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Potential therapeutic application of small molecule with sulfonamide for chondrogenic differentiation and articular cartilage repair

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ABSTRACT

The restoration of damaged articular cartilage is a long-pursued goal in regenerative medicine. Chondrocyte-specific differentiation of mesenchymal stem cells (MSCs) may be an effective means of repairing damaged cartilage. We identified small molecule **6** with sulfonamide as an agent that promotes specific chondrogenic differentiation of human adipose-derived MSCs (hASCs). Unlike other chondrogenic differentiation media composed of various defined components, simply adding compound **6** into culture medium was sufficient to induce chondrogenesis in this study. In an animal osteoarthritis model, both the small molecule **6** and the **6**-treated hASCs exhibited enhanced recovery of injured articular cartilage. This work provides new insight into MSC differentiation induced by small molecules and potential new therapeutic approaches for articular cartilage injury.

Chondrogenesis is a primary process in skeletal development and is regulated by numerous growth factors and a network of transcription factors.[1,2] Chondrocytes are a major element of articular cartilage, a simple and dense connective tissue that has the potential to maintain the cushion between bones and joints.[3] Articular cartilage injuries cause severe pain, the loss of chondrocytes and their function, and an irreversible debilitation. However, damaged articular cartilage is incapable of self-recovery.[4] As temporary expedients, exercise, non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular hyaluronic acid injection, and replacement with artificial joints are used for treatment and pain relief.[5] For more effective and successful treatment, the fields of regenerative medicine and tissue engineering have utilized chondrocytes and/or mesenchymal stem cells (MSCs).[6] To date, strategies using druggable small molecules in adult stem cell therapies have focused on identifying molecules for more specific and selective control of MSC proliferation and differentiation.[7] Nevertheless, only a handful of studies in which small molecules induced potent and specific chondrogenic differentiation of MSCs have been published.[8-10] Therefore, the identification of a small molecule that promotes chondrogenesis is important for the development of a novel therapeutic agent to treat damaged articular cartilage. Herein, we report the identification and characterization of a small molecule containing sulfonamide that promotes direct human adipose-derived MSC (hASC) differentiation into chondrocytes.

In our previous study, we demonstrated that protein kinases play crucial roles in modulating stem cell fate. In that particular study, we screened our house protein kinase inhibitor library composed of six major subfamilies, and the results indicated that a protein kinase A (PKA) inhibitor H-89 (*N*-[2-(*p*-bromo-cinnamylamino) ethyl]-5-isoquinolinesulfonamide • 2HCl, Fig. 1) had the potential to induce chondrogenic differentiation in MSCs.[11] In the present study, we further validated that a chemical compound can be used to induce chondrogenesis in MSCs.

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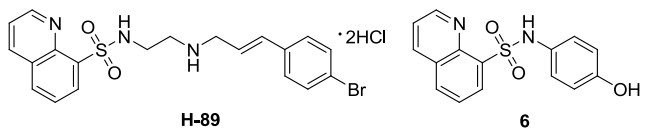
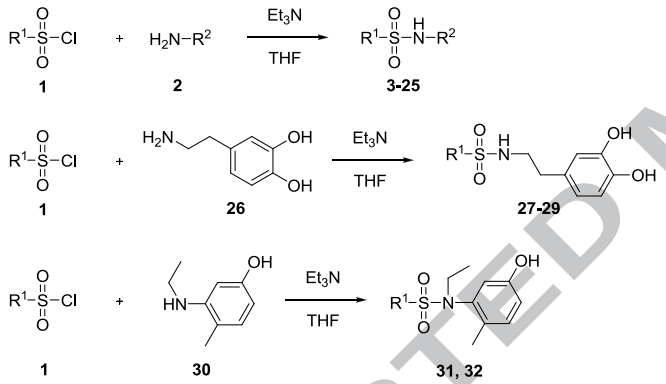


Figure 1. Structure of H-89 and compound **6**.

In an effort to identify a novel synthetic small molecule with chondrogenic potential, we designed and synthesized a series of sulfonamide analogues based on the structure of H-89. The synthetic procedure of sulfonamide analogues involved a simple and one-step reaction, the coupling of a sulfonyl chloride with a primary or secondary amine,[12-14] and these analogues have two substituents, R^1 and R^2. The R^1 substituents consisted of quinoline, naphthyl, biphenyl, 4-*tert*-butyl, or 4-methylbenzene, and the R^2 substituents were composed of benzene, naphthyl, or cyclohexyl with substituents (Scheme 1 and all synthetic products can be found in Supplementary data). Generally, sulfonamide compounds are well kwon for antibiotics,[15,16] and then among our synthesized compounds, some compounds reported that they have potential medicinal applications including antibacterial,[17] antiviral,[18] and antitumor activity.[19,20] However, chondrogenic properties of sulfonamides remain to be uncovered.



Scheme 1. General synthesis of sulfonamide derivatives.

To examine the chondrogenic activity of the synthesized sulfonamide analogues, hASCs were cultured in a complete growth medium with 1 μM or 10 μM of sulfonamide analogues for 11 days. The expression of aggrecan, a chondrogenesis marker, was determined by sandwich enzyme-linked immunosorbent assay (ELISA) and was used to evaluate the chondrogenic potential of analogues. The aggrecan expression analyses indicated that six analogues (**6**-**11**) increased aggrecan expression approximately 2-fold compared with the control at both concentrations used (1 μM and 10 μM). Other analogues failed to induce the expression of aggrecan at the lower concentration used (Fig. 2a).

Using sandwich ELISA, a structure-activity relationship (SAR) analysis was conducted. Quinoline was the most active, whereas 4-methylbenzen was less active than other substituents in R^1 (**5** vs. **7**). Neither having substituent (2-hydroxyethyl) cyclohexyl in R^2 position (**23**-**25**), having a long carbon chain between sulfonamide and R^2 (**27**-**29**), nor introducing *N*-ethyl in sulfonamide (**31** and **32**) affected aggrecan expression at 1 μM and 10 μM. In the case of naphthalenol in R^2, the position of hydroxyl influenced the activity; 4-hydroxy (**11**) > 5-hydroxy (**4**) > 7-hydroxy (**5**) and hydroxy- and chloro- (**15**), fluoro- (**18**) or nitro- (**22**) substituted benzene were more active than only hydroxy-substituted benzene (**3**) in R^2 at 10 μM. This suggested that chondrogenic activity was more influenced by R^1 substituents than R^2 substituents. Although the number of analogues exhibited good aggrecan-inducing ability, we focused on compound **6** (Fig. 1) because it exhibited the highest aggrecan expression at 1 μM and was an appropriate chondrogenic inducer of hASCs based on SAR analysis.

Lineage-specific differentiation of hASCs in the presence of compound **6** was further confirmed by alcian blue staining. The intensity of alcian blue staining increased approximately 3-fold in the hASCs treated with compound **6** for 11 days (Fig. 2b). Additionally, aggrecan expression was dose-dependently increased with compound **6** treatment (Fig. 2c). During chondrogenic differentiation by compound **6**, we observed a time-dependent increase of ERK phosphorylation (Fig. 2d). The inhibition of the ERK signaling pathway by a selective MEK inhibitor U0126 suppressed aggrecan expression even in the presence of compound **6** (Fig. 2c, d).

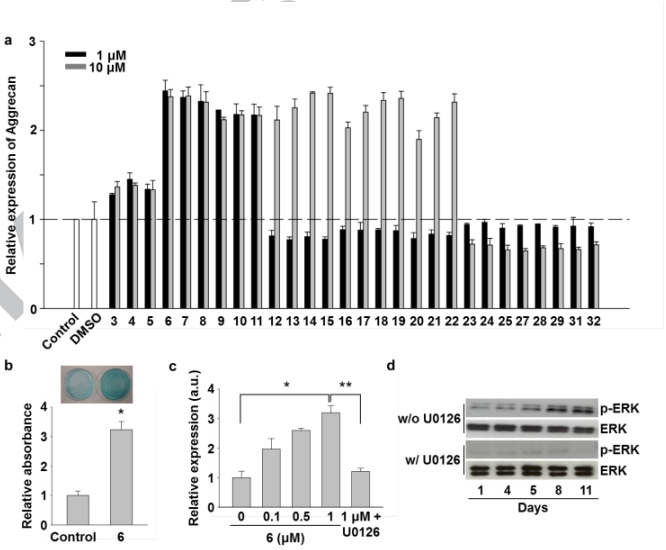


Figure 2. Compound **6** induces chondrocyte differentiation in hASCs. a) The measurement of aggrecan contents as a chondrogenic marker at day 11; b) Compound **6** upregulates aggrecan expression; c) Compound **6** upregulates aggrecan expression in a dose-dependent manner, but ERK inhibitor (U0126) inhibits aggrecan expression; d) The chondrogenic effect of compound **6** is mediated by the ERK pathway.

To evaluate the effects of compound **6**-treated hASCs and compound **6** alone in vivo, we used the collagenase type II-induced chronic joint injury (osteoarthritis; OA) model.[21,22] Knee joints from each group (intra-articular injection of 1) hASCs, 2) hASCs treated with compound **6** or 3) compound **6** in 50 μl of PBS on days 7 and 21) were harvested and prepared for sectioning and immunohistochemical analysis 6 weeks later.

The sections were strained with safranin O/Fast Green for detecting sulfated glycosaminoglycans (GAGs). The thickness of articular cartilage significantly decreased in the OA group compared with the normal group (Fig. 3a, b). However, thickness recovered in the group injected with compound **6**-treated hASCs as well as in the group injected with compound **6** alone (Fig. 3d, e). In addition, the proteoglycan contents were increased in the compound **6**-treated hASCs-injected group compared with the OA and hASCs-injected group (Fig. 3b-d). The compound **6** alone-injected group exhibited similar recovery effects to that of the compound **6**-treated hASCs-injected group (Fig. 3e). The results of safranin O/Fast Green staining are also correlated with aggrecan immunohistochemistry. Compared with normal

articular cartilage, the OA model exhibited low aggrecan expression levels (Fig. 4a, b), whereas the hASC injection group exhibited slightly increased levels of aggrecan (Fig. 4c) and treatment with compound **6**-induced chondrogenic-differentiated hASCs dramatically increased aggrecan expression (Fig. 4d) compared with the OA group. Notably, the compound **6** injection group also exhibited higher expression levels of aggrecan (Fig. 4e) relative to the OA group. These results implied that compound **6** induces naïve chondrocyte properties in hASCs and raises the possibility that both compound **6**-treated hASCs and compound **6** itself impart protective effects against the damaged articular cartilage.

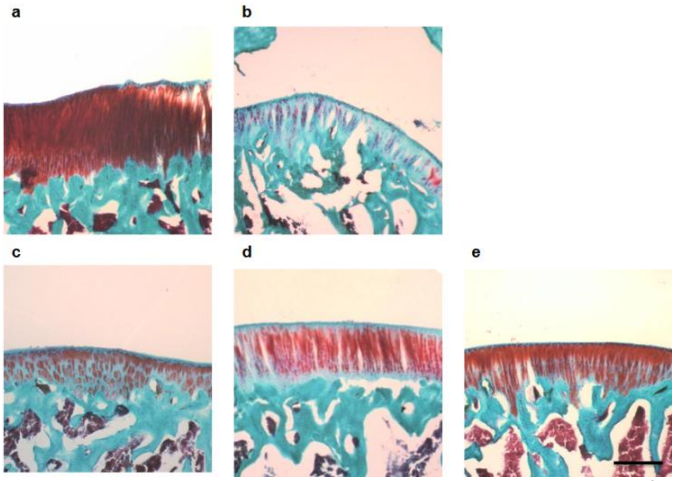


Figure 3. Chondrogenically differentiated-hASCs by compound **6**. Compound **6** promotes cartilage repair in collagenase II-induced osteoarthritis models in rats. a) Naïve rat knee joints; b) 6 weeks after intra-articular injection of collagenase II on day 1 and day 4; c) 4 x 10^5 hASCs; d) 4 x 10^5 cells from chondrogenically differentiated hASCs; e) 10 μM compound **6** on days 7 and 21. Scale bars=250 μm.

Stem cell-based approaches for the treatment of OA have focused on cellular regenerative properties, including the proliferation and differentiation of chondrocytes. However, these studies exhibit some limitations, such as poor cell survival and regeneration potential.[23] Furthermore, the major chondrogenic agent TGF-β can cause nonspecific differentiation and undesired phenotype changes, such as hypertrophic maturation and osteophyte formation, which leads to the defunctionalization of joints and is unsuitable for cartilage regeneration.[24-26] To overcome these limitations, in the current study, we focused on compound **6** with a sulfonamide moiety as a therapeutic reagent that has chondrogenic activity and confirmed that it restores articular cartilage in an OA rat model. Although many signaling pathways are related to chondrogenesis[27] and there is controversy regarding whether the MEK/ERK signaling cascade is necessary for chondrogenic differentiation,[28] we confirmed that the chondrogenic differentiation mechanism of compound **6** is associated with an increase in the ERK phosphorylation state. Certainly, undifferentiated hASCs can partially repair cartilage degradation; however, we observed that chondrogenically differentiated hASCs by compound **6** could be more easily appropriated for cartilage regeneration. Perhaps the chondrogenically differentiated states of hASCs have characteristics that are more similar to those of naïve chondrocytes, such as active cellular signaling molecules and biological properties.[29,30] Future studies are still necessary to elucidate the in vivo mechanism of action of compound 6-induced chondrogenically differentiated hASCs on impeding cartilage degradation. However, based on the overall in vitro and in vivo chondrogenic capacity and protective efficacy of cartilage damage by compound **6**, we suggest that compound **6** is a new potential disease-modifying OA drug candidate.[31] Moreover, in this study, we provide new insights and strategies in regenerative medicine and novel therapeutic methods for the treatment of degenerative diseases, including cartilage defects.

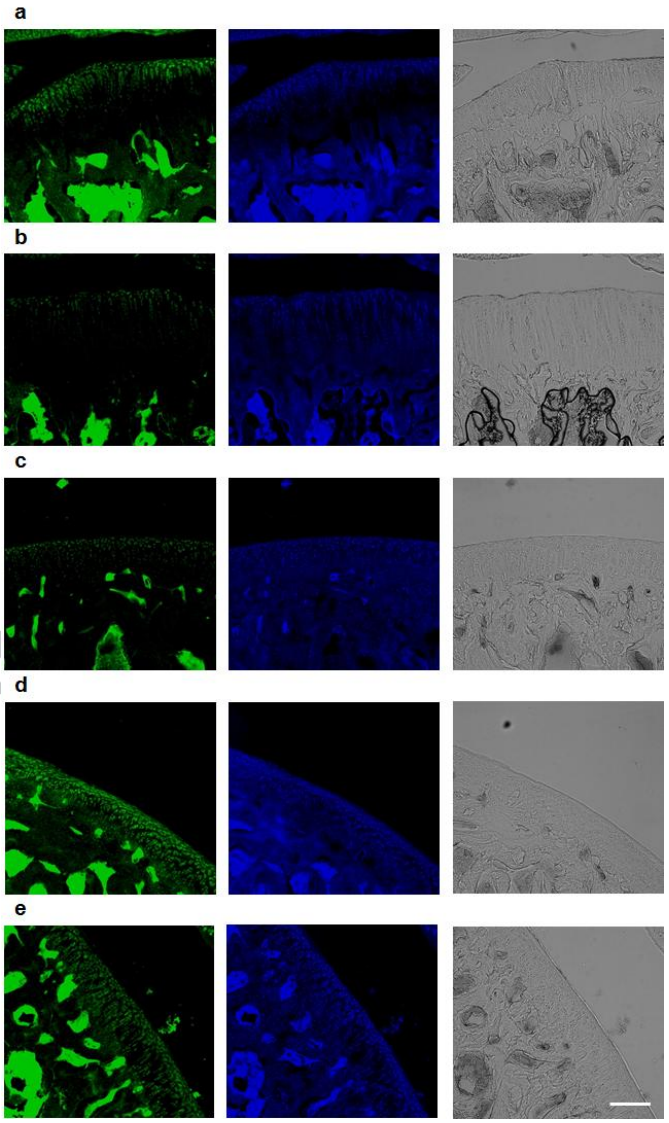


Figure 4. Articular cartilage of knees treated with chondrogenically differentiated hASCs by compound **6** or compound **6** alone exhibited increased expression of aggrecan relative to the OA group. a) Naïve rat knee joints; b) 6 weeks after intra-articular injection of collagenase II on day 1 and day 4; c) 4 x 10^5 cells of hASCs; d) 4 x 10^5 cells of chondrogenically differentiated hASCs with compound **6**; e) 10 μM compound **6** on days 7 and 21. Aggrecan: green; Cell nuclei: DAPI, blue. Scale bar=200 μm.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/

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