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Purmorphamine Induces Osteogenesis by Activation of the Hedgehog Signaling Pathway

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Summary

Previously, a small molecule, purmorphamine, was identified that selectively induces osteogenesis in multipotent mesenchymal progenitor cells. In order to gain insights into the mechanism of action of purmorphamine, high-density oligonucleotide microarrays were used to profile gene expression in multipotent mesenchymal progenitor cells treated with either purmorphamine or bone morphogenetic protein-4 (BMP-4). In contrast to BMP-4 treatment, purmorphamine activates the Hedgehog (Hh) signaling pathway, resulting in the up- and downregulation of its downstream target genes, including *Gli1* and *Patched*. Moreover, the known Hh signaling antagonists, cyclopamine and forskolin, completely block the osteogenesis and Glimediated transcription induced by purmorphamine. These results demonstrate that purmorphamine is a small molecule agonist of Hedgehog signaling, and it may ultimately be useful in the treatment of bone-related disease and neurodegenerative disease.

Introduction

Mesenchymal stem cells (MSC) are capable of differentiating into a number of cell lineages, including bone, cartilage, adipose, muscle, stroma, and tendon [1]. Among them, bone is a highly specialized connective tissue that undergoes constant remodeling by osteoblasts and osteoclasts, which are responsible for bone formation and resorption, respectively. Misregulation of the differentiation of mesenchymal precursor cells into osteoblasts is involved in several bone-related diseases, including osteoporosis, which affects an estimated 44 million Americans [2]. Identification of small molecules that selectively induce osteoblast differentiation from MSCs would provide useful chemical tools to study the molecular mechanisms of osteoblast differentiation and ultimately might lead to useful therapeutics agents for the treatment of bone diseases.

Previously, by screening a combinatorial chemical library of more than 100,000 compounds, the small molecule purmorphamine was discovered, which can induce osteogenesis in mouse mesenchymal progenitor cells (C3H10T1/2). C3H10T1/2 cells are multipotent mesenchymal progenitor cells that have the potential to differentiate into adipocytes, osteoblasts, chondrocytes, and myocytes, and have been widely used as a model system to study the differentiation of osteoblasts. Purmorphamine induces alkaline phosphatase (ALP) and Cbfa1/Runx2 gene expression in C3H10T1/2 cells with an EC50 of 1 μM [3]. It also induces osteogenesis in pre-osteoblasts (MC3T3-E1), and when combined with BMP-4, can trans-differentiate pre-adipocytes (3T3-L1) and myoblasts (C2C12) into osteoblasts [3]. Figure 1 shows the effects of purmorphamine on induction of the expression of osteogenesis markers in C3H10T1/2 cells.

Several signaling pathways, including bone morphogenetic protein (BMP), Wnt, and Hedgehog signaling, are known to be involved in osteoblast differentiation. Bone morphogenetic proteins belong to the transforming growth factor-β (TGF-β) superfamily. BMP induces osteogenesis by binding to its transmembrane receptors and activating Smad proteins, which translocate into the nucleus and mediate the expression of downstream target genes [4] that promote the differentiation of osteoblasts and chondrocytes. When C3H10T1/2 cells are treated with BMP, the majority of cells differentiate into osteoblasts. However, a significant number of cells also differentiate into adipocytes [5], indicating that BMP-induced differentiation is not lineage specific. In addition to BMP, canonical Wnt/β-catenin signaling is also involved in osteogenesis. Several Wnt proteins, including Wnt3a, induce expression of the osteoblast marker, alkaline phosphatase (ALP) in C3H10T1/2 cells [6]. Loss of function in the Wnt signaling coreceptor LRP5 leads to osteoporosis, and a gain-of-function mutation in this receptor results in a high bone mass phenotype in mice [7]. Hedgehog proteins, like Wnt proteins, are important signaling molecules which play critical roles in the control of pattern formation and cellular proliferation/differentiation [8]. In vitro, Sonic Hedgehog (Shh) induces ALP expression [9, 10], and also increases selectivity in the differentiation of multipotent mesenchymal cells into the osteoblast lineage [11]. Thus, osteogenesis is a complex process that can likely be modulated by small molecules acting on a number of signaling pathways.

High density oligonucleotide microarrays are a useful tool for analyzing the transcriptional effects of small molecules [12, 13]. To gain insights into the cellular response associated with purmorphamine treatment, we carried out transcriptional analysis of C3H10T1/2 cells after treatment with purmorphamine and bone morphogenetic protein 4 (BMP-4). Clustering analysis revealed that the gene expression pattern arising from purmorphamine treatment differs significantly from that of BMP-4 treatment. A cluster of genes was found to be uniquely upregulated by purmorphamine treatment; pathway analysis of genes in this cluster revealed that Hedgehog signaling is activated by purmorphamine treatment. These results were confirmed by demonstra-

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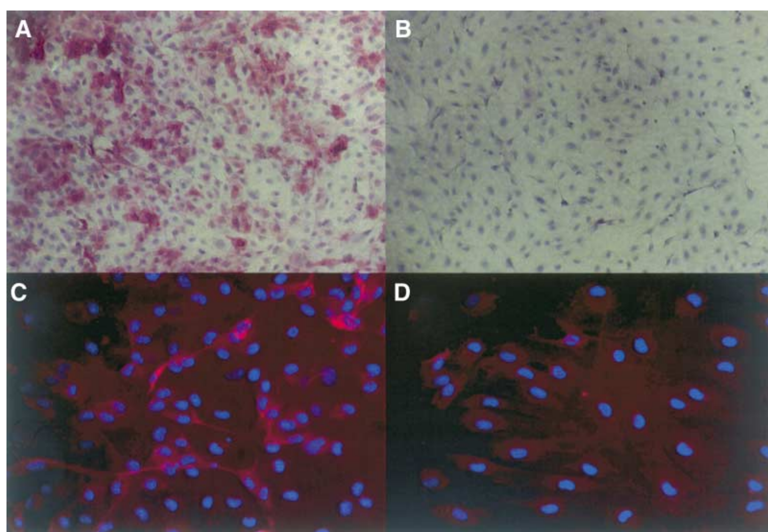


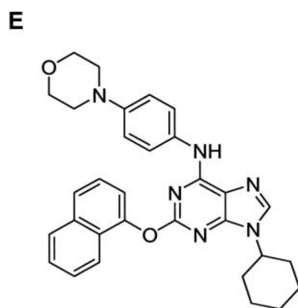


Figure 1. Purmorphamine Induces Osteogenesis in Pluripotent Mesenchymal Progenitor Cells

C3H10T1/2 cells were treated with 2 μM of purmorphamine (A and C) or 0.5% DMSO control (B and D) for 4 days and stained for alkaline phosphatase (A and B) and osteopontin (Red, [C] and [D]). The chemical structure of purmorphamine is shown in (E).

ting that antagonists of Hedgehog signaling block the activity of purmorphamine.

Results and Discussion

Transcriptional Analysis of Purmorphamine and BMP-4 in Pluripotent Mesenchymal Progenitor Cells

C3H10T1/2 cells were treated independently with 2 μM purmorphamine, 100 ng/mL of BMP-4, and 1% DMSO (control) for either 12 hr, 24 hr, 2 days, 4 days, or 6 days. mRNAs were isolated and biotinylated cRNAs were prepared, fragmented, and subsequently hybridized to Affymetrix mouse genome U74Av2 GeneChips, which represent all genes (~6000) in the mouse UniGene Database that have been annotated, as well as ~6000 expressed sequence tag (EST) clusters. Each RNA sample was hybridized onto duplicate sets of microarrays. For each gene and EST, the difference in expression level between purmorphamine, BMP-4-treated cells and control cells were calculated. Genes and ESTs with changes in expression levels higher than 2-fold (up- or downregulation) were analyzed.

Out of the ~12000 genes and ESTs represented on the microarray, only a very small percentage of genes (0.5% to 2%) were differentially expressed. At early time points (24 hr), purmorphamine treatment led to the upregulation of cell cycle control genes, including *Cyclin B2* (6-fold), *Cyclin D1* (2.2-fold), *Cyclin B1* (2.9-fold), *Cyclin A2* (3.5-fold), and *CDK2* (2.3-fold). Genes responsible for DNA synthesis, including *ribonucleotide reductase M2* (3.7-fold) and *M1* subunits (3.2-fold), were also upregulated. These data indicate that purmorphamine treatment stimulates cellular proliferation at early stages, and is consistent with our observation that purmorphamine-treated cells proliferate faster and have a higher cell density after 2 days relative to control cells. Once confluent, cells stop proliferating and differentiate into osteoblasts. We found that after 2 days of treatment with purmorphamine, multiple osteogenesis genes were upregulated, including the essential osteogenesis transcription factor *Cbfa1/Runx2* (2.0-fold); the *core binding protein* β (*Cbf*-β, 2.4-fold), a required cofactor for Cbfa1/Runx2-mediated skeletal development [14]; and other osteoblast marker genes including *alkaline phosphatase* (3.6-fold) and *osteomodulin* (2.2-fold) [15]. Purmorphamine treatment also abolishes spontaneous adipogenesis of C3H10T1/2 cells. Consistent with the observation, genes involved in adipogenesis, including *apolipoprotein E* (−29-fold), *adiponectin/Acrp30* (−6.7-fold), *lipoprotein lipase* (−3.4-fold) and *adipose differentiation related protein* (*Adrp*, −3.1-fold) were downregulated after 24 hr of purmorphamine treatment [16–19]. *CCAAT/enhancer binding protein*-β (*CEBP*-β) and *peroxisome proliferator-activated receptor*-γ (*PPAR*-γ), transcription factors involved in adipogenesis, were also downregulated 3-fold

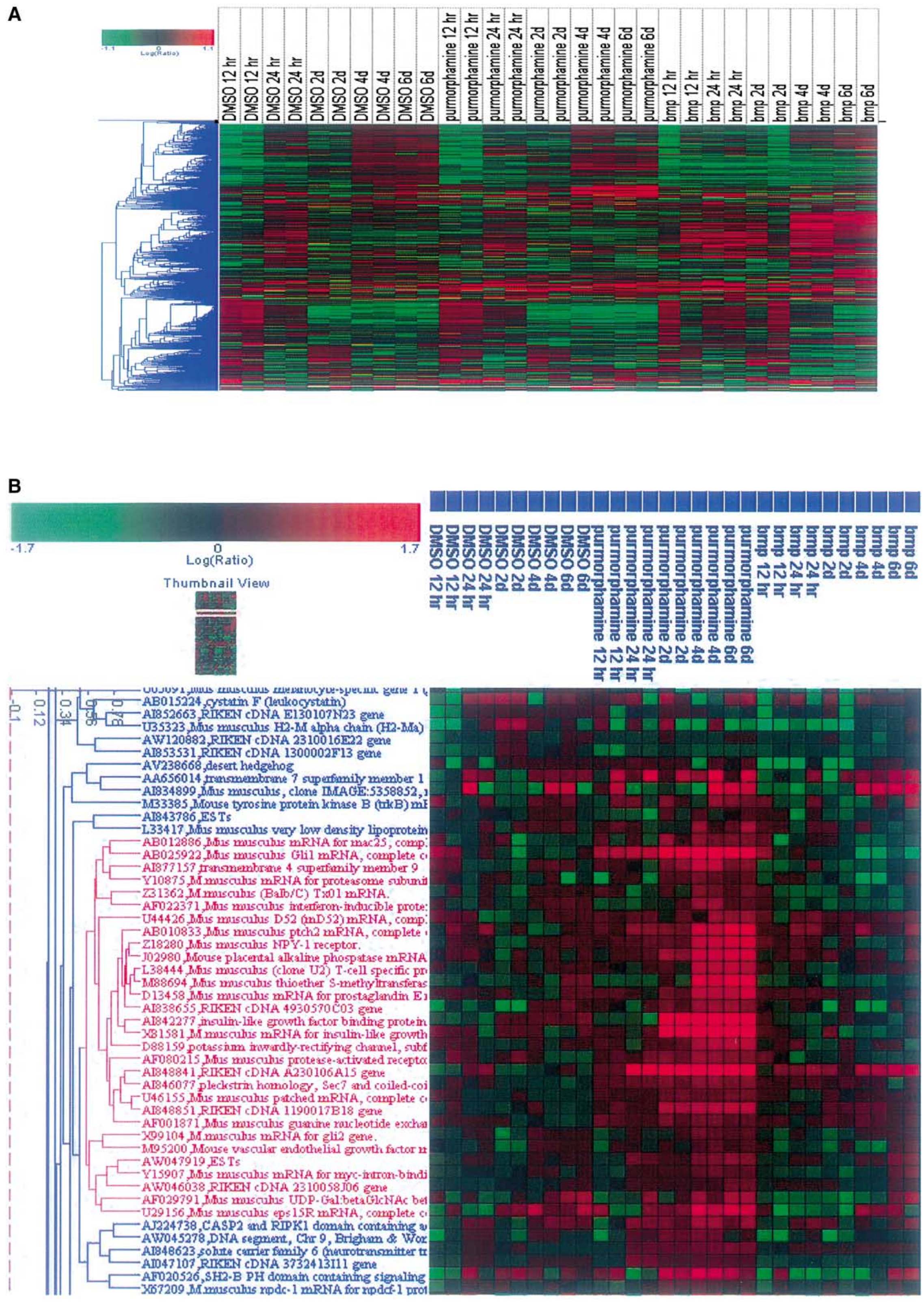


Figure 2. Clustering Analysis of Microarray data

Global clustering analysis of expression data after the treatment of DMSO (control), Purmorphamine, and BMP-4 (A) and 29 genes which are upregulated uniquely by purmorphamine are displayed in a cluster (B).

Table 1. List of 29 Genes which Are Upregulated by Purmorphamine Treatment as Identified from Clustering Analysis

Probe Set	Gene/Description	Fold Change (at 4 Days)
94191_at	Mus musculus protease-activated receptor 4 (Par4) mRNA, complete cds.	2.0
103520_at	Mouse vascular endothelial growth factor mRNA, complete cds.	2.0
95627_at	RIKEN cDNA 2310058J06 gene	2.0
160249_at	Mus musculus D52 (mD52) mRNA, complete cds.	2.0
98497_at	Mus musculus eps15R mRNA, complete cds.	2.2
100395_at	M. musculus mRNA for gli2 gene	2.3
160527_at	Mus musculus mRNA for mac25, complete cds.	2.3
92484_at	Mus musculus mRNA for myc-intron-binding protein-1	2.9
101486_at	M. musculus mRNA for proteasome subunit MECL-1	2.9
94504_at	RIKEN cDNA 4930570C03 gene	3.3
104031_at	Mus musculus patched mRNA, complete cds.	3.3
93451_at	ESTs	3.3
103433_at	pleckstrin homology, Sec7 and coiled-coil domains 3	3.8
103434_at	Mus musculus guanine nucleotide exchange factor and integrin binding protein homolog GRP1 mRNA, complete cds.	3.8
102780_at	M. musculus (Balb/C) Tx01 mRNA	4.1
92426_at	transmembrane 4 superfamily member 9	5.2
93321_at	Mus musculus interferon-inducible protein 203 (Ifi203) mRNA, complete cds.	5.4
93007_at	Mus musculus NPY-1 receptor	5.4
102849_at	potassium inwardly-rectifying channel, subfamily J, member 8	5.6
95418_at	RIKEN cDNA 1190017B18 gene	5.7
103362_at	Mus musculus mRNA for prostaglandin E receptor EP2 subtype, complete cds.	5.8
96557_at	Mus musculus ptch2 mRNA, complete cds.	5.9
97402_at	Mus musculus thioether S-methyltransferase mRNA, complete cds.	6.0
92796_at	Mouse placental alkaline phospatase mRNA, complete cds.	6.1
94097_at	Mus musculus Gli1 mRNA, complete cds.	6.9
102906_at	Mus musculus (clone U2) T-cell specific protein mRNA, complete cds.	9.4
104030_at	RIKEN cDNA A230106A15 gene	17.7
95082_at	insulin-like growth factor binding protein 3	89.9
95083_at	M.musculus mRNA for insulin-like growth factor binding protein-3	100

and 2.2-fold, respectively. CEBP-β is known to activate the expression of CEBP-α and PPAR-γ, which then transcriptionally activate genes that produce the adipocyte phenotype [20].

BMP-4 treatment also induces cellular proliferation at early stages (24 hr), and like purmorphamine, causes upregulation of genes involved in cell cycle control and DNA synthesis, including *Cyclin B2* (5.8-fold), *Cyclin A2* (5.1-fold), *Cyclin B1* (3.6-fold), and the *ribonucleotide reductase M2* (5.0-fold) and *M1* subunits (5.7-fold). This suggests that initial cell number expansion might be an important component of the differentiation process. Several known target genes of BMP signaling, including *inhibitor of DNA binding-1* (*Id-1*) (2.0-fold) and *Id-3* (2.1-fold), were upregulated after 24 hr of treatment with BMP-4 [21]. In contrast, purmorphamine did not induce these genes' expression, indicating that purmorphamine does not activate BMP signaling, and suggesting that expression of Id genes may not be essential for osteogenesis [22]. After treatment with BMP-4 for 4 days, several adipogenesis genes were upregulated, including *adipose differentiation related protein* (*Adrp*, 3.8-fold), *apolipoprotein E* (2.9-fold), *fatty acid synthase* (2.9-fold), and *PPARγ* (2.4-fold). These data are consistent with previous studies of expression analysis of BMP signaling in mesenchymal cells [22–24], and suggest that BMP is not an osteogenesis-specific inducing factor—cells can differentiate into either osteoblasts or adipocytes when treated with BMPs.

Clustering and Pathway Analysis Reveals that Purmorphamine Activates the Hedgehog Signaling Pathway

The gene expression data suggests that purmorphamine has a distinct biological activity relative to BMP in C3H10T1/2 cells. However, this analysis did not provide enough information to determine which signaling pathways purmorphamine affects. Hierarchical clustering can be used to group genes with similar patterns of expression, and allows systematic analysis of the large amounts of information obtained from genome-wide expression profiles [25, 26]. Therefore, those genes that were induced or repressed by treatment with purmorphamine across all time points were clustered by Z score with an agglomerative algorithm using the Rosetta Resolver software (Figure 2A). Among the ~12,000 genes and ESTs in the MG_U74Av2 array, only one cluster of 29 genes was found to have low expression levels (low Z scores) under control conditions (DMSO treatment), and high expression levels (high Z scores) after treatment with purmorphamine at all time points. The clustering diagram and list of the 29 genes in this cluster are shown in Figure 2B and Table 1. No cluster of significantly downregulated genes was found from this analysis. Among the genes in the upregulated cluster, we found several transcription factors, including *Gli2* (2.3-fold), *Gli1* (6.9-fold), and *c-Myc intron binding protein-1* (2.8-fold) that are upregulated by treatment with purmorphamine, but not by treatment with BMP-4. Similarly, membrane-bound receptors and signal transduction

Figure 3. Purmorphamine Activates the Hedgehog Signaling Pathway

Genes in the biological interaction network related to Hedgehog signaling pathway as displayed using the Ingenuity Pathway Analysis software ([A], see http://www.ingenuity.com for detailed explanations of the symbols). Genes whose expression is increased by purmorphamine are displayed by shaded symbols. The expression levels of *Gli1, Patched (Ptc), Shh, Ihh, Dhh*, and *GAPDH* at different time points after the treatment of 2 μM of purmorphamine (+) or DMSO control (−) are determined by semiquantitative RT-PCR (B).

molecules were also upregulated upon treatment with purmorphamine but not BMP-4, including *Patched 2* (5.9-fold), *Patched* (3.3-fold), and *insulin like growth factor binding protein 3* (*IGFBP-3*, 90-fold).

To determine which biological pathways are affected by purmorphamine, the cluster of 29 upregulated genes was subjected to Ingenuity Pathway Analysis (Ingenuity Systems). Hedgehog signaling pathway is the top scored biological network, suggesting that the osteogenesis activity of purmorphamine might be associated with modulation of this pathway (Figure 3A). Genes involved in Hedgehog signaling that are significantly induced by purmorphamine treatment include *Gli1* (6.9-fold), *Gli2* (2.3-fold), *Patched* (3.3-fold), *Patched 2* (5.9-fold), and *IGF binding protein-3* (90-fold). These same genes were identified in previous microarray expression analysis experiments as downstream targets of Hedgehog signaling [27–30]. Secreted frizzled-related protein-1 (Sfrp1) and Sfrp-2 are known antagonists of Wnt signaling, and are known to be strongly repressed by Hedgehog signaling [28]. Consistent with this observation, treatment with purmorphamine also strongly downregulates expression of *Sfrp-1* (–3.2-fold) and *Sfrp-2* (−15.1-fold) (Table 2). Because Wnt signaling is also known to promote osteogenesis, suppression of the expression of Wnt antagonists by purmorphamine may contribute to its osteogenesis activity. Taken together, the above analysis suggested that purmorphamine may act as a small molecule agonist of the Hedgehog signaling pathway.

Hedgehog signaling plays an important role during

Table 2. Expression Levels of Hedgehog Genes (*Shh*, *Ihh*, and *Dhh*) and Some Known Hedgehog Signaling Downstream Targets

Probe Set	Gene/Description	Fold Change (P Value)				
		12 hr	24 hr	2 days	4 days	6 days
94097_at	Mus musculus Gli1 mRNA,	2.1 (0.018)	2.7 (8.8E-13)	5.5 (1.6E-39)	6.9 (0)	6.9 (0)
100395_at	GLI-Kruppel family member GLI2	Absent (0.5)	1.2 (1)	1.1 (0.9)	2.3 (1.8E-5)	1.2 (0.034)
104031_at	Mus musculus patched mRNA	1.2 (0.7)	1.6 (0.08)	3.1 (1.6E-12)	3.3 (3E-17)	3.9 (1.4E-23)
96557_at	Mus musculus patch2 mRNA	Absent (1)	Absent (0.7)	2.7 (2.7E-6)	5.9 (2.6E-11)	9.4 (3.2E-22)
95082_at	insulin-like growth factor binding protein 3	Absent (0.8)	Absent (0.2)	20.1 (0)	89.9 (0)	86.9 (0)
98623_g_at	M. musculus gene for IGF-II, exon 6.	1.1 (0.1)	1.2 (0.2)	1.2 (0.6)	4.1 (7.4E-14)	18.8 (1.8E-14)
93503_at	Mus musculus secreted frizzled related protein sFRP-2 (Sfrp2) mRNA	−14 (9.5E-13)	−2.1 (0.01)	−13.4 (1.5E-12)	−15.2 (7.5E-11)	−15.7 (4.5E-11)
97997_at	Mus musculus secreted frizzled related protein sFRP-1 (Sfrp1) mRNA	−6.5 (4.4E-4)	−2.6 (0.037)	−4.4 (0.0066)	−2.8 (0.026)	−2.8 (0.028)
101831_at	M. musculus Shh mRNA	1.2 (0.51)	−1.2 (0.58)	−1.1 (0.67)	−1.0 (0.92)	1.1 (0.66)
103949_at	M. musculus Ihh mRNA	−1.0 (0.96)	1.1 (0.86)	−1.1 (0.69)	1.1 (0.72)	1.1 (0.82)
99861_at	M. musculus Dhh gene	−1.1 (0.47)	1 (0.98)	1.1 (0.69)	−1.1 (0.69)	1.0 (0.8)

Fold changes and P values of differential expression compared with DMSO controls are shown. Positive values show upregulation and negative values show downregulation.

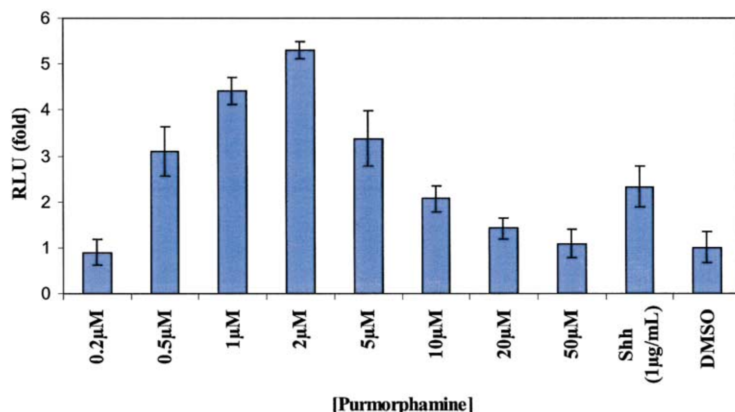
the development of a wide range of tissues and organs, including lung, bone, CNS, limb, and hair follicles. In vertebrates, the hedgehog family consists of three secreted proteins: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh) [8]. Response to the Hedgehog (Hh) signal is controlled by two transmembrane proteins, Patched (Ptc) and Smoothened (Smo). In the resting state, Smo activity is suppressed by Ptc; Hh stimulation releases the inhibitory effect of Ptc and subsequently activates Smo. Activated Smo then activates downstream signaling molecules, leading to the activation of the Gli family of transcription factors, including Gli1, Gli2, and Gli3. The transmembrane receptor Ptc and the transcription factor Gli1 are also direct downstream targets of Hedgehog signaling, leading to both negative and positive feedback regulation [8].

Recently, members of the hedgehog gene family have also been shown to regulate the development of vertebrate skeleton [31–34]. Ihh was first identified as a regulator of chondrocyte differentiation, and has also been shown to play an important role in osteoblast differentiation [31]. Mice lacking Shh cannot form vertebrae and display severe defects of distal limb skeletal elements [35]. Shh protein can also induce the expression of alkaline phosphatase (ALP) in multipotent mesenchymal cells and pre-osteoblast cells and inhibit adipogenesis in vitro [11]. Therefore, it is reasonable that the osteogenesis effects of purmorphamine result from the activation of Hedgehog signaling.

Purmorphamine Is a Small Molecule Agonist of Hedgehog Signaling

To confirm that purmorphamine activates expression of genes in the Hedgehog signaling pathway, semiquantitative RT-PCR experiments were carried out on the genes *Patched (Ptc)*, *Gli1*, and all three Hedgehog genes (*Shh, Ihh*, and *Dhh*). As shown in Figure 3B, the expression levels of *Ptc* and *Gli1* were upregulated significantly as early as 12 hr after treatment with purmorphamine, suggesting that activation of Hedgehog signaling is an early and primary response to purmorphamine. The expression of *Shh* and *Dhh* is not detectable by RT-PCR. *Ihh* is endogenously expressed, but its expression level does not change throughout all time points of purmorphamine treatment, suggesting that the activation of Hedgehog signaling is not the result of the upregulation of the expression of hedgehog proteins. These results are consistent with our microarray data, which showed that the P values of differential expression of all three Hedgehog genes are high. Therefore, the probability of differential expression of these genes (compared with controls) is very low (Table 2). To further demonstrate that purmorphamine activates Hedgehog signaling, a Hedgehog signaling reporter assay was carried out. A luciferase reporter plasmid containing multiple Gli binding sites and a minimal promoter upstream of a firefly luciferase gene was transfected into C3H10T1/2 cells [36–39]. As a positive control, addition of Sonic Hedgehog protein (expressed in *E. coli*, 1 μg/mL) resulted in a 2- to 3-fold increase in luciferase activity. Luciferase reporter activity increased up to 6-fold when cells were treated with 2 μM of purmorphamine (Figure 4A). The EC50 of purmorphamine in this assay is 0.5 μM. These results are consistent with the notion that purmorphamine acts as a small molecule agonist for Hedgehog signaling [37, 39]. Furthermore, anti-Shh antibody (5E1) cannot inhibit the Gli activity induced by purmorphamine (Figure 4B), suggesting purmorphamine does not activate Hedgehog signaling pathway by inducing the expression of Hedgehog protein.

The effects of two well-known Hedgehog antagonists, cyclopamine and forskolin, on the osteogenesis activity of purmorphamine and BMP-4 were then examined. Cyclopamine is a plant-derived, steroidal alkaloid which



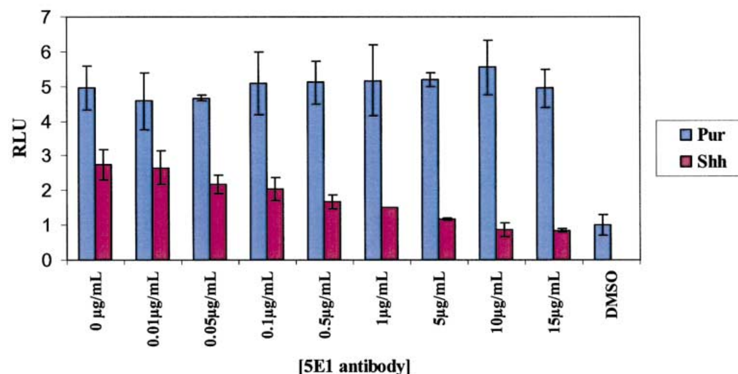


Figure 4. Purmorphamine Could Activate Hedgehog Signaling Independent of Hedgehog Ligand

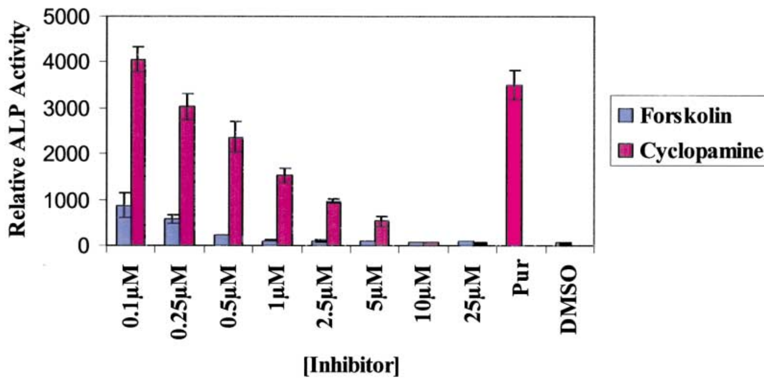
Activation of Gli-mediated transcription by purmorphamine (A) and anti-Shh antibody (5E1) has no inhibitory effect on Gli activity induced by 2 μM of purmorphamine (Pur), but inhibits the activity of recombinant Shh protein (1 μg/mL) (B).

blocks Hedgehog signaling by inhibiting the membrane protein Smoothened [39–41]. Forskolin is an adenylate cylase/protein kinase A (PKA) activator [42], which blocks Hedgehog (Hh) signaling by stimulating the degradation of members of the Gli family of transcriptional activators [43]. It has been reported that forskolin has synergistic effects on BMP-induced osteogenesis [44].

As shown in Figure 5A, after 4 days, ALP activity of purmorphamine (2 μM) treated cells is 70-fold higher than the control (0.5% DMSO-treated cells). The osteogenesis activity of purmorphamine (2 μM) is inhibited by cyclopamine with an IC_{50} of 0.8 μM, consistent with the notion that purmorphamine activates Hedgehog signaling. At concentrations of cyclopamine greater than 10 μM, osteogenesis is completely blocked. Forskolin also has a very strong inhibitory effect on the osteogenesis activity of purmorphamine. Treatment with 100 nM forskolin inhibits the ALP activity induced by purmorphamine (2 μM) by more than 70%; at concentrations greater than 1 μM, forskolin completely blocks ALP expression. By contrast, forskolin acts synergistically with BMPs−10 μM forskolin enhances ALP expression induced by BMP by 5-fold [44]. This result further confirms that purmorphamine induces osteogenesis primarily by activating Hedgehog (Hh) signaling, and functions by a different mechanism than BMP. The effects of cyclopamine and forskolin on the activation of Hedgehog signaling by purmorphamine were confirmed with the Hedgehog signaling reporter assay described above. As shown in Figure 5B, cyclopamine and forskolin can both block the transcriptional activity of Gli induced by 2 μM of purmorphamine, with IC_{50} values of 2.5 μM and 5 μM, respectively. At concentrations higher than 10 μM, these inhibitors completely block the transcriptional activity of Gli. Cyclopamine blocks Hedgehog signaling at the level of Smoothened, suggesting that purmorphamine activates either Smoothened or a protein upstream of it in the Hedgehog signaling pathway.

Significance

Understanding the molecular mechanisms by which small molecules affect cellular activity remains a challenge. Genome-wide expression analysis can provide insights into which biological pathways are affected by small molecules. In this study, expression analysis by microarrays suggested that purmorphamine acts as a Hedgehog signaling agonist. This was confirmed in cellular reporter assays and by using known antagonists of the Hedgehog signaling pathway. Because Hedgehog signaling is known to play an important role in the development of many tissue and organs [8, 45–49], activation of this pathway may have therapeutic value. Indeed, recent studies have demonstrated



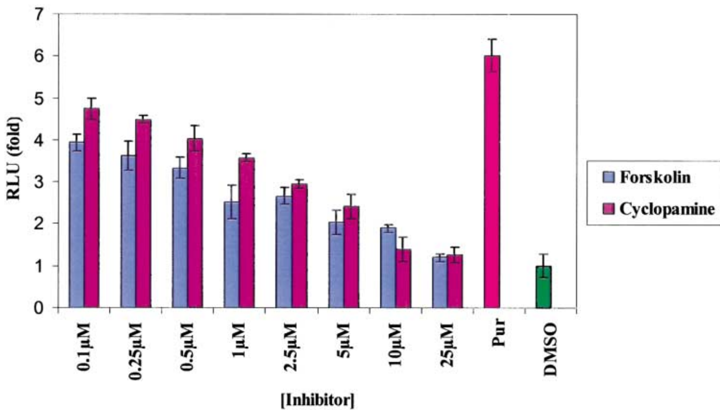


Figure 5. Hedgehog Antagonists Could Inhibit Purmorphamine Function

Hedgehog (Hh) signaling antagonists, cyclopamine and forskolin, inhibit osteogenesis (A) and Gli-mediated transcription (B) induced by 2 μM purmorphamine (Pur).

positive effects of Hedgehog proteins in the treatment of Parkinson's disease and peripheral nerve damage [50, 51]. Therefore, small molecules which modulate Hedgehog signaling might be useful in the development of new medicines for the treatment of diseases, such as osteoporosis and neurodegenerative disease [37, 39].

Experimental Procedures

Cell Culture

Mouse embryonic mesoderm fibroblasts C3H10T1/2 cells (obtained from American Type Culture Collection, ATCC, Manassas, VA) were cultured in MEM-α medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Gibco/Invitrogen, Carlsbad, CA), 50 units/mL penicillin and 50 μg/mL of streptomycin (Gibco/Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2 in air atmosphere. To induce differentiation, cells were plated in 10 cm dishes at a density of 10^6 cells in 10 ml of culture medium (80% confluence). After 12 hr, 2 μM of purmorphamine (dissolved in DMSO to make 10 mM solution) and 100 ng/mL of BMP-4 or 0.02% DMSO was added into culture media.

Histochemical and Immunofluorescent Staining

Cells were fixed with 10% formalin solution (Sigma) for 15 min in 96-well plate. Fixed cells were washed with PBS (200 μL, 3 times) and stained with the Alkaline Phosphatase Staining Kit 86R (Sigma Diagnostics) following the manufacturer's protocol. Immunostains were performed in PBS (Gibco) with 0.3% Triton X-100 and 6% horse serum. Primary goat anti-osteopontin antibody was used at a 1:250 dilution (Santa Cruz Biotechnologies). Secondary antibody was Cy3-conjugated anti-goat antibody (Jackson ImmunoResearch, 1:500). Cell nuclei were stained with DAPI (Roche). Images were recorded on a Nikon Eclipse TE300 microscope with 200× magnification. Double- or triple-labeled images were assembled in Metamorph.

Total RNA Extraction, cDNA Preparation, and GeneChip Hybridization

Total RNA from different samples was isolated and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA). Double-stranded cDNA was synthesized from 5 μg of total RNA with the SuperScript Choice System (Invitrogen, Carlsbad, CA) and T7-$(dT)_{24}$ primer (Geneset, La Jolla, CA). In vitro transcription was carried out with an Ambion in vitro transcription kit using double-stranded cDNA as the template in the presence of biotinylated UTP and CTP. Biotin-labeled cRNA was purified, fragmented, and hybridized to Murine genome U74Av2 arrays (Affymetrix Incorporated, Santa Clara, CA) according to the protocol suggested by the manufacturer. Chips were scanned, and the hybridization data were acquired using Affymetrix Suite 5.0. Each RNA sample was hybridized onto duplicate sets of microarrays to minimize experimental error.

Microarray Data Analysis

To find transcripts differentially expressed between purmorphamine and control conditions, samples across all time points were clustered by Z score with an agglomerative algorithm using the Rosetta

Resolver software (Kirkland, WA). This algorithm used the following parameters: average link, Pearson correlation, and individual probes were error weighted. A Euclidean distance metric was used to cluster all arrays. Sequences were used if their detection p-values were less than 0.01, and they were scored as "present" for at least 2 arrays across the entire dataset, and their coefficients of variation across the set were at least 0.5.

For pathway analysis, 29 sequences that represented transcripts increased in expression in the purmorphamine samples relative to DMSO, but not the BMP samples, were imported into the Ingenuity Pathway Analysis (Ingenuity Systems, Mountain View, CA) software. The magnitude of expression change between all DMSO conditions and purmorphamine at 4 days, for the 29 input genes, was examined by ratio ANOVA in the Rosetta Resolver software package (Table 1). Resolver ANOVA analysis is similar to standard ANOVA but instead uses two inputs, expression measurement quantity and estimated error of measurement quantity. This additional input provides more reliable variance measurements, when the number of replicates is small [52]. This error estimate also brings extra degrees of freedom to the analysis, allowing for fewer false positives and false negatives (see http://www.rosettabio.com/publications/default.htm for additional references).

Semiquantitative RT-PCR

Semiquantitative RT-PCR was used to quantify the expression levels of *Patched*, *Gli1*, and the Hedgehog genes (*Shh*, *Ihh*, and *Dhh*) in purmorphamine-treated cells. After extraction, total RNA was transcribed into single-stranded cDNA and then amplified by PCR using Titan One Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN). *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as an endogenous amplification standard. The amount of total RNAs and PCR conditions were optimized so that amplification of both *GAPDH* and the cDNAs of interest were in the exponential phase. The primers used in PCRs are as follows: *Patched* (*Ptc*) 5′-GTCT CAGGGTAGCTCTCATA-3′ and 5′-GCATTCTGGCCCTAGCAATA-3′; *Gli1* 5′-TGTGGCGAATAGACAGAGGT-3′ and 5′-TGCCAGATATGCT TCAGCCA-3′; *Shh* 5′-GGCAGATATGAAGGGAAGAT-3′ and 5′-ACT GCTCGACCCTCATAGTG-3′; *Ihh* 5′-GTCTCTTGCTAGAAGAGAGC-3′ and 5′-GCCTGCAGGGAAGGTCATGT-3′; *Dhh* 5′-GTGCGCAAGC AACTTGTGCC-3′ and 5′-GAATCCTGTGCGTGGTGGCC-3′; and *GAPDH* 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-GCATGGAC TGTGGTCATGAG-3′.

Alkaline Phosphatase Activity Assay for Osteoblast Differentiation and Gli-Reporter Assay

C3H10T1/2 cells were plated into 96-well plates with a density of 10^4 cells/well. After different drug treatments, the cells were lysed with 20 μl of passive lysis buffer (Promega, Madison, WI) for 10 min and 20 μl alkaline phosphatase substrate solution (1 mM in pH 10 buffer, 2′-[2′-benzothiazoyl]-6′-hydroxybenzothiazole phosphate, commercially named AttoPhos, purchased from Promega, Madison, WI) was then added to each well. After incubating 15 min at room temperature, the plates were read on an Acquest high-throughput plate reader (Molecular Devices, Sunnyvale, CA) following the manufacturer's protocol. Total protein was quantified using a BCA protein assay kit from Pierce. To assay the activation of Gli-mediated transcription, a reporter plasmid with multiple copies of the Gli recognition element and a minimal promoter upstream of the firefly luciferase gene was used. C3H10T1/2 cells in 10 cm dishes were cotransfected with 6 μg of Gli-reporter plasmid and 3 μg of Renilla luciferase control reporter (Promega, Madison, WI) with FuGENE6 (Roche Diagnostics, Indianapolis, IN) following the manufacturer's protocol. After 12 hr, cells were trypsinized and replated into a 96-well plate and treated with purmorphamine; 48 hr later, firefly luciferase and Renilla luciferase activities were assayed with the Dual-Glo Luciferase Assay System (Promega, Madison, WI). The increase of firefly luciferase activity was normalized to Renilla luciferase activity and represents the average of three experiments. Sonic Hedgehog protein (expressed in *E. coli*) was purchased from R&D systems (Minneapolis, MN) or kindly provided by Dr. Ulrich Mueller (The Scripps Research Institute). Cyclopamine was purchased from Biomol (Plymouth Meeting, PA), forskolin was purchased from Sigma (St. Louis, MO), and 5E1 antibody was purchased from the Developmental Hybridoma Bank (Iowa City, IA).

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