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A Synthetic Small Molecule That Induces Neuronal Differentiation of Adult Hippocampal Neural Progenitor Cells**

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Neural stem/progenitor cells have been identified in several regions of the adult brain, including the subventricular zone and the dentate gyrus of the hippocampus. They are multipotent in vitro (i.e., they can give arise to neurons, astrocytes, and oligodendrocytes) and have been shown to contribute to neurogenesis in adulthood.[1,2] The discovery of neural stem/progenitor cells (NPCs) in the adult central nervous system has created considerable interest in the development of stem-cell-based therapies for neurodegenerative disease.[2–4] How-

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ever, the molecular mechanisms that underlie stem-cell-fate specification are still poorly understood, and we lack robust and selective methods to control the differentiation programs of NPCs. Although several agents, including retinoic acid (RA),[1] leukemia inhibitory factor (LIF),[5] and insulin-like growth factor-1 (IGF-1),[6] have been found to direct neuronal, astroglial, and oligodendrocytic differentiation, they either are pleiotropic or have poor in vivo activities. Moreover, relatively little is known about the endogenous molecules that control stem-cell fate. Consequently, the generation of small molecules that can direct differentiation of adult NPCs could provide useful chemical tools to probe signaling pathways that control neuronal specification and could ultimately facilitate therapeutic application of NPCs. Herein, we report the identification and preliminary characterization of a novel small molecule, neuropathiazol, that selectively induces neuronal differentiation of multipotent hippocampal neural progenitor cells.

To screen for small-molecule inducers of neuronal differentiation, a primary neural progenitor (HCN) cells isolated from adult rat hippocampus were used. Although it has been shown that HCN cells can differentiate into neurons, astrocytes, and oligodendrocytes in vitro and can functionally integrate into existing neuronal networks in vivo, HCN cells very rarely differentiate into neuronal and astroglial lineages without treatment with exogenous factors, for example, with RA or LIF in the absence of basic fibroblast growth factor (bFGF): In contrast, a relatively large percentage of HCN cells are induced to differentiate into oligodendrocytes at high cell density by the insulin present in N2 supplemented medium. The extremely low frequency of 'spontaneous' (cell autonomous) neuronal and astroglial differentiation of HCN cells makes them a good in vitro model system to screen for novel chemical regulators.

HCN cells were expanded and maintained in an undifferentiated state on a polyornithine/laminin-coated dish in a defined serum-free growth medium (DMEM/F12 supplemented with N2 and bFGF (20 ng mL^{-1})) in a homogeneous monolayer. Neuronal and astroglial differentiation were analyzed directly with double immunofluorescence staining of βIII tubulin (TuJ1) as a neuronal marker and of glial fibrillary acidic protein (GFAP) as an astroglial marker. A combinatorial heterocycle library of 50000 compounds was screened by using undifferentiated, early passage HCN cells, which were plated onto polyornithine/laminin-coated 384-well plates at a density of 6000 cells/well in complete growth medium. After overnight incubation, the medium was exchanged with bFGF-free basal medium, and the compounds (final concentration of 5 μM) were added to each well. After treatment with the compounds for four days, the cells were fixed and stained with βIII tubulin/TuJ1 and GFAP antibodies, the morphology of positively stained cells was analyzed, and the number of cells with the desired phenotypes were counted.

A class of 4-aminothiazoles, which induces specific neuronal differentiation in a dose-dependent manner, was identified. To improve the potency and specificity of these compounds further, a sublibrary of 4-aminothiazole compounds was generated by the reaction of α-chloroacetylanilines and thiobenzamides (Scheme 1) and their activity was assayed. This structure–activity relationship (SAR) study revealed that: 1) aminomethylation (i.e., $R^3 = Me$) significantly enhanced activity relative to other substituents at the R^3 position, 2) substitution of R^1 with 4-Cl and 4-CF_3 leads to a decrease in activity, and 3) R^2 substitution at the *meta* position or the presence of a hydrogen-bond donor dramatically decreases activity. Compound **1**, neuropathiazol (Scheme 1), has the highest activity among the analogues tested (representative analogues and their relative activity are provided in the Supporting Information).

Treatment of HCN cells with neuropathiazol significantly slowed cell proliferation (Figure 1) without visible cytotoxic

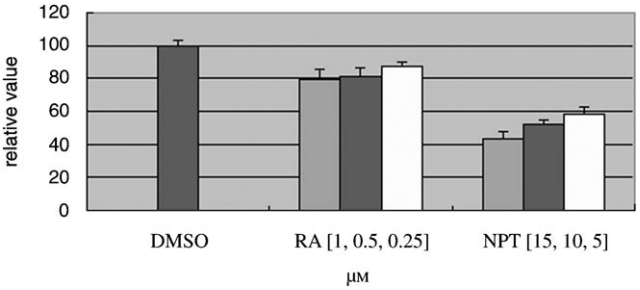
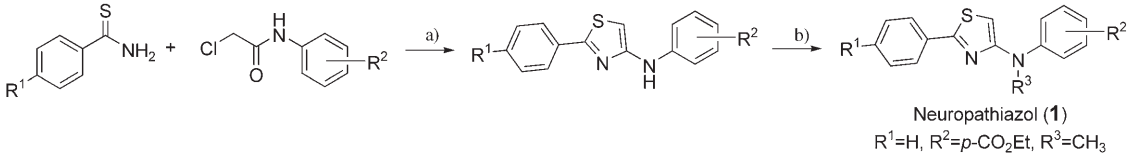


Figure 1. BrdU proliferation assay. Neuropathiazol inhibits proliferation of HCN neural progenitor cells. HCN cells treated with RA (1 μM, 0.5 μM, and 0.25 μM) or neuropathiazol (NPT) (15 μM, 10 μM, and 5 μM) for 5 h in bFGF-free N2 medium were labeled with BrdU for 2 h, and BrdU-positive cells were counted.

effects; more than 90% of the cells differentiated into neuronal cells as determined by immunostaining with TuJ1 and the characteristic neuronal morphology. RA, however, showed weaker antiproliferation activity and significant cytotoxicity over 2 μM in growth factor (bFGF or LIF/BMP2)-free basal medium. GFAP-positive cells were very rarely detected after neuropathiazol treatment, indicating that neuropathiazol specifically induces a neuronal lineage (Figure 2B). In contrast, relatively large numbers of both neuronal and astroglial cells were found with RA-induced differentiation of HCN cells, which indicates that RA is not very specific (Figure 2A). Longer treatment of HCN cells (7 days) with neuropathiazol led to positive staining for later-



Scheme 1. Synthesis of 4-aminothiazole analogues. Reagents: a) DMF (or EtOH), 80°C, overnight; b) NaH, RX, room temperature, 2 h.

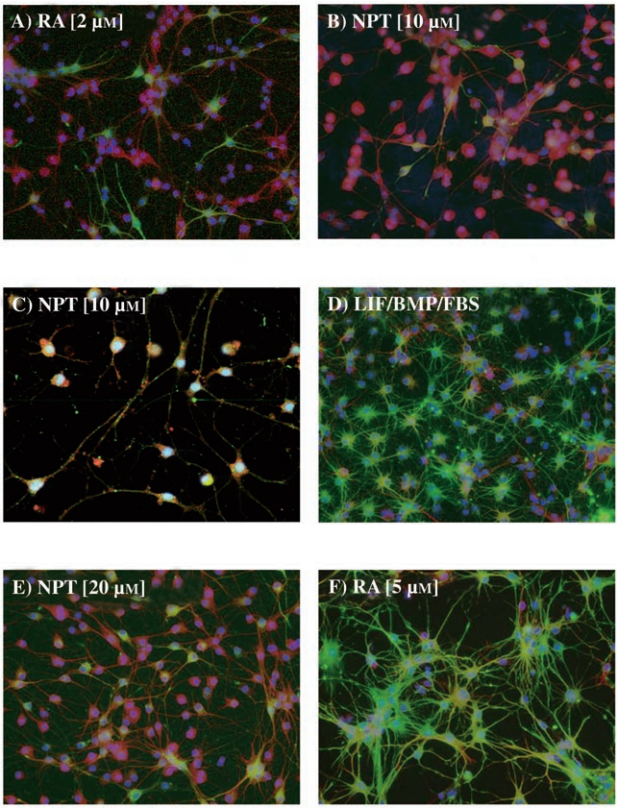


Figure 2. Neuropathiazol specifically induces neuronal differentiation of adult hippocampal neural progenitor (HCN) cells. HCN cells in N2 media were treated with A) retinoic acid (RA; 2 μM) or B) neuropathiazol (10 μM) for four days and immunostained with TuJ1 (red) and GFAP (green). C) Mature neurons were observed with Map2ab (red) and neurofilament-H (green) antibodies after treatment with neuropathiazol (10 μM) for 7 days. D) Astroglial differentiation of HCN cells was strongly induced by LIF/BMP/FBS treatment (LIF (50 ng mL^{-1}), BMP2 (50 ng mL^{-1}), and FBS (0.5%)). E) Astroglial differentiation can be inhibited by neuropathiazol (20 μM), but not by RA (5 μM; F). Cells were stained with markers for neurons (TuJ1, red) and astrocytes (GFAP, green), and also with DAPI (blue) for nuclei.

stage neuronal markers including neurofilament-H and Map2(a+b), which indicates that neuropathiazol-treated HCN cells can differentiate into mature neurons (Figure 2C).

Moreover, RT-PCR of marker genes showed that Sox2 (a neural progenitor marker)[7] was downregulated and NeuroD1 (a neuronal cell marker)[8] was upregulated upon treatment with neuropathiazol (Figure 3). Interestingly, neuropathiazol can also inhibit astroglial differentiation that was induced by LIF and BMP2 (Figure 2E), whereas RA cannot (Figure 2F). This suggests that neuropathiazol functions by a different mechanism and has a more specific neurogenic-inducing activity than RA.

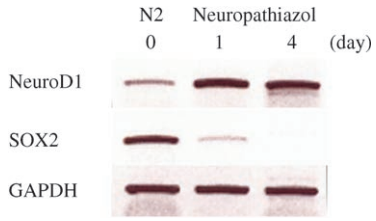


Figure 3. HCN cells were incubated with neuropathiazol (10 μM) or in compound-free N2 medium (N2; supplemented growth media without bFGF) and mRNA levels were determined by semiquantitative RT-PCR analysis.

In conclusion, the small molecule neuropathiazol, which induces neuronal differentiation of multipotent adult hippocampal neural progenitor cells, was identified by a high-throughput image-based screen. Neuropathiazol is a more selective inducer of neuronal differentiation than RA and can competitively suppress astrogliogenesis by LIF/BMP2/FBS in a dose-dependent manner. Further experiments are required, however, to determine the precise molecular target of neuropathiazol. Nonetheless, neuropathiazol will be a useful tool for studying the molecular mechanisms that determine cell fate with the ultimate goal of stem-cell therapy.

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