenesis of NIH3T3 cells induced by Nzl. NIH3T3 cells were incubated with 4 µM Nzl in the presence or absence of 15 µM NSC668036, 20 nM PKF118-310, 25 µM Cur61414, and 20 µM MK0752 for 8 days. The cells were stained with NF200 antibody. Bottom images are of DAPI stained cells (bar: 50 µm).
Imidazole-based synthetic small molecules promote neurogenesis in readily available and simply manageable neuroblastoma and fibroblast cells.