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A Small Molecule with Osteogenesis-Inducing Activity in Multipotent Mesenchymal Progenitor Cells

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Stem cells are self-renewing and multipotent cells which can differentiate into various cell types. Mesenchymal stem cells (MSC) are capable of differentiating into the mesenchymal cell lineages, such as bone, cartilage, adipose, muscle, stroma, and tendon, and play important roles in repair and regeneration.[1] Misregulation of the differentiation of mesenchymal precursor cells into osteoblasts may account for several bone-related diseases.[2] Potent and specific small-molecule modulators of stem cell differentiation could provide useful chemical tools to study the molecular mechanisms of stem cell differentiation and ultimately might lead to useful new therapeutics for the treatment of disease. Here we report the identification of a 2,6,9-trisubstituted purine, purmorphamine **1**, which can differentiate multipotent mesenchymal progenitor cells into an osteoblast lineage.

Mouse embryonic mesoderm fibroblast C3H10T1/2 cells were used for this study. C3H10T1/2 cells, like MSCs, are multipotent mesenchymal progenitor cells which can differentiate into various mesenchymal cells and have been widely used as a model system for studies of osteoblast differentiation.[3,4] Upon treatment with bone morphorgenetic protein 4 (BMP-4), C3H10T1/2 cells differentiate into osteoblasts.[5] During differentiation, osteogenesis transcription factor Cbfa1/Runx2 and several osteoblast specific genes, such as alkaline phosphatase (ALP), collagen type I, osteocalcin, and osteopontin are highly expressed.[2]

To screen small-molecule libraries for compounds that might induce osteogenesis of C3H10T1/2 cells, we assayed the expression of the osteogenesis marker gene ALP, whose expression is correlated with osteogenesis.[1] ALP hydrolyzes inorganic pyrophosphates to phosphates and promotes the formation of hydroxyapatite crystals in bone matrix. Deactivating mutations of ALP cause osteomalacia, characterized by poorly mineralized bones and frequent bone factures, indicating that ALP plays an essential role in bone formation.[6] ALP is also a highly active and stable enzyme, making direct assays of its enzymatic activity feasible. The ALP substrate 2′-[2′-benzothiazoyl]-6′-hydroxybenzothiazole phosphate (BBTP), upon hydrolysis, affords the highly fluorescent-active product BBT anion,[7] providing a highly sensitive and reliable assay to measure ALP expression level in cells (see Supporting Information for details).

Recently, we reported the "convergent" synthesis of a heterocycle combinatorial library consisting of roughly 50 000 compounds with purine, pyrimidine, quinazoline, pyrazine, phthalazine, pyrazine, and quinoxaline-based scaffolds.[8] Since these and structurally related compounds have been shown to have diverse biological functions,[9−11] we chose to screen this heterocycle library for small molecules with osteogenesis-inducing activity.[12] One 2,6,9-trisubstituted purine was found to have significant activity in the ALP enzymatic assay. This compound, which has morpholinoaniline substitution at the C6 position of purine nucleus, was named purmorphamine **1**. The morphology of cells treated with purmorphamine changes from fibroblast (long and spindle-shaped) to osteocyte (small and round, see Supporting Information). Further studies indicated that the EC_{50} (based on ALP expression) for purmorphamine is 1 μM in C3H10T1/2 cells; moreover, this compound can lead to greater than a 50-fold increase in ALP after treatment for 4 days as compared with 1% DMSO treatment. (Figure 1). Purmorphamine shows minor cytotoxicity in C3H10T1/2 cells at concentrations higher than 20 μM.

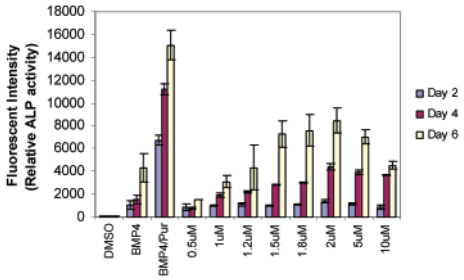
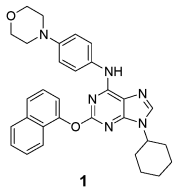


Figure 1. Purmorphamine is a potent inducer of osteogenesis in multipotent C3H10T1/2 cells. C3H10T1/2 cells were treated with DMSO (control), BMP-4 (300 ng/mL), BMP-4 (100 ng/mL) and purmorphamine (1 μM), and various concentrations of purmorphamine alone, and the ALP activities were measured.



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To confirm that C3H10T1/2 cells are induced to differentiate into osteoblasts upon treatment with purmorphamine, a reporter gene assay for bone-specific transcription factor Cbfa1/Runx2 was carried out.[13] Cbfa1/Runx2 plays an essential role in osteoblast differentiation[14]—mice lacking the Cbfa1/Runx2 gene die shortly after birth due to lack of bone formation.[15] Treatment of C3H10T1/2 cells with 10 μM purmorphamine for 4 days led to a more than 6-fold increase in the reporter activity in transient transfection experiments (Figure 2), indicating the Cbfa1/Runx2 gene is up-regulated. This result is consistent with the differentiation of C3H10T1/2 cells into an osteoblast lineage.

Histochemical staining of endogenous ALP indicated that more than 80% of the cells expressed ALP after purmorphamine treatment

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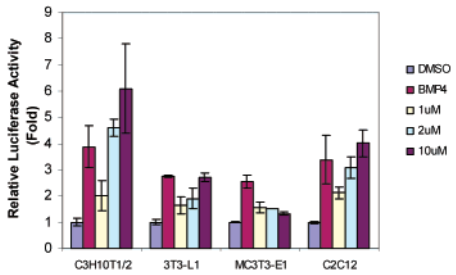

Figure 2. Luciferase reporter gene assay shows the induction of Cbfa1/Runx2 gene by DMSO (control), BMP-4 (300 ng/mL) and purmorphamine in various mesenchymal cells.

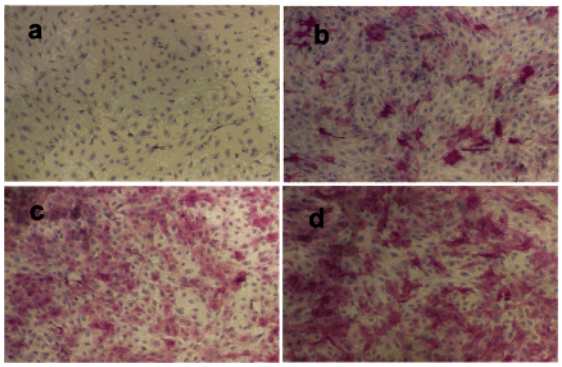

Figure 3. Histochemical staining of ALP expression in C3H10T1/2 cells. C3H10T1/2 cells were treated with DMSO (a), 300 ng/mL of BMP-4 (b), 2 μM of purmorphamine (c), and 100 ng/mL of BMP and 1 μM of purmorphamine (d) for 4 days and stained for ALP activity. ALP-positive cells were stained red and the cell nuclei were stained blue.

(2 μM for 4 days), while only 40% of BMP-4 treated cells stained positive for ALP[16] (Figure 3). Interestingly, although fewer cells were induced by BMP-4, those induced cells had a higher expression level of ALP compared to that in cells treated with purmorphamine. Our observation suggested that purmorphamine induced the majority of the cell population to commit into an osteoblast linage. Purmorphamine also showed a synergistic effect with BMP-4 in inducing the differentiation of C3H10T1/2 cells. When cells were treated with both BMP-4 and purmorphamine, the induction of ALP activity was approximately 3-fold greater than the simple additive effect of the individual molecules, suggesting that purmorphamine does not act as a BMP-4 analogue.

Mouse MC3T3-E1 cells are progenitor cells committed to an osteoblast lineage. Both BMP-4 and purmorphamine promote the terminal differentiation of MC3T3-E1 cells. Since MC3T3-E1 cells already have high levels of endogenous Cbfa1/Runx2,[17] slight up-regulation of this gene may be sufficient to promote terminal differentiation.[18] Indeed, the increased change of Cbfa1/Runx2 reporter activity in stably transfected MC3T3-E1 cells is not dramatic when treated with purmorphamine. Interestingly, 3T3-L1 preadipocyte cells, which are progenitor cells committed to an adipocyte lineage, can be transdifferentiated into an osteoblast lineage when treated with purmorphamine and BMP-4. Purmorphamine induces Cbfa1/Runx2 expression in preadipocyte cells (Figure 2) and increases the ALP level 9-fold at an optimal concentration of 5 μM, while BMP-4 induces ALP expression more than 40-fold. Purmorphamine (1 μM) and BMP-4 (100 ng/mL) together increase ALP activity more than 90-fold in 3T3-L1 cells (see Supporting Information). Similarly, C2C12 cells, which are progenitor cells committed to a skeletal muscle lineage, can be induced by purmorphamine to express the Cbfa1/Runx2 gene (Figure 2). However, treatment with purmorphamine did not inhibit myotube formation in C2C12 cells (see Supporting Information). This observation is consistent with a previous report that transient up-regulation of Cbfa1/Runx2 is required, but not sufficient, to transdifferentiate C2C12 cells into osteoblasts,[19] while in multipotent mesenchymal cells (C3H10T1/2), up-regulation of Cbfa1/Runx2 is sufficient for osteogenesis.[14]

In summary, we have discovered the 2,6,9-trisubstituted purine, purmorphamine **1**, which can induce osteoblast differentiation of multipotent mesenchymal progenitor cells and lineage-committed preosteoblasts. The synergistic effects of purmorphamine and BMP-4 can also induce the transdifferentiation of preadipocytes and myoblasts. We are currently attempting to identify the molecular basis for the activity of this novel molecule using both affinity-based and genomics tools in an effort to reveal new insights into the mechanisms that control cellular differentiation.

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Supporting Information Available: Detailed experimental procedures, compound characterization, and additional figures (PDF). This material is available free of charge via the Internet at http://pub.acs.org.

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(12) Generally, 2500 cells were plated in each well of a 384-well plate with 100 μL of medium, and 500 nL of the 1 mM compound solutions were added. After incubating for 4 days, cells were lysed, and ALP activities were assayed (see Supporting Information for details).

(13) The promoter region of Cbfa1/Runx2 gene (1.8 kb) was cloned and inserted directly upstream of the luciferase gene. Cells were transfected with reporter plamid and plated in 96-well plates. After incubating for 4 days, luciferase activity was assayed using Bright-Glo Luciferase reagent (see Supporting Information for details).

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(16) Cells were fixed with 10% formalin solution and stained with an ALP staining kit from Sigma Diagnostics (see Supporting Information for details).

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