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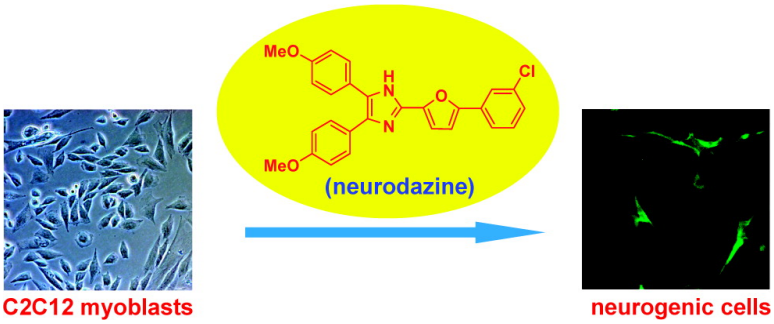

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Synthetic Small Molecules that Induce Neurogenesis in Skeletal Muscle

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Neurodegenerative diseases such as stroke, Parkinson's, and Alzheimer's diseases result from the loss of neurons and are prevalent throughout the world. Recent advances in stem cell biology offer the prospect of a new therapeutic approach for the treatment of these diseases.[1] However, this approach requires a sufficient source of stem cells, a precise control over differentiation, a suppression of host rejection against allogenic cells, and the prevention of tumor formation by undifferentiated cells.[2] These technical problems as well as ethical issues concerning the use of discarded embryos restrict stem-cell-based therapies. Adult stem cells have the potential to differentiate into several cell types and thus could be also used for cell therapies.[3] However, the adult stem cells have reduced efficiency to grow and differentiate into various types of cells compared to embryonic stem cells.

A more convenient and attractive approach for the generation of neurons is the use of small molecules that induce the neuronal differentiation of easily available cells or tissues. Generation of neurogenic cells from non-stem cells using a small molecule inducer can avoid the above-mentioned problems that arise from stem cells therapies. Retinoic acid (RA) is a widely used small molecule for the induction of neuronal differentiation.[4] However, this molecule also has other biological activities, such as the regulation of embryogenesis, the development of bone, and the maintenance of epithelium.[5] Recently, synthetic small molecules that induce neuronal differentiation were also developed.[6] These molecules induce the conversion of pluripotent murine embryonal carcinoma cells and embryonic stem cells to neurogenic cells. However, more interesting small molecules would be those with the capacity to induce neurogenesis in easily available cells or tissues, such as myoblasts and muscle tissues. Herein, we describe the first small molecule with neurogenesis-inducing activity in non-pluripotent myoblasts and the cells derived from skeletal muscle.

Various imidazole derivatives show a broad range of bioactivities, such as antineoplastic, immunosuppressive, and anti-inflammatory activities.[7] Thus, we synthesized an imidazole library with diverse substituents at the positions of 1, 2, 4, or 5 on a solid support to identify active compounds inducing interesting differentiation in cells, such as neurogenesis (Figure 1a).[8] Amine-conjugated diethylene gycol was inserted into this library for the facile preparation. We could obtain about 300 imidazole derivatives with more than 70% purity (based on HPLC analysis).

C2C12 myoblasts have the advantage of being well-characterized and possess the potential to be differentiated into non-muscle cell types, such as adipocytes and osteoblasts.[9] In addition, these cells were converted to a physiologically active neuronal phenotype by genetic manipulation, such as disruption of the function of a single genetic element.[10] Therefore, C2C12 cells were chosen for primary screening. We adopted a high-throughput screening approach to select imidazole-based chemical inducers of neuronal differentiation of these cells. We utilized the fluorescent dye FM1-43 (Figure 1b), which is employed to detect depolarization-induced synaptic vesicle recycling that is a neuron-specific property.[11] This dye enters the

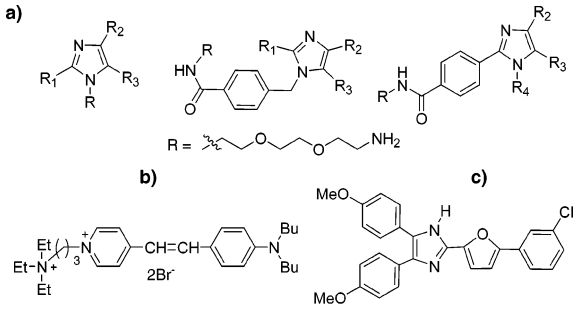

Figure 1. Structures of (a) an imidazole library (see Supporting Information for substituents R_1-R_4), (b) FM1-43, and (c) neurodazine (Nz).

differentiated neurons in the presence of a high extracellular concentration of KCl when the synaptic vesicles are recycled back into the neurons after depolarization. Thus, our screening method using FM1-43 allows active compounds inducing neurogenesis to be rapidly identified by observing the fluorescent intensity of treated cells. Associated neurite outgrowth of cells treated with neurogenesis-inducing agents was further examined by light microscopy.

About 300 imidazole derivatives (3 μM) were incubated with a relatively low density of C2C12 myoblasts (10^4 cells/mL) in culture media supplemented with 2% fetal bovine serum to suppress the onset of terminal myogenesis. Fluorescence analysis showed that four compounds exhibited relatively high depolarization-induced fluorescent intensity in comparison with untreated cells. In addition, phenotypic analysis using light microscopy indicated that these compounds induced the development of membrane outgrowth resembling a prominent axonal development. To confirm the neurogenesis-inducing effect of these compounds in C2C12 myoblasts, four active compounds without a diethylene gycol linker were resynthesized in solution. On the basis of our neurogenesis activity test using FM1-43, neurodazine (Nz) exhibited the highest fluorescence intensity after treatment with FM1-43 and was selected as a neurogenesis-inducing agent for further studies (Figure 1c).

To ascertain that the C2C12 cells differentiated by treatment with Nz indeed have neurogenic properties, we performed immunocytochemical and Western blot analyses with antibodies against neuron-specific proteins, such as neuron-specific βIII tubulin (NST, TuJ1), neuron-specific enolase (NSE), neurofilament 200 (NF200), GAP-43 (growth associated protein-43, a marker of axon development), choline acetyltransferase (CAT), microtubule-associated protein 2 (MAP-2), and synapsin. These analyses showed that Nz induced the expression of neuron-specific markers in treated C2C12 cells (Figure 2). We also investigated synaptic vesicle recycling of the cells differentiated by Nz as a function of external K^+ concentration since synaptic vesicle recycling is a neuron-specific property. Synaptic vesicle recycling detected by FM1-43 became apparent in the cells derived from Nz-treated C2C12 myoblasts when the concentration of KCl approached 90 mM (see Supporting Information). The degree of synaptic vesicle recycling was similar to that displayed by the cells derived from rat PC12 cells treated

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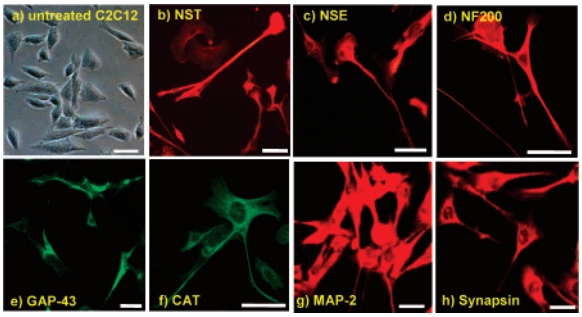

Figure 2. Neurogenesis of C2C12 myoblasts by Nz. C2C12 cells were treated (a) without and (b−h) with 2 μM Nz for 7 days. The upregulation of neuron-specific markers in Nz-treated C2C12 cells is visualized by immunocytochemistry (bar = 50 μm) (see Supporting Information for Western blot analysis).

with 100 ng/mL nerve growth factor (a well-characterized model of neurogenesis).[12] On the basis of immunocytochemical analysis and FM1-43 studies, 40−50% myoblasts were converted to a neuronal phenotype by Nz.

The conversion of differentiated myotubes to neurogenic cells is an attractive prospect because it would suggest that neurogenic cells can be derived from adult skeletal muscle fibers. The differentiation of C2C12 myoblasts into myotubes is an in vitro model of skeletal muscle myogenesis. Since C2C12 myotubes are not directly converted to neurogenic cells by Nz, we attempted a two-step procedure to differentiate myotubes into neurogenic cells. Myotubes differentiated from C2C12 myoblasts were first treated with 10 μM myoservin to induce cellularization of myotubes.[13] Mononucleates isolated from cellularized myotubes were then incubated with 2 μM Nz for 7 days in the absence of myoseverin. The differentiated cells were confirmed to have neurogenic properties by immunocytochemical and Western blot analyses of neuron-specific protein expression and synaptic vesicle recycling studies using FM1-43 (see Supporting Information). These studies show that the Nz-treated mononucleates derived from C2C12 myotubes possess a similar neurogenic potential to Nz-treated C2C12 myoblasts.

The results obtained from experiments using mouse myotubes prompted us to test if Nz can be used to generate neurogenic cells from human skeletal muscle. For these studies, single muscle fibers were isolated from the abductor hallucis muscle.[14] The single muscle fibers were treated with 15 μM myoseverin for 20 h in order to obtain mononucleates. The isolated mononucleates were further incubated with 2 μM Nz for 7 days. Alternatively, the single muscle fibers were cultured to produce satellite cells which are quiescent, mononuclear muscle cells residing between the sarcolemma and basement membrane of the muscle fibers. The satellite cells were treated with 2 μM Nz for 7 days. Mononucleates and the satellite cells treated with Nz developed a neuronal phenotype on the basis of immunocytochemical analysis and synaptic vesicle recycling studies (Figure 3).

Understanding the mode of action of Nz should give insight into the mechanisms involved in neurogenesis and assist the development of other novel agents to induce neuronal differentiation. We undertook gene expression profiling in C2C12 myoblasts treated with Nz to assess the effects of Nz using mouse 20K DNA chips. It is apparent that Nz preferentially upregulates genes involved in neurogenesis over other differentiation pathways (see Supporting Information). In particular, B-cell translocation gene 2 (Btg2), which

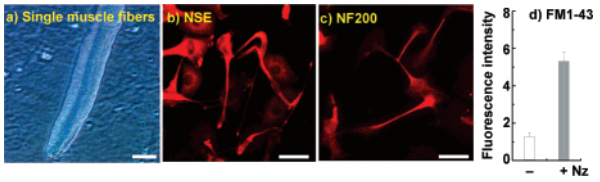

Figure 3. Neurogenesis of human skeletal muscle fibers by Nz. (a) Single muscle fibers isolated from the abductor hallucis muscle and (b-c) immunocytochemical analysis of mononucleates and satellite cells after treatment with 2 μM Nz for 7 days (bar = 50 μm). (d) Synaptic vesicle recycling of mononucleates after treatment with 2 μM Nz for 7 days using FM1-43 (error = SD).

is known to be involved in inducing neuronal differentiation in rat PC12 and neuroepithelial cells,[15] was upregulated by Nz. However, the expression of key regulators of myogenesis, such as MyoD, Myf-5, and myogenin,[16] remained unchanged during the early stages of Nz treatment, suggesting that C2C12 cells develop a neuronal phenotype without suppressing their own potential to differentiate into muscle fibers under permissive conditions, such as fusion with neighboring cells in culture.

In conclusion, we have developed the first small molecule that can induce neurogenesis of non-pluripotent myoblasts and the cells derived from mature, human skeletal muscle. These studies build upon recent research illustrating the value of chemical approaches for providing tools that differentiate lineage-committed cells into other cell types.[9c]

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Supporting Information Available: Preparation of a library and cell experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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