Bioorganic & Medicinal Chemistry Letters 21 (2011) 7050–7053

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Discovery of small molecules that enhance astrocyte differentiation in rat fetal neural stem cells

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ARTICLE INFO

Article history:
Received 23 July 2011
Revised 14 September 2011
Accepted 23 September 2011
Available online 29 September 2011

Keywords:
Neural stem cell
Small molecule
Astrocyte
Differentiation
1,3,4-Oxadiazole

ABSTRACT

1,3,4-Oxadiazole derivatives were found to enhance astrocyte differentiation in rat fetal neural stem cells (NSCs). Differentiation activity was assessed by immunocytochemistry and analysis of mRNA expression of astrocyte markers, *GFAP* and *S100*. Compounds **7** and **8** showed approximately a two-fold increase in astrocyte differentiation without engagement of neuronal differentiation and detectable cytotoxicity.

Stem cells have unlimited proliferative properties and the ability to produce functional differentiated progeny.[1] Neural stem cells (NSCs) also have the ability to self-renew in the nervous system and to differentiate into specific cell types, including neurons and glia.[2] NSCs have been identified in the subventricular zone and the hippocampus in the adult as well as in the developing nervous system.[3] Recent works have suggested that NSCs may be suitable for transplantation to replace neurons lost in many neurodegenerative diseases such as Parkinson's disease.[4]

Intrinsic regulators, such as transcription factors, can control the fate of NSCs,[5] in addition to extrinsic factors, such as retinoic acid and ciliary neurotrophic factor (CNTF)/leukemia inhibitory factor (LIF).[6] Interestingly, NSCs isolated from different regions of developing embryos showed specific characteristics in proliferation and differentiation and the cell fate could be modulated by ectopic expression of transcription factors.[7] In addition, small molecules can regulate stem cell fate efficiently and conveniently; they also could be useful probes in the identification of key factors involved in controlling cell fate. It has been reported that small molecules targeting endogenous signaling pathways can modulate NSCs differentiation.[8] The pyrimidine compound KHS101, which was found to specifically bind transforming acidic coiled-coil-containing protein 3 (TACC3),[9] and synthetic 4-aminothiazole compounds termed neuropathiazols (KHS2)[10] have been implicated in the induction of neuronal differentiation from adult rat hippocampus-derived NSCs. Small molecule inhibitors of glycogen synthase kinase-3 (GSK-3), such as SB-216763 and kenpaullone, are also known to increase neurogenesis of human NSCs.[11] Moreover, many trials have been undertaken to find small molecules for differentiation into neurons from other stem cells.[8] Thus, it would be of great interest to identify additional small molecules that can precisely regulate NSCs or stem cell fate.

The study of astrocyte differentiation might greatly contribute to understand how the cell fate of NSCs is regulated. In addition, much attention has been paid to the role of astrocytes in neuropathological conditions.[12] An in-house chemical library was therefore screened to see if any could induce astrocyte differentiation of NSCs. An image-based screen of about a thousand chemicals has resulted in the identification of potential compounds for astrocyte differentiation of NSCs. We carried out additional studies with the best hit from the primary screen. Herein, we describe the identification of small molecule regulators for astrocytogenesis of NSCs derived from fetal rat cortex along with the brief structure–activity relationship study.

All tested compounds were prepared by the coupling reaction of phenyl(5-phenyl-1,3,4-oxadiazole-2-yl)methanamine (**5**) or (4-fluorophenyl)(5-phenyl-1,3,4-oxadiazole-2-yl)methanamine

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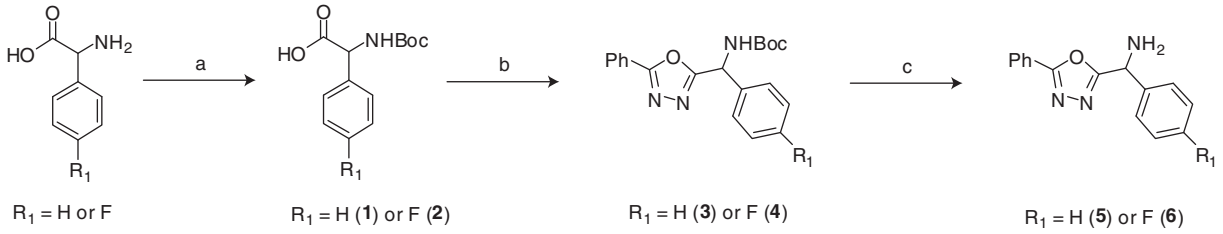
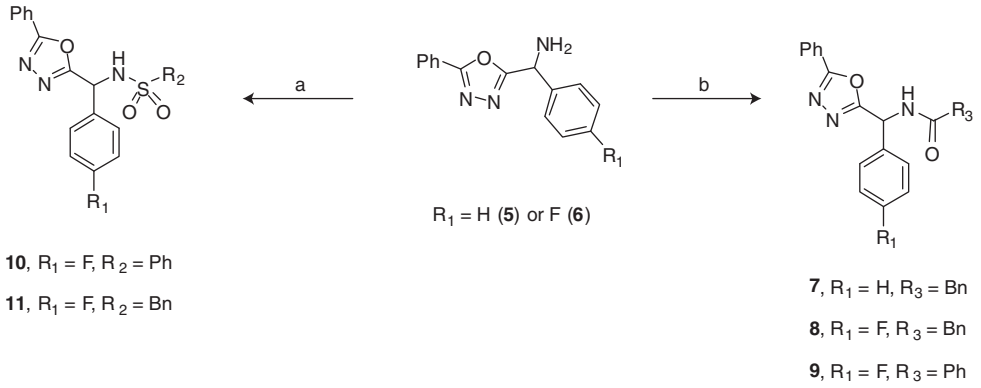
doi:10.1016/j.bmcl.2011.09.099



Scheme 1. Synthesis of disubstituted methanamines with 1,3,4-oxadiazole-core (**5**, **6**). Reagents and conditions: (a) Boc_2O, $NaHCO_3$, water–dioxane; (b) benzohydrazide, CDI, CBr_4, PPh_3, DCM, 0 °C; (c) TFA, DCM, rt.



Scheme 2. Synthesis of sulfonamide and amide analogues with a 1,3,4-oxadiazole-core (**7**–**11**). Reagents and conditions: (a) Et_3N, sulfonyl chlorides, DCM, 0 °C; (b) EDCI, DMAP, carboxylic acids, DCM, rt.

(**6**) and the appropriate sulfonyl chlorides or carboxylic acids. The synthesis of the methanamine with a 1,3,4-oxadiazole-core is described in Scheme 1. The amino group for the ultimate amide coupling was protected with a Boc group by Boc_2O and $NaHCO_3$ in water–dioxane solvent.[13] Then, we applied one pot synthesis of 2,5-disubstituted 1,3,4-oxadiazole (**3**, **4**) from Boc-protected amino acid (**1**, **2**) and benzohydrazide using carbonyl diimidazole (CDI) for activation of the carboxylic group and CBr_4 and PPh_3 for dehydration,[14] where the *N*-Boc group was then deprotected by TFA to give phenyl(5-phenyl-1,3,4-oxadiazole-2-yl)methanamine (**5**) or (4-fluorophenyl) (5-phenyl-1,3,4-oxadiazol-2-yl)methanamine (**6**).

The synthesis of the final sulfonamide and amide with a 1,3,4-oxadiazole-core is shown in Scheme 2. The ultimate coupling of the appropriate sulfonyl chlorides using Et_3N to the intermediate **5** or **6** gave the corresponding sulfonamides (**10**–**11**), and the EDCI mediated coupling of the appropriate carboxylic acids toward the final product gave the corresponding amides (**7–9**).

To explore the potential roles of a series of small molecules in NSCs differentiation, NSCs derived from the developing rat cortex at embryonic day (E) 14 were treated with 5 μM of oxadiazole compounds. For in vitro differentiation, NSCs were cultured as neurospheres and dissociated as single cells for plating onto poly-D-lysine-coated dishes.[15] Four days after treatment, cells were fixed with 4% paraformaldehyde and immunocytochemistry assays were performed using cell type specific antibodies.[16] To identify neurons and astrocytes, anti-βIII tubulin (TuJ1) and anti-GFAP were used, respectively. As shown in Figure 1, oxadiazole compounds significantly increased generation of astrocytes when compared with vehicle treated control in NSCs (p <0.05). Among them, compounds **7**, **8**, and **9** appeared to be more effective than compound **10**, suggesting that an amide group may be favored over a sulfonamide group for differentiation activity. Interestingly, the number of neurons (Tuj1-positive cells) was almost not affected by active compounds. These results demonstrate that these oxadiazole compounds specifically differentiate NSCs into astrocytes. We further evaluated compound **7** since it effectively induced astrocytogenesis without cell death as examined by morphology and nuclei counting. Reverse transcriptase PCR (RT PCR) revealed that 1 day of compound **7** treatment significantly induced transcription of astroglial marker, *GFAP* and *S100*.[17] Interestingly, no difference was observed in transcription of neuronal gene *βIII tubulin*. When the rest of the oxadiazole derivatives were tested, they all induced *GFAP* and *S100* mRNA expression without affecting *βIII tubulin* expression (Fig. 1). These results indicate that oxadiazole derivatives control NSCs fate to astrocytes.

In summary, oxadiazole derivatives could enhance astrocytogenesis in fetal NSCs derived from rat cortex without engagement of neuronal differentiation. Compounds **7** and **8** showed the best activity, approximately a two-fold increase, compared with untreated NSCs, in astrocyte differentiation, when determined by immunocytochemistry and RT-PCR. Although further investigation is required to reveal the molecular targets of the described compounds, we believe that those chemicals would be useful in understanding the molecular mechanisms of the cell fate determination of NSCs.

Acknowledgment

This work was supported by the R&BD program of Seoul Metropolitan Government (ST090791).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.099.

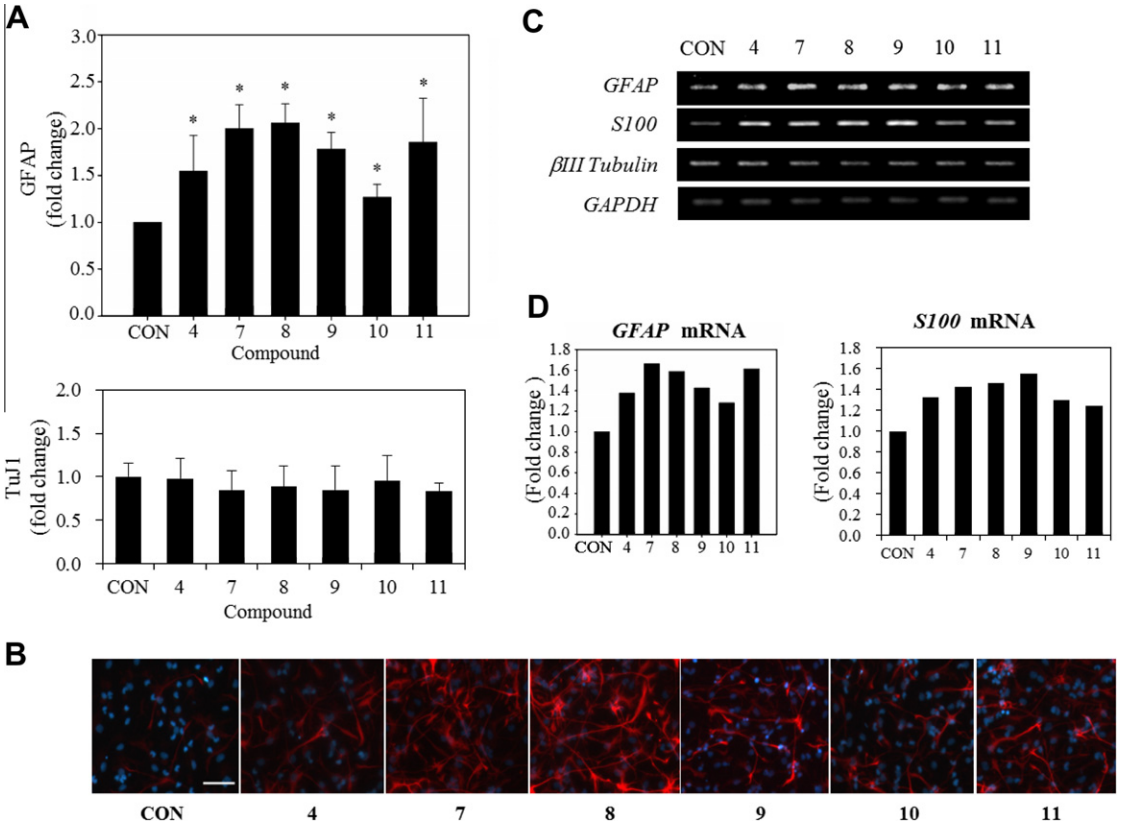


Figure 1. Induction of astrogliogenesis by oxadiazole compounds. Dissociated cells of NSCs were plated and treated with either DMSO (CON) or compound **4**, **7**, **8**, **9**, **10** or **11**. Four days after treatment cells were fixed and immunostained with anti-GFAP and Cy3-conjugated secondary antibody. To count the total cell number, 4,6-diamidino-2-phenylindole (DAPI) staining was performed. (A) Quantification of GFAP and TuJ1 positive cells in the control and oxadiazole compounds treated NSCs. GFAP-positive and TuJ1-positive cells were counted, and the ratio of positive cells to total cells was calculated and divided to that of CON to yield fold changes. The values are presented as the mean ± standard error of mean from independent experiments ($n = 6$). Statistical analysis was carried out by Student's t-test. *The values are significantly different from the control group ($P < 0.05$). (B) Representative immunofluorescence images of GFAP (astrocyte marker, red) and DAPI (nuclei, blue) of control (0.05% DMSO treated) and indicated compound (5 μM) treated NSCs. Scale bar = 50 μm. (C) Increased *GFAP* and *S100* mRNA expression by oxadiazole compounds in NSCs. NSCs were cultured with either DMSO (CON) or indicated compound (5 μM) for 2 days. Total RNA was prepared, and cDNA was synthesized, and subjected to semiquantitative RT-PCR with *GFAP*, *S100*, and *βIII-tubulin*. The transcripts of *GAPDH* were used to normalize the loading amount. (D) Graphs show the relative intensities of *GFAP* and *S100* mRNA levels analyzed by band densitometry.

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15. *NSCs culture*. We cultured NSCs from cortices of rat (Sprague–Dawley rats) embryos at E14. The NSCs (200,000/ml) were expanded as neurospheres for 7 days in Dulbecco's modified Eagle's medium (DMEM)/F12 media (Gibco, Carlsbad, CA) supplemented with 2% B27 (Invitrogen), 20 ng/ml epidermal growth factor (EGF; Chemicon, CA), and 20 ng/ml FGF-2 (Chemicon, CA). The media was replaced every 2 days. For differentiation, neurospheres were dispersed into a single-cell suspension with accutase for 10 min at 37 °C, plated onto 0.01% poly-D-lysine and 10 μg/ml laminin (Sigma-Aldrich, MO) with no growth factors but supplemented with 2% B27. The cells treated with chemicals or DMSO were maintained at 37 °C in 5% CO_2 incubators and cell differentiation was assessed 4 days thereafter.
16. *Immunocytochemistry and cell counting*. For immunocytochemistry, cell cultures were fixed with 4% paraformaldehyde for 30 min and washed with phosphate-buffered saline (PBS). Fixed cells were blocked with 5% normal goat serum and 0.2% Triton X-100 in PBS and incubated with TuJ1(monoclonal; 1:1000; Sigma-Aldrich, MO), GFAP (polyclonal; 1:1000; Dako, Carpinteria, CA). Following rinsing with PBS, the cells were incubated for 30 min with secondary antibodies conjugated to Cy3 (1:1000, Molecular Probes, CA). DAPI (1:10,000 in PBS) was added for 5 min after completion of the secondary antibody incubation as a nuclear stain. The images were obtained with an inverse fluorescence microscopy (DMIL; Leica, Wetzlar). To avoid biased results, we took photos randomly and either GFAP or TuJ1 positive cells were counted from 3–6 independent experiments. To calculate the total cell number, DAPI stained nuclei were use. The numbers of either GFAP-positive cells or TuJ1 positive cells were divided by DAPI positive cells to get the percentage. The percentage values of each chemical treated groups were divided by that of control to get the fold increase. All cell-count data were expressed as means ± SEM and statistics were analyzed using the Student's t-test.
17. *Semi-quantitative RT-PCR*. The total RNA was extracted using TRIzol reagent (Invitrogen, MD). First-strand cDNA was synthesized from 2 μg of total RNA in

a 20 μl reaction with oligo (dT) primers and RevertAid reverse transcriptase (Fermentas). The following primer sets were used to amplify cDNA. for *βIII tubulin*, agccctctacgacatctgct (forward) and attgagctgaccagggaatc (reverse); for GFAP, agcggctctgagagagattc (forward) and agcaacgtctgtgaggtctg (reverse); for GAPDH, agttcaacggcacagtcaag (forward) and gtggtgaagacgccagtaga (reverse). The housekeeping gene *GFAP* was used for the internal control. cDNA was amplified by an initial incubation at 94 °C for 5 min followed by 26 cycles of 94 °C for 0.5 min, 55–58 °C for 0.5 min, 72 °C for 0.5 min, and a final extension at 72 °C for 10 min. The PCR products were then separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.