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The first Hes1 dimer inhibitors from natural products

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ABSTRACT

In the present study, we developed a high-throughput screening system for small molecule-inhibitors of the basic helix-loop-helix (bHLH) transcriptional repressor factor Hes1. Successful dimerization of Hes1 immobilized on a microplate and fluorophore (Cy3)-labelled Hes1 was confirmed. Using this system, several natural products were identified as the first Hes1 dimer inhibitors. Of these, two compounds which were isolated from myxomycetes (true slime molds) inhibited Hes1 from N box-dependent suppression of the gene expression in C3H10T1/2 cells.

Basic-helix-loop-helix (bHLH) repressor and activator genes play an essential role in embryogenesis, neurogenesis and the development of many organs.[1,2] bHLH activators, such as Mash1 and E47, control the differentiation of neural stem cells (NSCs) into neurons by activation of neural-specific gene expression. Hes factors (Hes1, Hes3 and Hes5) regulate cell proliferation and differentiation in the nervous system, acting as bHLH repressors by either active or passive suppression of bHLH activator activity. Active suppression comprises Hes binding to N box (CACNAG) as homodimers to suppress bHLH activator transcription (Fig. 1). Passive suppression is caused by the formation of heterodimers of Hes and bHLH activators (e.g., E47) that cannot bind to DNA. In the present paper, we focused on Hes1 active suppression. We hypothesized that inhibition of Hes1 dimer formation would result in acceleration of bHLH activator transcription, which may promote differentiation of NSCs into neurons. Small molecules that can modulate the activity of Hes1 would be candidates for medicine of neural disease, especially *regenerative medicine* for new neurons after stroke. Recent studies have revealed that the adult mammalian brain has some capacity for neuronal regeneration after insult.[3–5] Therefore NSCs are of great clinical interest for the treatment of neural diseases.

To date, there exist only a few reports of small molecules that activate NSC differentiation or lead to selective differentiation into neural cells.[6–8] As the precise mechanisms of NSC proliferation and differentiation contain many unknown factors, the small molecules that control the roles of bHLH factors are potentially important

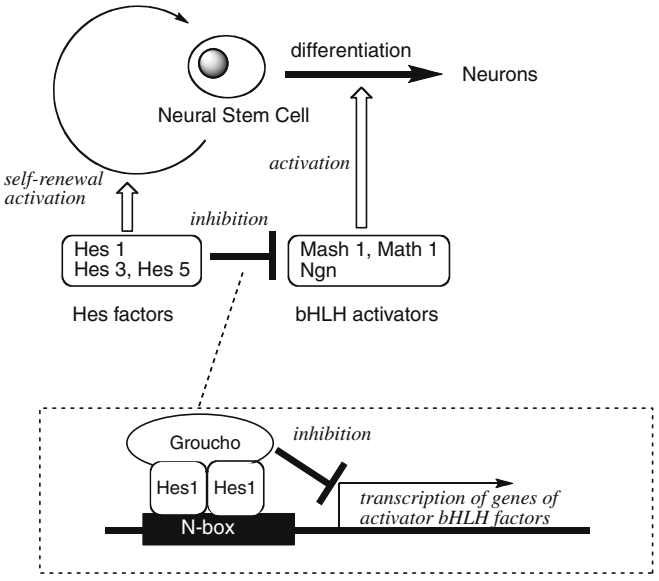

Figure 1. The roles of Hes factors and bHLH activators in the differentiation of neural stem cells. Hes factors, which act as bHLH repressors, suppress bHLH activator activity. A Hes1 dimer bound to an N box suppresses transcription of the bHLH activator.

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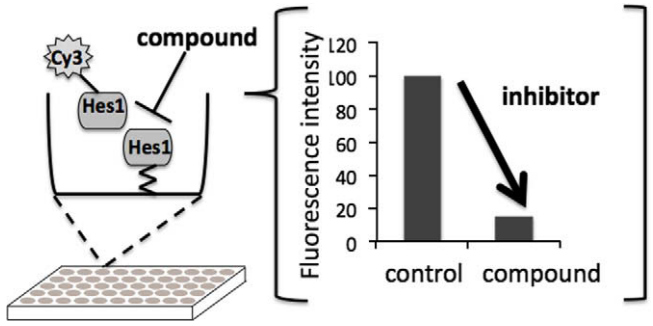

Figure 2. High-throughput screening system for Hes1 dimer inhibitors. In the presence of the Hes1 dimer inhibitors, there was decreased fluorescence from dimer complexes between Hes1 bound on the microplate and Cy3-labelled-Hes1.

chemical tools. Moreover, such small molecules might be lead compounds for support medicines that derive new neural cells from endogenous NSCs.

In the present study, we describe a rapid in vitro high-throughput screening (HTS) for identifying Hes1 inhibitors using fluorophore-labelled Hes1 and Hes1 immobilized on microplates (Fig. 2). In the presence of an inhibitor compound, the level of Hes1 dimerization was reduced, detected by a corresponding reduction in fluorescence. Using this system, we identified the first Hes1 dimer inhibitors from our natural products library. Moreover, using a cell-based reporter gene assay, it was revealed that two of these natural compounds also inhibit Hes1 intracellularly.

A mouse Hes1 construct (3–281 aa, full length) in pGEX-6p-1 was prepared to obtain recombinant Hes1 protein. Glutathione-*S*-transferase-fused-Hes1 (GST-Hes1) was expressed in *Escherichia coli*, purified with glutathione sepharose 4B, and cleaved of GST by PreScission protease on beads. Hes1 protein (3–278 aa) was

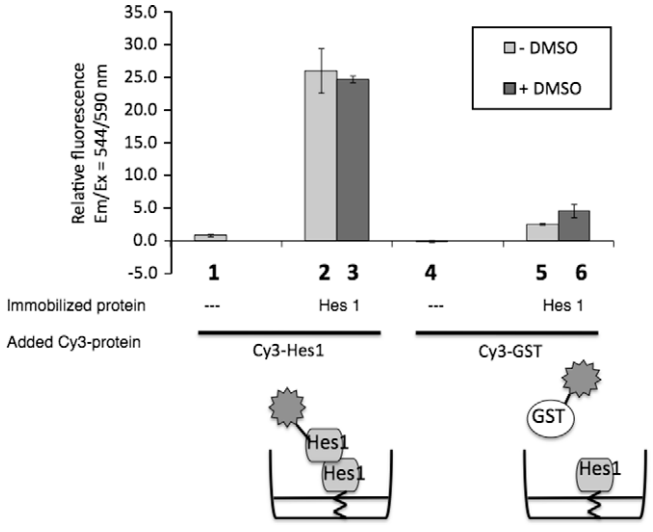

Figure 3. Hes1 dimer formation on the microplate. All wells were treated with ethanolamine, then incubated with Cy3-proteins followed by washing with buffer. The background value (without Hes1) was subtracted. Excitation was 530 nm and emission was 590 nm. **1**, Cy3-Hes1 without immobilized Hes1: No dimer was detected (control); **2**, Cy3-Hes1 with immobilized Hes1: Hes1/Cy3-Hes1 dimer was detected; **3**, DMSO addition: no effect of DMSO on Hes1/Cy3-Hes1 dimerization was observed; **4**, Cy3-GST without immobilized Hes1: No dimer was detected; **5**, Cy3-GST with immobilized Hes1: No Hes1/Cy3-GST dimer was detected; **6**, DMSO addition: no effect of DMSO on Hes1/Cy3-GST dimerization was observed.

then immobilized on a microplate by chemical linkage.[9] To optimize the immobilization, we applied increasing concentrations of Hes1 to a 96-well microplate (Nunc Immobilizer™ Amino Plate, Nalge Nunc Int., NY, USA) followed by incubation for 2 h at room temperature. After incubation, the plates were washed with buffer, and Hes1 immobilization was confirmed by measuring the fluorescence intensity of Cy3 after addition of Hes1 antibody and Cy3-conjugated secondary antibody. It was found that 10 μg/ml of Hes1 resulted in sufficient immobilization on the plate (data not shown).

Cy3-dye-labelled Hes1 was prepared by incubating Hes1 with dye reagents (GE Healthcare, Buckinghamshire, UK) for 1 h at 4 °C. The buffer, incubation time, temperature and Hes1 concentration for the formation of Hes1 dimers were also investigated. It was found that use of a NET-N buffer (20 mM Tris–HCl, pH 7.5; 200 mM NaCl; 1 mM EDTA; 0.05% Nonidet P-40) generated a better Hes1-Cy3-Hes1 interaction. To form the homodimer of Hes1, the dye-labelled Hes1 was added to the Hes1-immobilized well. After 1 h incubation at room temperature, the well was washed and the fluorescence was measured by microplate reader. The dimer complex between Hes1 and Cy3-Hes1 was detected successfully (Fig. 3, **2**). As the compounds were prepared in DMSO solution, the solvent effect was investigated. Fluorescence value of the dimer complex was demonstrated to be unaffected by addition of DMSO (Fig. 3, **3**). Cy3-labelled GST was prepared to assess the interaction between the immobilized Hes1 and Cy3-GST and it was demonstrated that Cy3-GST did not bind to Hes1 specifically (Fig. 3, **5**, **6**). These results indicated that non-specific protein interaction does not occur and that the dimer of Hes1 with Cy3-Hes1 was specific interaction.

The compounds of our natural product library were evaluated using this constructed HTS system. This library consists of natural compounds isolated by our research group, including terpenoids, flavonoids, phenylpropanoids, their glycosides and bisindole alkaloids. The natural products were added to the Hes1 dimer complex on the microplate and incubated for 1 h. The microplate was washed gently once with 200 μl of NET-N buffer and dried at room temperature for 1 h in the dark under reduced pressure. The fluorescence intensity was then measured using a microplate reader. We reported previously on the development of a HTS of vitamin D receptor–co activator interaction using vitamin D derivatives.[9] In the study, we found HCHO-fixation method was effective to detect the protein–protein interaction. Although the fluorescence intensity of the Hes1 dimer complex was greatly improved with HCHO-fixation, risk of structural modification of some natural compounds, especially quinone and hydroquinone type compounds, was observed. Therefore we did not use the HCHO-fixation method in the present study. We identified six natural compounds that act as inhibitors of Hes1 dimer formation (Fig. 4) with the following IC_{50} values: lindbladione (**1**),[10] IC_{50}, 4.1 μM isolated from the myxomycetes *Lindbladia tubulina*; two bisindole alkaloids, lycogarubin B (**2**),[11] 16.5 μM and lycogaric acid A (**3**),[11a,b,12] 6.0 μM isolated from the myxomycetes *Lycogala epidendrum*; demethy-2"-epifraxamoside (**4**),[13] 7.8 μM isolated from *Jasminum grandiflorum*; and, two lignan glycosids, lyoniside (**5**),[14] 48.1 μM and nudiposide (**6**),[15] 9.6 μM isolated from *Saraca asoca*. Interestingly, the stereochemistry in **5** and **6** affected their activity; compound **6** had 5 times higher inhibitory activity than compound **5**. Compounds **1**–**3** are natural products isolated from myxomycetes (true slime molds), which are an unusual group of primitive organisms that can be assigned to one of the lowest classes of eukaryotes. We also examined several bisindole alkaloids (Fig. 5): lycogarubin C (**7**),[11b] staurospolinone (**8**)[16] and lycogarubic acid methyl ester (**9**),[11d] which were less active than compounds **1**–**6** even at 100 μM.

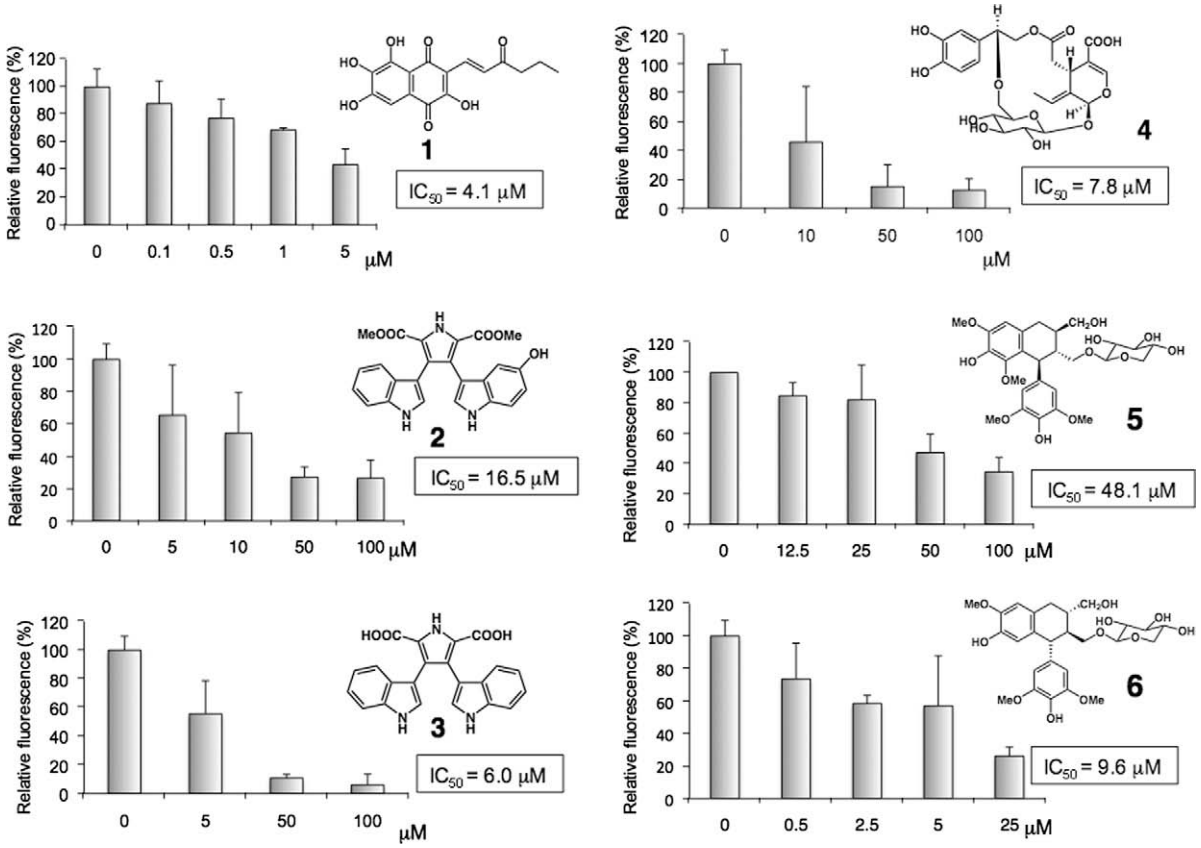

Figure 4. Evaluation of the inhibitory activity of natural products on Hes1 dimer formation in a microplate. All experiments were performed in a microplate containing immobilized Hes1. Excitation was 530 nm and emission was 590 nm. The background value (without Hes1) was subtracted. Fluorescence intensity of the blank (DMSO) was normalized to 1.

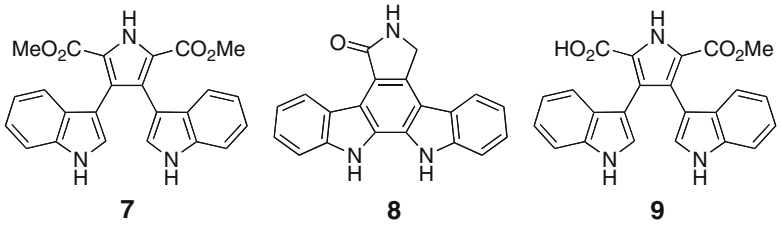

Figure 5. Chemical structures of less active bisindole alkaloids.

We subsequently examined the intracellular inhibition activity of these compounds. As shown in Figure 1, Hes1 dimer binds to N box sequences in the target promoter of bHLH activators, thus suppressing their transcription. A cell-based reporter assay for measuring the effect on Hes1-induced repression[11b] was performed in C3H10T1/2 cells. Findings indicated that a decrease in reporter activity depends on the production of endogenous Hes1 protein (Fig. 6). Of the inhibitors identified by protein-based screening, **1** and **3** showed dose-dependent inhibition of the Hes1-mediated suppression of gene expression. With compounds **1** and **3** transcription recovered to around 60% at 50 μM. As these compounds inhibit Hes1 in vitro, the in vivo molecular target would be intracellular Hes1 dimer formation.

In the present study, we developed a novel rapid system for screening for inhibitors of Hes1 dimer formation. Using this system, the first Hes1 dimer inhibitors were found from our natural products library. Two of the identified compounds, lindbladione (**1**) and lycogaric acid A (**3**) isolated from the myxomycetes *L. tubulina* and *L. epidendrum*, respectively, inhibited Hes1-mediated suppression of the transcription in C3H10T1/2 cells. This HTS system has potential uses in biochemical research and to identify Hes1 dimer inhibitors as lead compounds for NSC activation. As ongoing research, we are seeking other Hes1 inhibitors and isolating novel compounds from natural products for investigation. We believe these results would contribute to finding of biotools and regenerative medicine of neural disease.

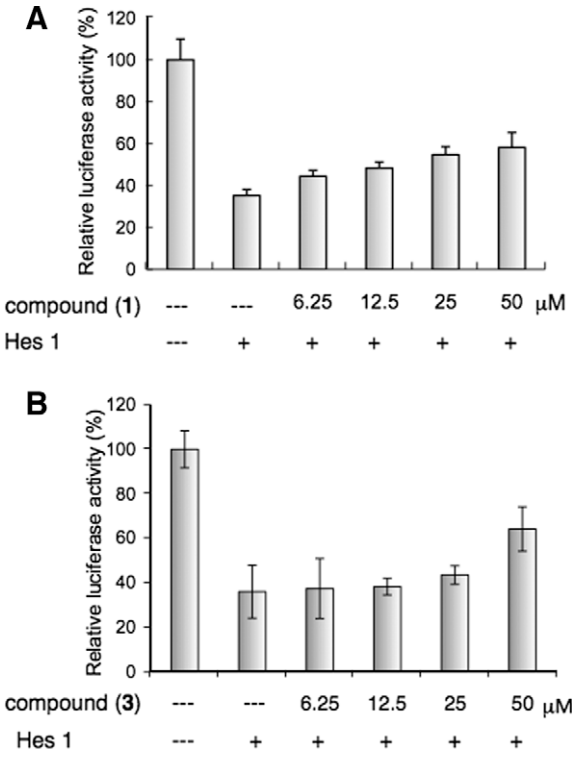

Figure 6. Inhibitory effects of natural product on Hes1-dependent gene repression. Mouse C3H10T1/2 cells were transfected with pN6-βA-luc, pCL-Hes1 and pRL-SV40 in 24-well plate. Compounds were added to the medium 3 h after transfection. After 24 h, luciferase activity was determined. The mean and SD of three individual wells were calculated.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.146.

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