Purmorphamine as a Novel Osteoinductive Molecule: A Systematic Review

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Introduction: Small molecules are active substances which are used in bone tissue engineering. They present great characteristics including induction of developmental genes, bioavailability and easy metabolism. Purmorphamine is a small molecule which has been demonstrated to exert osteogenic effects. In the present study we aimed to review present literature regarding the osteogenic effect of purmorphamine. **Materials and Methods:** The MEDLINE (NCBI PubMed and PMC), Google Scholar and Scopus were searched by the following keywords "purmorphamine" AND "osteogenesis" OR "osteogenic differentiation" OR "bone formation". According to PRISMA statement, all in vivo and in vitro studies conducted on osteogenic effect of purmorphamine were included. Search was limited to English-language studies up to February 2020. **Results:** Finally, 16 studies were included and the data were extracted. Data were categorized by the studied cell type, purmorphamine dosage and treatment groups, scaffolds and results. **Conclusion:** It is demonstrated that purmorphamine may be effective in osteogenic differentiation of various cells but this effect may vary by applied dosage and duration.

Keywords: Bone regeneration; Osteogenesis; Purmorphamine

Introduction

Bone tissue engineering is a new merged treatment to promote healing in the defects resulted from trauma, cancers and other diseases (1). The important triad which exists in tissue engineering consists of a group of multipotent progenitor stem cells, scaffolds and signaling molecules (2-4). Several osteogenic growth factors such as bone morphogenic protein (BMPs), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) have been demonstrated to be able to induce differentiation on osteoprogenitor cells (2, 5, 6). However, their clinical applications face some limitations. Not targeting the specific tissue specially for BMP-4 (7, 8), side effects including inflammation, ectopic formation, low quality bone with fatty marrow, unsteady structure, toxicity and high production costs can be mentioned as disadvantages of growth factors (2, 9-11). Small molecules are biologically active substances which have some advantages for application in biological and clinical settings. In fact, small molecules can not only induce the expression of critical developmental genes, but also be easily removed from the culture system (12, 13). They are produced by enzymatic activity in uncoded genome (14) and comparing to proteins, they show reduced risk of host immune reaction (2), can be synthesized simply with lower costs (15) and may exert instant effects due to rapid release and are easily metabolized in the culture medium (12). Moreover, since the small molecules have been considered to have higher bioavailability compared to growth factors, they can be used as a substitute for bone regeneration process (3). 2-(1-Naphthoxy)-6-(morpholinoanilino)-9-cyclohexylpurine, known as purmorphamine (Pur) is a small molecule recognized not only for the ability to increase alkaline phosphatase activity and accelerate the formation of bone-like structures in stem cell-derived osteoblasts, but also for inducing hedgehog pathway (3, 16). Hedgehog (Hh) signaling is known to be important in stem cell growth and development (15, 17). Though some studies demonstrated the purmorphamine's ability to activate Hh signaling, a study indicated an inhibitory effect of purmorphamine in osteoblast differentiation of human multipotent adipose-derived stem cells (14, 15, 18-20).

Due to the shortcoming of protein growth factors, small molecules have become the topic of interest to overcome the challenges associated with current materials. As a hedgehog signaling pathway stimulator, it is assumed that purmorphamine

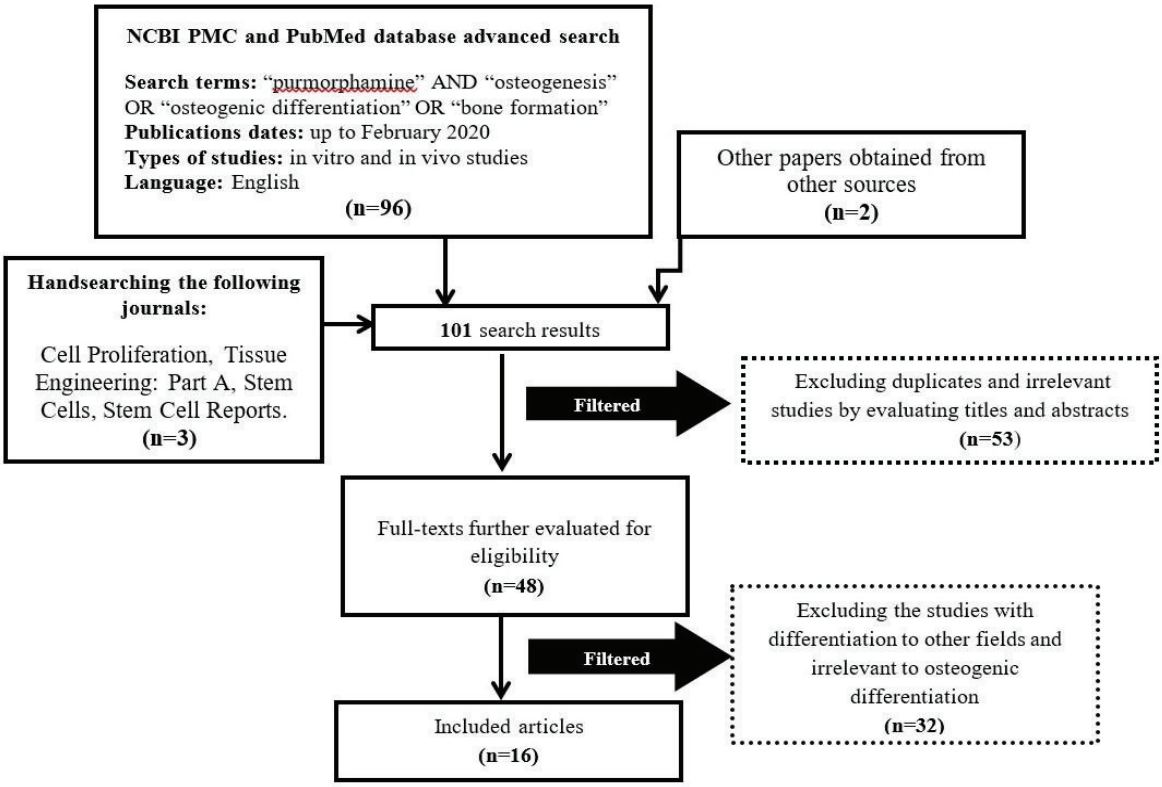

Figure 1. Flow chart of the search strategy used in this study

increases bone regeneration by activating alkaline phosphatase activity (ALP) in a step-wise manner (19). Considering the controversial studies on osteogenic effect of purmorphamine on stem cells, the present study aimed to systematically review the current available literature in this regard.

Materials and Methods

The systematic review was conducted according to the Cochrane Handbook PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) statement.

Types of studies

All in vivo and in vitro studies conducted on osteogenic effect of purmorphamine from 2000 to February 2020 were included. Included studies were limited to English-language ones. Abstracts, reviews, letters and irrelevant studies were excluded.

Type of participants

Studies that focused on the effect of different dosages of purmorphamine and on osteogenic differentiation of cells were included.

Type of interventions

Studies that had used purmorphamine alone or in combination with any other growth factor on any cell type were included. The studies which did not focus on the effect of purmorphamine on osteogenesis procedure were excluded.

Types of outcome measures

Osteogenesis was measured by alkaline phosphatase activity test (ALP), osteocalcin (OCN), runt-related transcription factor 2 (RUNX2), collagen-1(Col1) or bone sialoprotein (BSP) expression.

Information source

Our scientific electronic databases were MEDLINE (NCBI PubMed and PMC), Google Scholar and Scopus. Furthermore, a hand search was performed in the following journals: Cell Proliferation, Tissue Engineering: Part A, Stem Cells and Stem Cell Reports. The search was limited to English-language studies.

Search strategy

An electronic search was performed to select relevant studies to

osteogenic effect of purmorphamine with the following keywords: "purmorphamine" AND "osteogenesis" OR "osteogenic differentiation" OR "bone formation".

Study selection

Two independent reviewers performed the search and the study selection was based on the keywords. Initial screening of the titles and abstracts was performed based on the defined criteria. Disagreement between the reviewers was resolved following a discussion. The final review was performed on the full-texts of the selected papers.

Data collection process

Data extraction and review were performed according to the PRISMA guideline and flow diagram as illustrated in Figure 1. The full-texts of the selected articles were reviewed and the data were extracted and tabulated.

Data items

Data were extracted from the studies and were classified in a table with the following rows; (1) Aim of study, (2) Type of cells used, (3) Type of scaffolds used, (4) differentiation factors and dosages with application period, (5) Results.

Results

The primary search demonstrated 96 results, two papers from other sources and three studies by hand searching. Following removing duplicates, 48 studies were obtained. With respect to eligibility criteria and by reviewing the full-texts and according to the pre-defined criteria, the following studies were excluded; 2 studies for being review articles, 14 for studying the neurogenic differentiation, 11 for evaluating the molecular mechanisms and pathways, and 5 others on cancer and stem cells unrelated to bone regeneration and scope of the study. Finally, 16 articles were included in the final systematic review. The in vitro and in vivo examinations were studied separately (Table 1 and 2).

Cells used for studying the effect of purmorphamine

The most commonly cells used for studying osteogenic effect of purmorphamine were human bone marrow derived stem cells (hBMSCs) in five studies (18, 19, 21-23). C3H10T1/2 (14, 15, 18), human mesenchymal stem cells (hMSCs) (20, 24), C2C12 (17, 25), human dental pulp stem cells (hDPSCs) (2), human multipotent adipose- derived stem cells (hMADSCs) (18), light II reporter cells (26, 27), ICR- CD1 (27), human endometrial mesenchymal stem cells (hEnSCs) (3), mature osteoblasts (18) and rat mesenchymal stem cells (MSCs) (11) were also used in the included studies.

Other growth factors or mediums compared with purmorphamine

The most abundant combination was adding purmorphamine to osteogenic medium (OM) in seven studies (2, 3, 18, 20, 21, 23, 24), four studies compared the effect of BMP-4 alone or in combination with purmorphamine (14, 15, 17, 25), and the same comparison was done in one study for BMP-6 (27).

Dosage of purmorphamine

Majority of the studies had used purmorphamine at 2μM dosage (2, 11, 14, 15, 17-20, 24, 26, 27) and the greatest dosage was utilized in one study at 200 μM (27).

Scaffolds used in Purmorphamine treatment

Majority of the studies did not use any scaffolds but six studies used calcium tricalcium phosphate (TCP), βTCP/ poly(lactide–co-propylene glycol–co-lactide (PPLM), Calcium phosphate disc, collagen/hydroxyapatite(HA), βTCP, HA-porous beads, 3D collagen based scaffold, 2D static culture and commercially pure titanium(cpTi) (2, 3, 19, 23, 26, 27).

Osteogenic differentiation

Fourteen studies evaluated the levels of ALP as a measure of osteogenic differentiation (2, 3, 11, 14, 15, 17-25, 27) . Some studies evaluated osteocalcin (OCN), runt-related transcription factor 2 (RUNX2) and glioma-associated oncogene homologs (Gli) expression in addition to ALP (3, 11, 14, 15, 17, 18, 20, 24-27). A few studies evaluated the expression of bone sialoprotein (BSP) and collagen-1 (Col1) (3, 11, 19).

Discussion

In this systematic review, we aimed at evaluating the effect of an osteoinductive small molecule, purmorphamine, on osteoblastic differentiation. Purmorphamine has various advantages such as rapid effect, easy metabolism and lower costs which make it appropriate for osteogenesis procedure (2, 3, 21). It has been demonstrated that purmorphamine has no effect on the pattern of the proliferation of human bone marrow mesenchymal cells during three weeks, while it can influence the stimulation of cells with osteoprogenitor potential (19, 22).

Table 1. In vitro studies utilizing purmorphamine in osteogenesis

Author(s)/year	Aim of study	Cell	Scaffold	Groups, Induction(dose/time)	Results
Wu *et al.* /2002	Reporting the identification of Pur	C3H10T1/2	-	1. 10 μM Pur(4 days) 2. 2 μM Pur(4 days) 3. 1% DMSO (control) 5. BMP-4 (100 ng/mL) and Pur (1 μM) 4. 300 ng/ml BMP-4	Up- regulation of the Cbfa1/Runx2 gene Lower expression of ALP in Pur- treated cells compared to BMP-4 treated cells. 3-fold increase in ALP comparing to individual molecule.
Wu *et al.* /2004	Discovering the mechanism of action of Pur	C3H10T1/2	-	1.treatment with Pur(2 μM dissolved in DMSO to make10 mM solution+ BMP-4(100 ng/mL) or 0.02% DMSO 2. treatment with BMP-4 3. Treatment with 1% DMSO (control) All for 12h, 24h, 2days, 4days and 6 days	Pur addition: Significant up- regulation of Ptc, Gli1, Cbfa1, Runx2, Cbf-β, ALP, osteomodulin, Luciferase reporter activity, Gli2, Patched 2 and down- regulation of Sfrp-1 and Sfrp-2. Pur and BMP-4stimulates cellular proliferation at early stages (after 24 h).
Beloti *et al.*/ 2005 (1)	Investigation the osteogenesis induced by Pur in hBMSCs.	Human osteoblasts differentiated from hBMSCs	-	After incubation in OM till 1st passage: 1. Pur at dosages of 1, 2 or 3 μM 2.Medium supplemented with Vehicle(control) After 7, 14, and 21 days	Number of bone like nodules : Control <1μM =3μM <2 μM Average area of bone-like nodules: control=1μM=3 μM<2 μM ALP activity: control=1μM< 2μM=3μM
Beloti *et al.*/ 2005 (2)	Evaluation of the osteogenic potential of Pur on hBMSCs	Human osteoblast derived from hBMSCs	cpTi	1. Pur 3 μM(dissolved in dimethyl sulphoxide at a concentration of 10^{-2} M 2. Control (medium supplemented with vehicle). After 7, 14, and 21 days	ALP activity increased by Pur and affected by period of culture in the following order: 21 days<7 days<14 days. Bone like nodule was increased by Pur(21 days).
Lee *et al.*/2008	Identification of osteogenic Pur derivatives	C2C12	-	1. Pur and its derivatives (#3, #70), 0.5,1,2.5,5,5.1 μM+DMEMwith/without BMP4. 2.DMSO (control) After 2 days	Efficient expression of ALP, Gli3, Runx-2 in the combination of Pur/#3 and BMP-4. All three compounds expressed p53 gene.
Plaisant *et al.* /2009	Association between osteoblast differentiation of human mesenchymal stem cells and a decrease in Hh signaling	1.hMADSCs 2.mature osteoblasts 3.C3H10T1/2 4.hBMSCs	-	1. OM+ 2 μM Pur 2. oxysterols (5 μM each) or with Pur (2 μM)(15 days) 3. OM (control, 10 days) RNA was extracted after 5, 10, 15, or 21 days.	Pur treatment: Gli1 expression: An increase in mature osteoblasts and decrease in hMADSCs and hBMSCs (group 1). Decrease in the expression of ALP, Runx2, osteonectin, OPN, and osteoprotegerin in hMADSCs and hBMSCs (group 1 and 2).

ALP: Alkalin phosphatase, AsaP: ascorbate-2-phosphate, ß-GP: ß-Glycerophosphate, BMP: Bone morphogenetic protein, BMSC: Bone marrow mesenchymal stem cells, BSP: bone sialoprotein, CaP: calcium phosphate, Cbfa1: core binding

Kim *et al.*/ 2009	Examination the effects of three Pur derivatives (#3, #10 and #70)	C2C12	-	1. Growth medium+BMP-4 (10 ng/ml) a Pur or Pur derivatives (0.5-5 µM) 2. Growth medium+BMP-4 (10 ng/ml)(control)	ALP: Increase in Pur and #3, and decrease in #10 and #70 Up- regulated expression of OCN by #10 and #70, increased expression of Dlx2 and Runx2 in all treated groups.
Gellynck *et al.*/ 2011	Formulation of a degradable bone adhesive containing Pur and calcium phosphate filler	Light II reporter cells	β- TCP filled PPLM	1.2 µM Pur to growth medium 2.0.008 or 0.08 mg disc^{-1} to 75 wt.% filled composite	Addition of Pur: 2 µM to the culture medium: 7-fold, 0.008 mg disc^{-1} to composite: 2-fold and 0.08 mg disc^{-1} to composite: 5-fold increase in the Gli response.
Oliveria *et al.* /2012	Investigation the expression of Hh Pathway genes in presence of Pur.	hMSCs	-	1.non-OM 2.non-OM+ 2 µM Pur 3. Vehicle (dimethylsulfoxide) RNA extracted at 7 and 14 days	Pur treatment: Up-regulated expression of SMO, PTCH1, Gli1, Gli2, Runx2 and SOX9 and down-regulation and for SMO and PTCH1 (after 7 days), collagen type I, a1, ALP, and OC.
Granéli *et al.*/ 2013	Identifying small-molecule substances with the ability to enhance osteogenic differentiation of hMSCs.	hMSCs	-	1.OM+Pur or Pur analogs* (0.5, 1, 2 and 5 µM) dissolved in DMSO 2. OM+ DMSO (control)	Upregulated expression of OCN (2 µM) and Gli1. Pur + recombinant BMP-2: No increase in the ECM mineralization in comparison to Pur alone.
Faghihi *et al.*/ 2013	Evaluation the effect of two small molecules on promotion of osteogenesis	hBMSCs	-	1.OM+Pur (1, 3, 5, 10 and 20 µM) 2.OM (control group) After 7, 14 and 21 days	10 and 20 µM of Pur: decrease in the number of cells. ALP: increased activity at doses of 3 and 5 µM, decreased activity in cultures with 10 µM of Pur Gene expression: Up- regulation of Runx-2, OC (3 and 5 µM/ day 21) and Gli-(1 at 3 µM).
Gellynck *et al.*/ 2013	Examination the ability of Pur in accelerating the formation of bone	ICR-CD1 Light II reporter cells	CaP disc	neg. medium+10 mM ß-GP pos. medium+10 nM Dex pos. medium+100 ng/ml BMP-6 pos. medium+2 µM Pur pos. medium+2 µM Pur+100 ng/ml BMP-6 Disc with/without 200 µM Pur adhered	Adding both BMP-6 and Pur showed a higher up-regulation of ALP than each on their own. More expression of luciferase or Gli in cells growing on the CaP coated discs with Pur.
Faghihi *et al.*/ 2014	Comparing the Effect of Pur on osteogenic differentiation of hMSCs within 2D static	hBMSCs	1.3D collagen-based scaffold 2.2D static culture system	1.OM 2.OM+3 µM Pur After14 and 21 days 1.OM	Deposition of the mineralized matrix in 3D culture in OM, with/ without Pur. Pur treatment: increased expression of ALP, OC, Runx2 and Gli after 4 and 21 days.

protein, Col 1: collagen 1, Cp Ti: commercially pure titanium, C2C12: immortalized mouse myoblast cell line, C3H10T1/2: mouse mesenchymal progenitor cells, Dex: dexamethasone, DMSO: dimethyl sulfoxide, Dlx2:distal-less homebox2,

	and 3D dynamic culture systems.		3.collagen	2.OM+3 µM Pur After 14 and 21 days Cells at passage 3 (control)	
Woltje *et al.*/ 2015	Influence of Pur on osteogenic differentiation of MSCs from rats	MSCs	-	Growth medium+10 nM Dex+10 mM ß-GP+0.2 mM AsaP+2 µM of Pur After 7,14 and 21 days	Lower cell numbers after 21 days in the presence of Pur compared to classical induction. Increased ALP activity in the presence of Pur from 14 to 10 days. Increased expression of osteopontin gene and osteocalcin gene (at day 7).
Rezai Rad *et al.*/ 2016	Investigation the osteogenic potential of Pur	hDPSCs	B- TCP	1. OM+ 2 µM of Pur 2. OM (control) After 7 and 14 days	Treatment with Pur: Increased expression of ALP(after 7 and 14 days), OC(14 days) and OPN(7 days) and a decrease for OPN(14 days).
Bahrami *et al*/ 2016	Setting the standards of quality and safety for the donation and processing of human tissues and cells	hEnSCs	Collagen/HA 2D group	1.DMEM+FBS 10%(control) 2. DMEM+FBS 10% +10nM Dex +ascorbic acid 50 µg/ml+β-GP10mM 3. DMEM+FBS 10%+3 µM Pur All for 21 days 1.OM (control) 2.OM+Pur After 7, 14 and 21 days	Lower expression of OC and BSP in the presence of Pur compared to control group. Expression of Col 1 and Runx 2: Scaffold groups> 2D groups ALP in 2D group: Control<Pur group<common OM

ECM: Extra cellular matrix, Gli: glioma-associated oncogene homologs, hBMSC: human bone marrow stem cell, hDPSCs: human dental pulp stem cells, hEnSCs: human endometrial stem cells, hMADSC: human multipotent adipose-derived stem cell, hMSC: human mesenchymal stem cell, hOSTs: Human primary osteoblasts, MSC: Mesenchymal stem cell, OCN: osteocalcin, OPN: osteopontin, OM: Osteogenic medium, PPLM: poly(lactide–co-propylene glycol– co-lactide) dimethacrylate, Ptc: patched, PTCH1: Patched 1, Pur: Purmorphamine, Runx2: Runt-related transcription factor 2, Shh: Sonic hedgehog, SMO: Smoothened, TCP: Tricalcium phosphate, Ti: titanium.
Pur analogs: Small molecules that are the result of a ligand-based virtual screening for substances similar to purmorphamine*

Table2. *In vivo* studies utilizing purmorphamine in bone regeneration

Author(s)/ year	Aim of study	Animal	Cell	Scaffold	Group(s)	Results
Faghihi et al/2013	Studying the osteopromotive property of two small molecules	9 male Wistar rats/ subcutaneously	hMSCs	TCP	1.Dex (10 nM) 2.Pur (3 µM) 3. Control (water). (Daily injection)	ALP and Col significantly up- regulated in Pur- treated group. OCN and Runx2: No significant difference between groups.
Gellynck et al/ 2013	Examination the ability of Pur in improving the integration of titanium implants	femur of fetal chick		HA Porous beads	1.HA- beads soaked in 200 µM Pur in PBS for 24 h 2. Control beads soaked in PBS Bone marrow of chicken embryo femurs in medium containing 10^{-8} M Dex, 100 ng/ml BMP-6, 0.1 M pamidronate or 2 µM Pur PTFE strip n(2 PTFE, 3 titanium coated PTFE 3, 3 titanium coated PTFE + Pur)	The proportion of trabecular bone to overall bone area was higher in Pur samples. ALP: Increased activity with addition of Pur or BMP-6 to medium. Trabecular bone was touching the implant on both sides in Ti coated and Ti coated Pur adhered strips.

ALP: Alkalin phosphatase, BMSC: Bone marrow mesenchymal stem cells, Col 1: collagen 1, Dex: dexamethasone, HA: hydroxyapatite, hBMSC: human bone marrow stem cell, hMSC: human mesenchymal stem cell, OCN: osteocalcin, PBS: phosphate-buffered saline, PTFE: polytetrafluoroethylene, Pur: Purmorphamine, TCP: Tricalcium phosphate, Ti: titani

It seems that purmorphamine is able to increase the number of bone- like nodules, the average area of nodules and also the degree of mineralization by two mechanisms: increasing the proliferation rate of osteoprogenitor cells and also number of formed nodules, and stimulating synthetic activity in these cells (19, 21, 22, 24). On the other hand, there is a controversial result indicating that cells show no increase in mineralization when they are treated with purmorphamine (23). Also, in one study it was shown that the effect of purmorphamine on hDPSC proliferation had been time-dependent (2). Two studies demonstrated that combination of purmorphamine and BMP-4 enhances ALP activity (14) and osteogenic differentiation (15). By working on hBMSCs, the greatest response of ALP activity was when the cells were treated with 2 μM or 3 μM of purmorphamine (20). In the presence of purmorphamine, the number of ALP positive cells increases, but at the same time, this increase differs based on the type of the scaffold that cells are cultured on. HMSCs show a better promotive activity on 2D cultures rather than 3D dynamic system in mid stage of osteogenesis (23). In a study by Bahrami *et al.*, addition of purmorphamine to 2D culture medium resulted in a higher ALP activity on days 7, 14 and 21 than negative control group and lower activity compared to osteogenic control group (3); while in a study by Rezia Rad *et al.*, the increase in ALP activity in the presence of purmorphamine was observed only on days 7 and 14 (2).

According to a study by Faghihi' *et al.*, purmorphamine has the ability to induce up- regulation of ALP at early and mid-phases of osteogenesis in hBMSCs particularly at the dosage of 3 μM and 5 μM of purmorphamine (3). This increase in ALP activity was also observed in C3H10T1/2 in the early period (15) and in rat MSCs on day 3, while adverse result was obtained in C2C12 cells (17). There is a controversial result indicating that the inhibition of ALP expression was detected in hMADSCs after 5 days of treatment and it lasted until 21st day of experiment. A 50% decrease in ALP activity and mRNA expression also happened when hBMSCs were treated with 2 μM of purmorphamine (18).

The important part is that the role of ALP can be considered significant when the alkaline mRNA translates into proteins and the level of enzymatic activity increases, which means that the amount of ALP mRNA does not have a great effect on the process of osteogenesis (23).

Expression of genes that are related to osteogenic differentiation differs between control group and Pur- treated group. Observation of hDPSCs cultured on β- TCP granules showed up- regulation of osteopontin and osteocalcin at early and mid-stages of differentiation, respectively (2). The down-regulation of osteopontin at day 14 may suggest that hDPSCs can no longer response to the same inductive molecules as they become differentiated. The increase in the up- regulation was observed for BSP and Col1 by EnSCs, Runx2, Ptch, Gli2 and MIBP by C2C12 cells (17), and Gli by light II reporter cells attached to PPLM (27). In a study by Plaisant *et al.*, by treating hMADSCs with 2 μM of purmorphamine, a decrease in Gli expression happened after 5 days (18).

On the other hand, the expression of Col1, BSP and PTCH1 seems to stay unchanged during osteogenic differentiation of hMSCs. Moreover, using 3D (dynamic) culture would result in an up- regulation of Gli1 during the early and late period, while in 2D (static) system, the up- regulation was only observed in the early period. In treating hBMSCs, the osteocalcin transcript showed a slight increase on day 21 (23).

With 3 μM and 5 μM of purmorphamine, a down- regulation of osteocalcin occurred at 21st day which was justified to happen because of the reduction in mRNA production during the maturation phase (21). In rat MSCs, not only a gradual increase in collagen gene expression was observed in the presence of 2μM of purmorphamine, but also osteopontin and osteocalcin genes were expressed on day 7 and day 14, respectively (11). In contrast to most of the results, hedgehog pathway activation in hMSCs inhibited the gene expression of Runx2, osteopontin, osteonectin and osteoprotegerin (18).

Based on in vivo experiments, up- regulation of osteocalcin and Runx2 was not affected by purmorphamine (21). Moreover, it was shown that purmorphamine cannot be a good choice for adjacency for implant placement in chick femurs (27).

Although a lot of in vitro experiments have been done on different kinds of cells, lack of data seems to exist and hence, several in vivo procedures is needed to study the effect of purmorphamine on proliferation and osteogenic differentiation of cells. By this, it may become possible to utilize purmorphamine in tissue engineering and reduce undesirable outcomes as much as possible.

Conclusion

Purmorphamine is one of the small molecules which can be used for bone tissue engineering. The efficiency of purmorphamine is proved by its capability in promoting adhesion, proliferation and osteogenic differentiation in different kinds of stem cells. The related events such as ALP activity and bone like nodule formation can be enhanced by purmorphamine, too. In applying purmorphamine, what should be noticed is the usage of scaffolds, duration and also the dosage of purmorphamine in order to prevent toxicity.

Conflict of Interest: 'None declared'.

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