A Comparison of ROCK Inhibitors on Human Bone Marrow-Derived Mesenchymal Stem Cell Differentiation into Neuron-Like Cells

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Abstract — Bone marrow-derived mesenchymal stem cells (BM-MSC) are a multipotent cell population that can differentiate into neuron-like cells. Previously it has been reported that murine BM-MSC can differentiate into neuron-like cells by co-treatment with a Rho-associated kinase (ROCK) inhibitor -Y27632 and CoCl₂. In this study, we compared several ROCK inhibitors for the ability to induce human BM-MSCs to differentiate into neuron-like cells in the presence of CoCl₂. Y27632 with high specificity for ROCK at 1-30 μM was best at inducing neuronal differentiation of MSCs. Compared to HA1077 and H1152, which also effectively induced morphological change into neuron-like cells, Y27632 showed less toxicity even at 100 μM, and resulted in longer multiple branching processes at a wide range of concentrations at 6 h and 72 h post-induction. H89, however, which has less specificity by inhibition of protein kinase A, S6 kinase 1 and MSK1 with similar or greater potency, was less effective at inducing neuronal differentiation of MSCs. Simvastatin, which can inhibit Rho, Ras, and Rac by blocking the synthesis of isoprenoid intermediates, showed little activity for inducing morphological changes of MSCs into neuron-like cells. Accordingly, the expression patterns for neuronal cell markers, including β-tubulin III, neuron-specific enolase, neurofilament, and microtubule-associated protein, were consistent with the pattern of the morphological changes. The data suggest that the ROCK inhibitors with higher specificity are more effective at inducing neuronal differentiation of MSCs.

Keywords: Human bone marrow, Mesenchymal stem cell, Neuronal differentiation, ROCK inhibitor

INTRODUCTION

Human bone marrow-derived mesenchymal stem cells (BM-MSC) are typically self-renewing, adherent, and fibroblast-like cells, which have the potential to differentiate into lineages of mesenchymal tissues including bone, cartilage, fat, tendon, muscle, and marrow stroma. In addition, BM-MSCs have shown a high plasticity to differentiate into skeletal and cardiac muscle cells, hepatocytes, keratinocytes, pancreatic islet cells and neuronal lineage cells (Pittenger et al., 1999; Grove et al., 2004). In particular, the ability to differentiate into neuron-like cells has been demonstrated in both human and rodent MSCs (Sanchez-Ramos et al., 2000; Woodbury et al., 2000). Since the therapeutic effects of BM-MSCs have been demonstrated in several animal models of neurological disorders such as stroke, spinal cord injuries and Parkinson’s disease (Li et al., 2001; Hofstetter et al., 2002; Dezawa et al., 2005), BM-MSCs may provide a promising autologous source of neurons.

Rho-kinase (ROCK) is a serine/threonine kinase, with isoforms of ROCK1 and ROCK2, and is one of the major downstream effectors of the small GTPase Rho. Rho GTPase is involved in the regulation of neuronal morphogenesis, including migration, polarity, axon growth and guidance by transducing signals from extracellular stimuli to the actin cytoskeleton (Luo, 2000; Van Aelst and Cline, 2004; Schmandke and Strittmatter, 2007). ROCK inhibitors have been shown to have therapeutic effects on cardiovascular diseases and central nervous system disorders (Liao et al., 2007; Kubo et al., 2008). ROCK also plays an important role in the regulation of apoptosis in various cell types, and a ROCK inhibitor, Y-27632, promotes the post-thaw viability and physiological function of cryopreserved human BM-MSCs (Shi and Wei, 2007; Olson, 2008; Heng, 2009).
In relation to neuronal differentiation, ROCK inhibition by the inhibitor Y27632 has been reported to potentiate HIF-1 (hypoxia inducible factor) activation-mimicking agent CoCl₂-induced MSC differentiation in particular into dopaminergic neuron-like cells (Pacary et al., 2006; Pacary et al., 2007; Pacary et al., 2008). This combination efficiently induced neurite-like extensions and morphological change in MSCs in vitro.

In this study, we compared the effects of several ROCK inhibitors (Fig. 1) on neuronal differentiation of human BM-MSCs when co-treated with CoCl₂, thereby elucidating the relationship between specificity of ROCK inhibitors and their efficacy in inducing neuronal differentiation of MSCs.

MATERIALS AND METHODS

Materials

ROCK inhibitors, including Y27632, HA1077, H1152, and H89, were purchased from Calbiochem (Merck Bioscience, San Diego, CA) and dissolved in ultra-pure water. Simvastatin was purchased from Calbiochem and dissolved in dimethyl sulfoxide (DMSO). HIF-1 activation-mimicking agent CoCl₂ was purchased from Sigma (St. Louis, MO) and dissolved in ultra-pure water.

Cell culture

Human bone marrow-derived mesenchymal stem cells (Cat. No. PT-2501, Batch. No. 8F3543, cryopreserved at the 2nd passage) were purchased from Lonza Inc. (Walkersville, MD). The cells were thawed and cultured further; three-four passages (P5-P6), in MSCGM® bullet kit (Cat No. PT-3001) purchased from Lonza Inc.

Neuronal differentiation in vitro

BM-MSCs (40-60 cell/mm²) were seeded onto plates and cultured in MSCGM® growth medium overnight. After washing with PBS, the cells were treated with 100 μM CoCl₂ and various concentrations of Y27632, HA1077, H1152, H89 or simvastatin and were incubated at 37°C for 72 h. During the incubation, the cells were photographed at four representative fields (Leica, Inverted Microscope, Germany). Images were analyzed using Image J (http://rsb.info.nih.gov/ij/) to determine branch lengths.

Immunofluorescence

Following induction of in vitro neuronal differentiation for 72 h, the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at RT. After washed with PBS, the cells were permeabilized by treatment with 0.1% Triton-X 100 in PBS. After twice washing with PBS, the cells were blocked by incubating with 5% normal goat serum (Vector Laboratories, Burlingame, CA) at RT for 1 h, and then incubated for 1 h with an antibody against neuron-specific class III β-tubulin (Tuj-1, 1:500, Cat. No. MMS-435P, Covance, Richmond, CA), neuron-specific enolase (NSE, 1:500, Cat. No. AB951, Chemicon, Temecula, CA), neurofilament-M (NF-M, 1:500, Cat. No. MAB1615, Chemicon), microtubule-associated protein (MAP2, 1:250, Cat. No. AB5622, Chemicon), or glial fibrillary acidic protein (GFAP, 1:500, Cat. No. Z0344, Dako, Glostrup, Denmark). After washing with PBS, the cells were incubated for 1 h with Cy3-conjugated anti-mouse IgG and anti-rabbit IgG (Cat. No. AP186C, AP182C, Chemicon). Finally, after washing with PBS, nuclei were stained with diamidino-phenylindole (DAPI, Sigma) for 5 min. The cells were observed under a fluorescent microscope (Carl Zeiss, Germany).

Western blot analysis

For the preparation of whole cell lysates, cells were lysed in a lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X 100, 1 mM Na₂VO₄, 1% Sodium dodecyl sulfate, 0.1% SDS, 1 μg/ml pepstatin A, 50 mM NaF, 0.5 mM EDTA, 1 mM EGTA and Protease inhibitor cocktail in distilled water) on ice for 20 min. After incubation, the lysate was centrifuged for 30 min at 13,000 rpm at 4°C. Then, the supernatant containing protein extract was resolved using SDS-PAGE gel, and transferred to a nitro-
cellulose membrane, which was blocked with 5% skim milk. The membrane was then incubated with antibody against Tuj-1 (Cat. No. MMS-435P, Convance), NSE (Cat. No. AB951, Chemicon), ROCK1 (Cat. No. 611136, BD bioscience, San Jose, CA), ROCK2 (Cat. No. 610623, BD bioscience) or β-actin (Cat. No. A5441, Sigma), followed by incubation with the corresponding secondary antibodies conjugated to horseradish peroxidase (Santa Cruz). All the primary antibodies used can detect both human and mouse-derived proteins. The bindings were detected by the enhanced chemiluminescence system (ECL kit, Amersham, Piscataway, NJ). Human neuroblastoma SH-SY5Y cells were used for positive controls.

Proliferation assay
MSCs were seeded at a density of 50 cells/mm² in a 96-well plate and cultured in MSCGM medium for 24 h. Then, the cells were replaced with fresh MSCGM medium, and treated with 30 μM Y27632, 30 μM HA1077, 3 μM H1152, 3 μM H89, 3 μM simvastatin and 100 μM CoCl₂, respectively. The cells were incubated for 1-3 days. After the change of medium, the cells were subject to an MTS assay according to the manufacturer’s protocol (Promega, Madison, WI).

Statistical analysis
Data were expressed as mean ± standard error or mean ± standard deviation. Statistical comparisons were performed using Student’s t-test (Sigma Plot Software). p-values smaller than 0.05 were considered to be statistically significant.

RESULTS

The effects of ROCK inhibitors on the morphological changes of MSCs into neuron-like cells

The synergistic effects of CoCl₂ and ROCK inhibition on neuronal differentiation have been previously shown in murine BM-derived mesenchymal stem cells (Pacary et al., 2006). Thus, we tested whether such synergistic effect of ROCK inhibition plus CoCl₂ could occur in human BM-MSCs. When human BM-MSCs were incubated in the presence of 100 μM CoCl₂ plus 30 μM Y27632 for 72 h using the same conditions as reported before, human BM-MSCs underwent dramatic morphological changes within 6 h. The human BM-MSCs demonstrated the synergistic effects of CoCl₂ and ROCK inhibition on cell morphology by forming neuron-like cells with long process extensions and multiple branches (Fig. 2). ROCK inhibition by Y27632 alone also showed a marked effect, but CoCl₂ alone did not cause morphological changes into neuron-like cells, which is in contrast to results with murine BM-MSCs. After 72 h, the synergistic effect in co-treated cells was more prominent, since neuron-like cells showing long processes became refractile cells having multiple branches with shorter processes compared to the cells treated with Y27632 alone.

Since combination treatment with CoCl₂ and ROCK inhibitor appeared the most effective in inducing MSCs into neuron-like cells, we compared several ROCK inhibitors with different selectivities for serine-threonine kinases (Fig. 2). Human BM-MSCs were incubated with 30 μM Y27632 and/or 100 μM CoCl₂ for 6 and 72 h, and then photographed under a microscope (objective ×200). Black box was magnified (×600).
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Fig. 3. Comparison of ROCK inhibitors on morphological changes into neuron-like cells of MSCs in the presence of CoCl₂. (A) MSCs were incubated in the presence of 100 μM CoCl₂ and various concentrations (1-100 μM) of ROCK inhibitors plus 100 μM CoCl₂ for 72 h. Within 6 h after co-treatment, the drastic morphological changes resulting in long thin process extensions and multiple branches occurred, except for treatment with simvastatin (Fig. 3A). During the initial 6 h, apparent toxicity was not observed except when cells were treated with H89 or simvastatin at concentrations > 30 μM, or with H1152 at 100 μM. Interestingly, treatment with Y27632, HA1077 and H1152 produced marked morphological changes into neuron-like cells over wide ranges of 3-100 μM, 3-100 μM, and 1-30 μM, respectively, without apparent toxicity during the 6 h period. H1152 showed more potency for inducing morphological changes compared to Y27632 and HA1077 at low concentrations (1-10 μM) and resulted in marked changes even at 1 μM. To

1), in order to examine which ROCK inhibitor is best for inducing neuronal differentiation of human BM-MSCs. Thus, human MSCs were subjected to neuronal differentiation at varying concentrations (1-100 μM) of ROCK inhibitors plus 100 μM CoCl₂ for 72 h. Within 6 h after co-treatment, the drastic morphological changes resulting in long thin process extensions and multiple branches occurred, except for treatment with simvastatin (Fig. 3A). During the initial 6 h, apparent toxicity was not observed except when cells were treated with H89 or simvastatin at concentrations > 30 μM, or with H1152 at 100 μM. Interestingly, treatment with Y27632, HA1077 and H1152 produced marked morphological changes into neuron-like cells over wide ranges of 3-100 μM, 3-100 μM, and 1-30 μM, respectively, without apparent toxicity during the 6 h period. H1152 showed more potency for inducing morphological changes compared to Y27632 and HA1077 at low concentrations (1-10 μM) and resulted in marked changes even at 1 μM. To
Fig. 4. Long-term induction of morphological changes of BM-MSCs into neuron-like cells by co-treatment with ROCK inhibitors plus CoCl$_2$. MSCs were incubated in the presence of CoCl$_2$ and various concentrations of each ROCK inhibitor for 72 h. The morphological changes of induced cells were examined under a microscope (objective ×200).

From these results, we concluded that Y27632, with comparably high specificity for ROCK, is the best ROCK inhibitor for inducing the morphological changes of MSCs into neuron-like cells without toxicity, and that HA1077 and H1152 are also effective inducers of neuronal morphology in vitro.

The analysis of neuronal marker expression in differentiated human BM-MSCs

We examined the expression of Tuj-1, NSE, NF-M, and MAP2 as neuronal markers and GFAP as a glial marker by immunofluorescence 72 h after co-treatment of human MSCs with ROCK inhibitor/CoCl$_2$. In Y27632-treated cells, Tuj-1, NSE, NF-M and MAP2 were positively stained as shown Fig. 5 and Table I, and a similar staining pattern was observed in HA1077-treated cells. H1152-treated cells were also positively stained for Tuj-1, NSE, NF-M and MAP2, however, at lower levels than Y27632 or HA1077- treated cells. Lower percentages of positive cells were shown in-H89 treated cells. Only small percentages of positive cells for Tuj-1 and NSE were observed in simvastatin-treated cells. No GFAP expression was detected in any inhibitor-treated cells, except untreated MSCs (0.61%± 0.5).

In parallel, we performed western blot analysis for detection of expression levels of two neuronal markers, (Tuj-1 and NSE), and ROCK isoforms (1 and 2). Both iso-

compare the relative potencies for inducing neuronal differentiation of MSCs in a semi-quantitative manner, we analyzed the average total length of branches/cell after 6 h of co-treatment of each ROCK inhibitor (Fig. 3B). In a dose range of 3-100 μM, Y27632 and HA1077 showed a dose-dependent increase of morphological changes, whereas H1152 showed a dose-dependent decrease of neuronal differentiation due to its potency. Compared to the specific ROCK inhibitors, treatment with H89, which inhibits protein kinase A (PKA), S6 kinase 1 and MSK1 as well as ROCK, produced a few neuron-like cells with multiple branch processes at 3 and 10 μM within 6 h, and treatment with simvastatin, a HMG-CoA reductase inhibitor, produced rare neuron-like cells, although it induced short bipolar branching processes at 3, 10 and 30 μM.

After 72 h of incubation, the cells treated with Y27632, HA1077 and H1152 became refractile cell bodies with more branching processes, thereby resulting in mature neuron-like cells (Fig. 4). When the cellular morphological changes and toxicity at 6 h and 72 h were considered, the optimum concentration for inducing cells to differentiate into neuron-like cells was 30 μM for Y27632 and HA1077, and 3 μM for H1152. H89 treatment produced a very few neuron-like cells and severe toxicity at concentrations > 10 μM. Simvastatin treatment showed no neuron-like cells possessing multiple branch processes and demonstrated severe toxicity at concentrations > 10 μM.
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Fig. 5. Analysis of neuronal marker expression by immunofluorescence. MSCs were induced into neuron-like cells using the optimal concentration of each ROCK inhibitor plus 100 μM of CoCl₂ for 72 h. Induced cells were stained for neuronal markers Tuj-1, NSE, NF-M, MAP2 for neuron, and GFAP for glia (in red), and nuclei were counterstained with DAPI (blue).

Table I. Analysis of expression level of neuronal markers

<table>
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<tr>
<th></th>
<th>Tuj-1 (%)</th>
<th>NSE (%)</th>
<th>NF-M (%)</th>
<th>MAP2 (%)</th>
<th>GFAP (%)</th>
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<tr>
<td>Untreated</td>
<td>13.54 ± 4.3</td>
<td>9.73 ± 7.4</td>
<td>1.70 ± 1.9</td>
<td>0.00 ± 0.0</td>
<td>0.61 ± 0.5</td>
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<tr>
<td>Y27632</td>
<td>60.56 ± 8.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.00 ± 10.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.04 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.66 ± 4.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00 ± 0.0</td>
</tr>
<tr>
<td>HA1077</td>
<td>66.78 ± 8.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.17 ± 7.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.94 ± 5.5</td>
<td>29.86 ± 10.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00 ± 0.0</td>
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<tr>
<td>H1152</td>
<td>42.26 ± 7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.63 ± 9.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.03 ± 2.7</td>
<td>11.80 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00 ± 0.0</td>
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<tr>
<td>H89</td>
<td>39.91 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.81 ± 3.3</td>
<td>3.25 ± 2.2</td>
<td>11.25 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00 ± 0.0</td>
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<tr>
<td>Simvastatin</td>
<td>29.79 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.82 ± 5.0</td>
<td>0.00 ± 0.0</td>
<td>0.00 ± 0.0</td>
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The positively stained cells were counted per 100 cells for Tuj-1, NSE, NF-M, MAP2 and per 1,000 cells for GFAP. The data are presented as the mean ± S.D. <sup>a</sup>p<0.05, <sup>b</sup>p<0.01 versus untreated, n=4.

forms were expressed in human BM-MSCs, and their expression was not significantly affected by inhibitors except simvastatin, which reduced expression (Fig. 6). Treatment with CoCl₂ alone has been shown to increase expression of NSE (Pacary et al., 2006). The co-treatment with Y27632, HA1077, or H1152 plus CoCl₂, resulted in slightly higher NSE expression than co-treatment using H89 or simvastatin. In case of Tuj-1 expression, the synergistic effect of ROCK inhibition and CoCl₂ was highest in the cells treated with Y27632. The co-treatment with HA1077, H1152, or simvastatin also increased Tuj-1 expression. However, H89 did not show a significant increase of Tuj-1
Fig. 6. Western blot analysis of neuronal marker expression. Total cell lysates were prepared from the MSCs treated with the optimal concentration of each inhibitor plus 100 μM of CoCl₂ for 72 h. Mouse brain and SH-SY5Y cell lines were used as positive controls and MSCs cultured in growth medium were used as a non-induced control.

The effect of ROCK inhibitors on human BM-MSC proliferation

In order to examine how ROCK inhibitors affect proliferation of MSCs at each optimal concentration for neuronal differentiation, we performed an MTS assay with MSCs treated with ROCK inhibitor or CoCl₂ alone at 24 or 72 h after treatment. As shown in Fig. 7A, the ROCK inhibitors tested showed no significant inhibition of cell proliferation, while CoCl₂ showed a severe inhibition, and simvastatin showed 18% inhibition at 72 h. The suppression of cell proliferation by CoCl₂ treatment was consistent with the previous report that CoCl₂ treatment increased the expression of the anti-proliferative gene BTG/PC3 and decreased cyclin D1 expression (Pacary et al., 2006). Interestingly, the cells treated with Y27632, HA1077, or H1152 showed bipolar or multi-polar morphology at 24 and 72 h even though cell proliferation occurred (Fig. 7B). From these results, we concluded that ROCK inhibitor alone induces morphological changes into neuron-like cells albeit not with complete shapes and has no effect on cell proliferation, which is contrast to CoCl₂.

DISCUSSION

BM-MSCs have the ability to differentiate into neuron-like cells, and following transplantation of these cells, therapeutic benefit has been shown in cerebral ischemia and trauma (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Chen et al., 2001; Mahmood et al., 2001). Thus, BM-MSCs have been considered as a valuable autologous source of neurons and it will be a challenging issue in this field to improve their efficacy. In this study, we examined ROCK inhibitors to determine if these inhibitors effectively induce neuronal differentiation of human BM-MSCs, in an attempt to develop a method to improve neuronal differentiation of MSCs. Interestingly, human BM-MSCs have a different response to the ROCK inhibitor Y27632, compared to murine BM-MSCs. Human BM-MSCs formed neuron-like cells, albeit with less branching processes, in the presence of Y27632 alone, whereas murine BM-MSCs developed long bipolar branching processes in the cells treated with CoCl₂ (single or combination), not in the cells treated with Y27632 alone. Human BM-MSCs showed much shorter bipolar processes when treated with CoCl₂ alone. However, it was not conclusive that it was only due to the difference in cell types (human vs. murine BM-MSCs), because different experimental conditions were employed; 1) different culture media were used because human BM-MSCs do not grow well in the 20% FBS-containing DMEM; and 2) cell density was different (40-60 cells/mm² for human MSCs and subconfluency for murine MSCs). However, both cell types showed identical synergistic effects of CoCl₂ and ROCK inhibition.

In the experiment comparing effects of ROCK inhibitors, MSCs were separately co-treated with CoCl₂ plus each inhibitor. Results for morphological changes and neuronal marker expression demonstrated that ROCK inhibitors, Y27632, HA1077 and H1152 showed greater potency for inducing neuronal differentiation of MSCs compared to H89, which also inhibited PKA, S6K and MSK1, or simvastatin - an HMG CoA reductase inhibitor. Among three ROCK inhibitors, Y27632, HA1077 and H1152 showed the best outcome for neuronal differentiation of MSCs, however, HA1077 also showed a marked effect on MSC neuronal differentiation. H1152 showed drastic effects on morphological changes into neuron-like cells at low doses (1-10 μM) during a short period of 6 hours, but
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Fig. 7. Effect of inhibitors on BM-MSC proliferation. (A) Proliferation of MSCs was determined using an MTS assay 24 and 72 h after incubation at the optimal concentration with each inhibitor, without co-treatment with CoCl₂. Data represent mean ± SE. *p < 0.05, **p < 0.01 versus control, n=4. (B) The morphology changes of treated cells were observed at 24 and 72 h by phase-contrast microscopy (objective ×200).

had less effect on neuronal marker expression after 72 hours, compared to Y27632 and HA1077. Surprisingly, compared to Y27632 and HA1077, H1152 demonstrates higher potency and selectivity, with Ki values of 1.6 nM for ROCK, 630 nM for PKA, and 9.27 μM for PKC (Sasaki et al., 2002). The lower efficacy of H1152 for inducing neuronal differentiation could possibly be explained by its inhibition of a broad spectrum of serine/threonine kinase PKA, which can be achieved at the concentration range of 1-3 μM. Inhibition of PKA by H1152 would inhibit the neuronal differentiation of MSCs induced by ROCK inhibition, particularly at a late stage of differentiation.

Interestingly, when compared to HA1077, ROCK selectivity is also greater for Y27632, with Ki values of 0.14 μM for ROCK, 25 μM for PKA, and 26 μM for PKC, compared to HA1077 with Ki values of 0.33 μM for ROCK, 1.0 μM for PKA, and 9.3 μM for PKC. Compared to HA1077, the much higher Ki values of Y27632 for broad spectrum serine/threonine kinases, PKA and PKC, may give rise to an advantage in inducing neuronal differentiation, since Y27632 is not likely to effectively inhibit PKA or PKC at the optimal concentration of 30 μM. This idea also correlates with the effect of H89 on neural differentiation, since it inhibits ROCK and even more potently inhibits PKA, and H89 exhibited toxicity at higher concentrations and had fewer effects on neuronal differentiation of MSCs compared to Y27632. Thus, it can be suggested that a ROCK inhibitor with higher specificity is more effective at inducing the morphological changes of MSCs into neuron-like cells and that the selectivity of inhibitors for ROCK and their Ki absolute values are important, especially when considering nonselective inhibition of broad spectrum serine/threonine kinases.

Although combination effects of ROCK inhibitors may
result from inhibition of ROCK, it could not be excluded that other kinases involved in neuronal differentiation of MSCs can also be blocked. Y27632 and HA1077, which inhibit both isoforms (1 and 2) of ROCK without selectivity, also inhibit PKC-related kinase-2 (PRK2) at an IC50 of 600 nM and 4 μM, respectively. Since the most effective ROCK inhibitor, Y27632, has a lower Ki value for PRK2 compared to ROCK (Davies et al., 2000), the possibility of PRK2 in involvement in neuronal differentiation remains to be elucidated. Additionally, it is plausible that simvastatin is not effective in neuronal differentiation, since it lacks selectivity by inhibiting Ras and Rac as well as Rho.

Since Rho-ROCK inhibition is a promising strategy for the treatment of cardiovascular disease and CNS disorders, based on our results, we can suggest a simple screening method for ROCK inhibitors employing MSC differentiation into neuron-like cells in vitro. The importance of ROCK inhibitors is not limited to application in the treatment of central nervous system disorders, such as spinal cord injuries, stroke and Alzheimer’s disease. ROCKs have also been implicated in the regulation of vascular tone, proliferation, inflammation, and oxidative stress (Liao et al., 2007). Despite the potential importance of ROCK inhibition, HA1077 (fasudil) is the only ROCK inhibitor approved for human use. HA1077 has been used in patients with cerebral vasospasm, angina or acute ischemic stroke. In addition to the present therapeutic use of HA1077, our results suggest that it might be applied for the cell therapy in neuronal regeneration, although further studies are necessary.

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