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Discovery of a New and Efficient Small Molecule for Neuronal Differentiation from Mesenchymal Stem Cell

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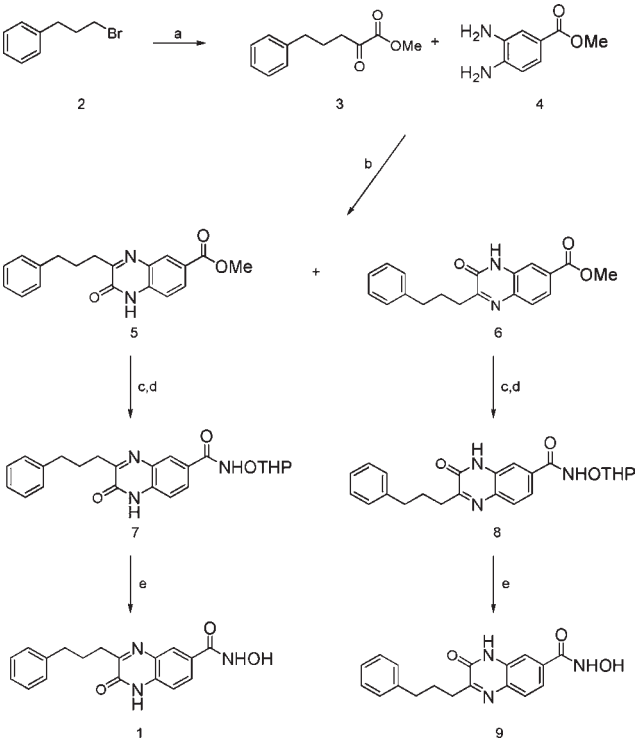
Abstract: A new synthetic small molecule, compound **1**, which induced a neuronal differentiation from mesenchymal stem cells (MSCs) with an excellent efficiency, was identified. Furthermore the differentiated cell by **1** showed the neural electrophysiological and cholinergic neuron properties.

Neuronal differentiation of stem cells has received much attention because of its potential for the treatment of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.[1] Moreover, small molecule inducers that can direct differentiation of stem cells are useful tools to facilitate therapeutic applications.[2] Recently, several small molecules have been reported to have neuronal differentiation ability,[3−9] but there is no report of single small molecule that can induce functional activity such as electrophysiological properties, so far.

To identify effective neuronal inducers, we screened more than 20 000 small molecules using the neuronal precursor-like PC12[10] cell lines as a primary screening and identified several novel small molecules. Among them, **1**, a quinoxaline based small molecule, revealed the most effective activity to show neurite outgrowth and increase neuronal markers (β-III tubulin and NSE[a]) in the PC12 cells (Supporting Information Figure S1). Thus, we considered **1** as a neuronal inducer prototype. Compound **1** and its derivatives were synthesized according to Scheme 1.

The 1-bromo-3-phenylpropane **2** was converted to Grignard reagent in the presence of I_2, followed by coupling with dimethyl oxalate to produce **3**. Keto ester **3** was cyclized with methyl 3,4-diaminobenzoate **4** in EtOH to give a mixture of **5** and **6** (~7:3 ratio), which were separated by crystallization. After ester hydrolysis under basic condition, NH_2OTHP was introduced to yield **7** and **8**, and finally deprotection using trifluoroacetic acid produced **1** and **9**, respectively.

Scheme 1. Synthesis of Compound **1** and Its Derivatives[a]



[a] Reagents and conditions: (a) Mg, I_2, THF, room temperature, 0.5 h, and then dimethyl oxalate, Et_2O, −78 to 0 °C 3 h; (b) EtOH, room temperature, 12 h; (c) LiOH, THF/MeOH/H_2O, 50 °C, 10 h; (d) EDCI, HOBt, NH_2OTHP, DMF, room temp, 3 h; (e) trifluoroacetic acid, MeOH, 35 °C, 12 h.

The possibility of neuronal differentiation in rat mesenchymal stem cells (MSCs) (Figure S2, MSCs characterization) with **1** and its derivatives (**5**, **6**, **7**, **8**, and **9**) was explored. The MSCs are highly important and easily accessible adult stem cells that could be differentiated into many cell types including osteoblasts, adipocytes, fibroblasts, myocytes, and neurons. The ability of MSCs to differentiate into neurons holds considerable promise because of their potential to serve as a possible unlimited source of stem cells to treat neurodegenerative diseases. Interestingly, **1** showed a significant phenotype change of MSCs, whereas the others including **9** did not induce any morphological change. After treatment of **1** (20 μM) for 2 days, **1** induced morphological change and increased the β-III tubulin expression without cytotoxicity (Figure 1a,b and Figure S3).

We also tested the neuron inducing properties of nerve growth factor (NGF),[11] representative neuronal differentiation inducers, and the neurophathiazole,[6] a small molecule that induces neuronal differentiation of neural progenitor cells; however, these agents did not induce any change in the MSCs system (Figures 1b and S4). Compound **1** dose- and time-dependently increased neuronal markers (β-III tubulin and NSE) (Figure 1c). On the basis of Nissl staining,[12] which is a simple and reliable method for neuron detection, it is estimated that >95% of MSCs were converted to a neuronal

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[a] Abbreviations: MSCs, mesenchymal stem cells; NGF, nerve growth factor; THF, tetrahydrofuan; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NH_2OTHP, tetrahydropyranyloxyamine; HOBt, hydroxybenzotriazole; DMF, dimethylformamide; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein; DAPI, 4′,6-diamidino-2-phenylindole; Chrna2, cholinergic receptor, nicotinic, α2; Chrnb2, cholinergic receptor, nicotinic, β2; Chrm4, cholinergic receptor, muscarinic 4; Fbxo2, F-box protein 2; Sizn 1, Smad-interacting zinc finger protein; RFU, relative fluorescence unit.

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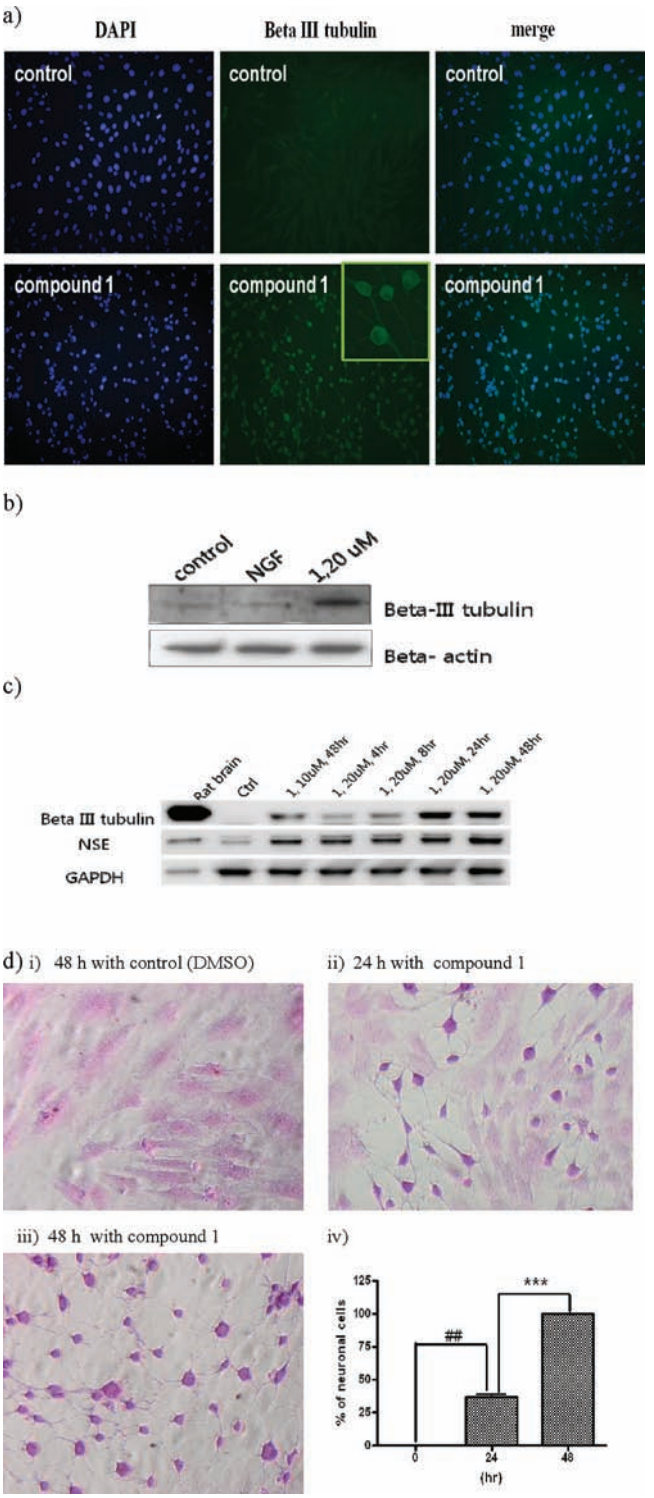

Figure 1. Morphological changes and β-III tubulin expression in bone marrow MSCs treated with **1**. (a) Immunocytochemistry. The treated cells were stained by anti-β-III tubulin and DAPI. (b) β-III tubulin expression was detected by Western blot. The MSCs were treated with 50 ng/mL NGF or 20 μM **1** for 2 days. (c) Compound **1** increased the expressions of β-III tubulin and NSE in rat bone marrow MSCs time-dependently. (d) Nissl staining of MSCs treated with 20 μM **1** for 2 days. Differentiation was estimated by cell counting. All experiments were done more than three times independently: (##) $p = 0.002$; (***) $p = 0.0008$.

phenotype after 48 h at 20 μM (Figure 1d). Astroglial differentiation by **1** was also monitored with Western blot of

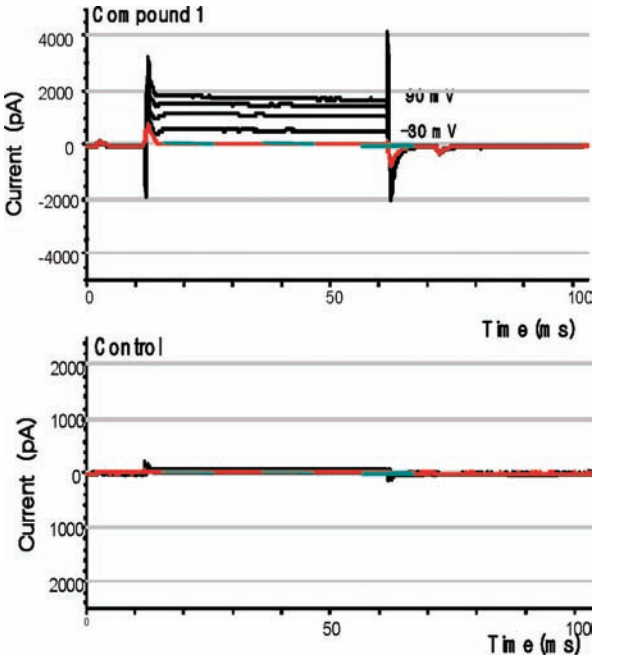

Figure 2. Electrophysiological properties of **1**-treated MSCs. The electrophysiological properties were determined by the patch clamp method. The data were analyzed by pClamp 8.1 software.

glial fibrillary acidic protein (GFAP), an astroglial marker; however, GFAP-positive cells were not detected (Figure S5).

To evaluate whether **1** treated MSCs really show neuronal electrophysiological property or not, we assessed the membrane current using the whole-cell patch clamp.[13] As shown in Figure 2, after 2 days, **1** treated MSCs (20 μM) showed the neural electrophysiological properties with the K^+ current by giving pluses ranging from -30 to 90 mV. In contrast, untreated MSCs did not show any detectable outward current under the same conditions.

Recently, several reports[14−18] have shown the possibility of neuronal induction from MSCs using small molecules. Woodbury[14,17] proposed that neural induction could be performed simply by the addition of chemical compound mixtures (β-mercaptoethanol, dimethyl sulfoxide, and butylated hydroxyanisole); however, the differentiated neuron-like cells did not have basic neuronal electrophysiological properties. Kim et al.[18] have also tried to differentiate MSCs using a small molecule, but they didn't show an electrophysiological property, and moreover the compound revealed cell cytotoxicity.

It is presumed that **1** is the first small molecule that can induce neuronal differentiation from MSCs with eletrophysiological property.

For the purpose of searching for neuronal differentiation-associated genes, we tested the changes of gene expression profiling by treatment with **1** using DNA microarray. Among the changed genes, interestingly, some cholinergic neuron receptors such as Chrna2, Chrnb2, and Chrm4 were significantly increased. In the quantitative PCR, the cholinergic neuron receptor genes also increased dose-dependently by **1** with some neuron specific genes such as Fbxo2,[19] a component of ubiquitine ligase expressed in neurons, and Sizn 1,[20] known as expression in the ventral forebrain and functions as a transcriptional coactivator necessary for cholinergic neuron-specific gene expression (Figure 3).

To further evaluate an effect of the increased cholinergic receptor genes, acetylcholine was added to **1** treated MSCs.

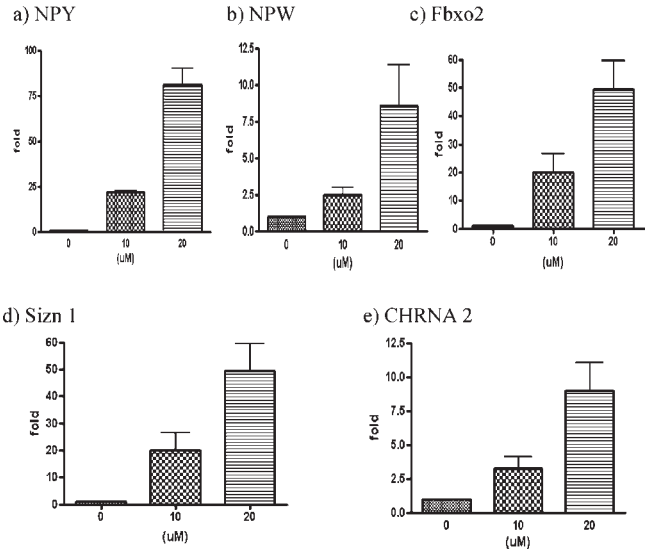

Figure 3. Quantitative PCR of some representative genes in **1** treated MSCs. The MSCs were incubated with **1** at 10 and 20 μM and Ctrl (DMSO) for 2 days, and the mRNA levels were determined by quantitative PCR analysis. The experiments were performed three times independently.

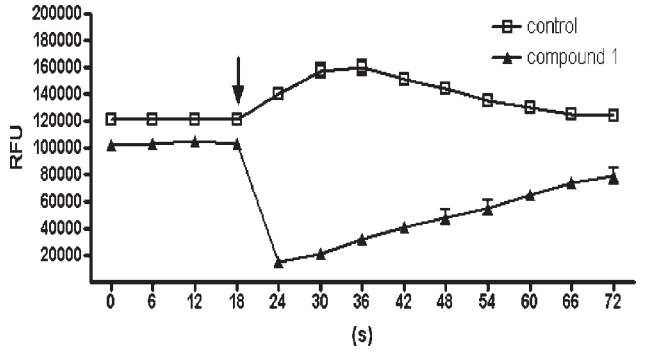

Figure 4. Membrane potential assay of MSCs with acetylcholine. MSCs were incubated with 20 μM **1** for 48 h. Membrane potential were detected by FLIPR assay kit. Data were analyzed using Flex station software. Open markers are control. Closed markers are **1** treatment. Arrow indicated treatment of 10 μM acetylcholine.

In the FLIPR assay, the membrane potential was changed on **1** treated MSCs (Figure 4). This result suggests that **1** can differentiate MSCs into a specific type of cholinergic neuronal cell.

In conclusion, we have developed a new synthetic small molecule (**1**) that can induce neuronal differentiation in MSCs. Compound **1** has a capacity to commit MSCs to neuronal fate, which was confirmed by morphological change and expression of neuronal markers (β-III tubulin and NSE). When treated with **1**, more than 95% of MSCs were converted to neuron-like cells. Moreover, quantitative PCR results showed that the expressions of neuropeptide, cholinergic receptors, and Fbxo2, which are a neuronal cell marker, were up-regulated by **1**. Importantly, **1** treated MSCs showed the neural electrophysiological and cholinergic neuron properties. Taken together, **1** will be a useful tool in studying cell therapy and the molecular mechanisms that determine cell fate, even if further biological evaluations and chemical derivatization to find better small molecules are now in progress.

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Supporting Information Available: Experimental procedures, biological assays, and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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