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OIC-A006 promotes osteogenesis *in vitro* and *in vivo*

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Bone morphogenesis proteins (BMPs) are one of the potent bone-forming factors. However, the safety, utility, and cost effectiveness of BMPs must be considered. Nowadays, there has been substantial interest in developing a chemical compound that safely promotes bone formation and facilitates fracture repair. Based on previous research with high throughout screening assay, we found one potent osteogenic inductive compound, named as OIC-A006 (Osteogenic inducible compound-active 006), which is classified in the amine family. In this study, we aimed to investigate the inducing effects of OIC-A006 on osteogenesis by bone marrow stem cells (BMSCs) *in vitro* and *in vivo*. We demonstrated that OIC-A006, at different concentrations, especially at optimal concentration of 6.25 μM, could stimulate BMSCs to express alkaline phosphatase (ALP), core-binding factor a1 (Cbfa1), osteopontin (OPN) and osteocalcin (OC), and to form calcified nodules *in vitro*. Under the bone tissue culture conditions, OIC-A006 also stimulated new bone formation of murine calvarial and metatarsal bone, indicating that OIC-A006 may exert positive effects on osteogenesis. Furthermore, to elucidate the *in vivo* osteogenic potential of OIC-A006, we used a rabbit skull defect model treated with sustained release microcapsules (OIC-A006/PLGA-MC) injected s.c. adjacent to the defect. These results revealed, for the first time, that OIC-A006 has the potential to promote osteogenesis *in vitro* and *in vivo*. This new compound may provide a new alterative agent for growth factors to promote bone healing and bone regeneration.

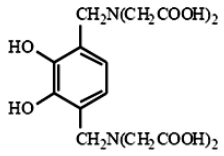
1. Introduction

Fracture healing involves a complex cascade of several cellular events, which include immediate injury response, intramembranous ossification, chondrogenesis and endochondral ossification, resulting in the formation of a fracture callus (Bolander 1992; Simmons 1985). Studies suggest that local growth factors, such as bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF-β), basic fibroblast growth factors (bFGFs) and platelet-derived growth factor (PDGF), may regulate cellular proliferation, differentiation and extracellular matrix synthesis in the initial and developmental stages of the fracture callus (Bourque et al. 1993; Heckmann et al. 1999; Nash et al. 1994). The local administration of these purified or recombinant molecules with chondrogenic and/or osteogenic capacities has been shown to induce bone regeneration and stimulate fracture healing in animal models (Lind 1996). These findings indicate that administration of growth factors may serve as a good strategy to improve fracture healing. However, the safety and cost effectiveness problems may hamper the large scale clinical use of these growth factors. Therefore, it is essential to develop some chemical compounds which have the same potency as growth factors to stimulate bone formation and at the same time exhibit more advantages in safety and cost-effectiveness.

Using a high throughout screening assay, scientists have synthesized an amine compound named CBMIDA [Catechol-3,6-bis(methyleiminodiaetic acid)] as a chelating agent (Henry et al. 2001; Fakuda 2005). Recently, we

Abbreviations: OIC-A006: Osteogenic inducible compound-active 006; BMPs, Bone morphogenesis proteins; BMSCs, bone marrow stem cells; OC, osteocalcin; ALP, alkaline phosphatase; OPG, osteoprotegerin; Cbfa1, Core-binding factor a1; TGF-β, transforming growth factor-β; bFGFs, basic fibroblast growth factors; PBS, phosphate-buffered saline.

have found that the compound can exert effects on bone formation through structure modification. In this study, we further evaluated the effects of the osteogenic compound OIC-A006 on osteogenesis *in vivo* and *in vitro*.



CBIMDA

Previous studies have demonstrated that many compounds stimulate the formation of bone-like nodules in bone marrow stromal cells cultured according to the method of Maniatopoulos et al. (1988). Whereas the marrow stromal population *in vitro* contains a diversity of cell types, including osteoblastic cells, fibroblastic cells, adipocytes and monocytes/macrophages, the rich cellular environment is similar to that in the marrow cavity (Aubin et al. 1992). In addition, the nodules formed *in vitro* exhibit features closely resembling woven bone *in vivo* (Lind 1996). Hence, this model has been extensively used for studies of bone cell lineage and of the regulation of osteogenesis by various agonists (Aubin et al. 1993). These findings prompted us to investigate potent bone formation stimulators using this culture system. Indeed, our *in vitro* study demonstrated that our newly discovered chemical compound, OIC-A006, can induce potent cellular ALP, OPN, OC, Cbfa1 activity in bone marrow stromal cell culture. Furthermore, OIC-A006 can induce ectopic ossification and skull defect repair suggesting its potential to stimulate bone formation *in vivo*.

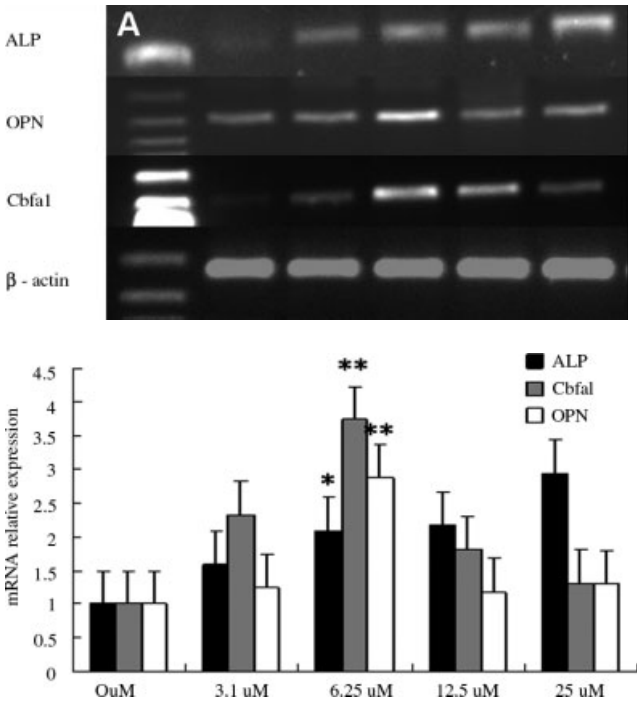


Fig. 1: OIC-A006 increases ALP, OPN, Cbfa1 Expression Levels. ALP, OPN, Cbfa1 mRNA expression can effect bone formation. To determine the osteogenesis of effect of OIC-A006 in BMBCs, the cells were treated with OIC-A006 for 5, 10, 15, 20, 25 days at different concentration of OIC-A006. A, RT-PCR showed that OIC-A006 can increase ALP, OPN, Cbfa1 mRNA expression. ALP, OPN, Cbfa1 mRNA expression at concentration of 3.1 μM, 6.25 μM, 12.5 μM, 25 μM was higher apparently than 0 μM. B, Real-time-PCR showed OIC-A006 can increase ALP, OPN, Cbfa1 mRNA expression. ALP, OPN, Cbfa1 mRNA expression at concentration of 3.1 μM, 6.25 μM, 12.5 μM, 25 μM was higher apparently than 0 μM. Data represent mean ± SD. * $P < 0.05$, ** $P < 0.01$

2. Investigations and results

2.1. OIC-A006 induce BMSCs to express ALP, Cbfa1, OPN

To determine the bone-forming effects of OIC-A006 in BMBCs, cells were treated with OIC-A006 for 5, 10, 15, 20, 25 days at concentrations of 0 μM, 3.1 μM, 6.25 μM, 12.5 μM and 25 μM, respectively. The osteogenic gene expression of ALP, OPN, Cbfa1 in BMSCs cultured with OIC-A006 was higher than that without OIC-A006 supplementation. After 10-day culture, RT-PCR expressed that OIC-A006 dose-dependently increased the mRNA expression of ALP in BMSCs but the maximal expression

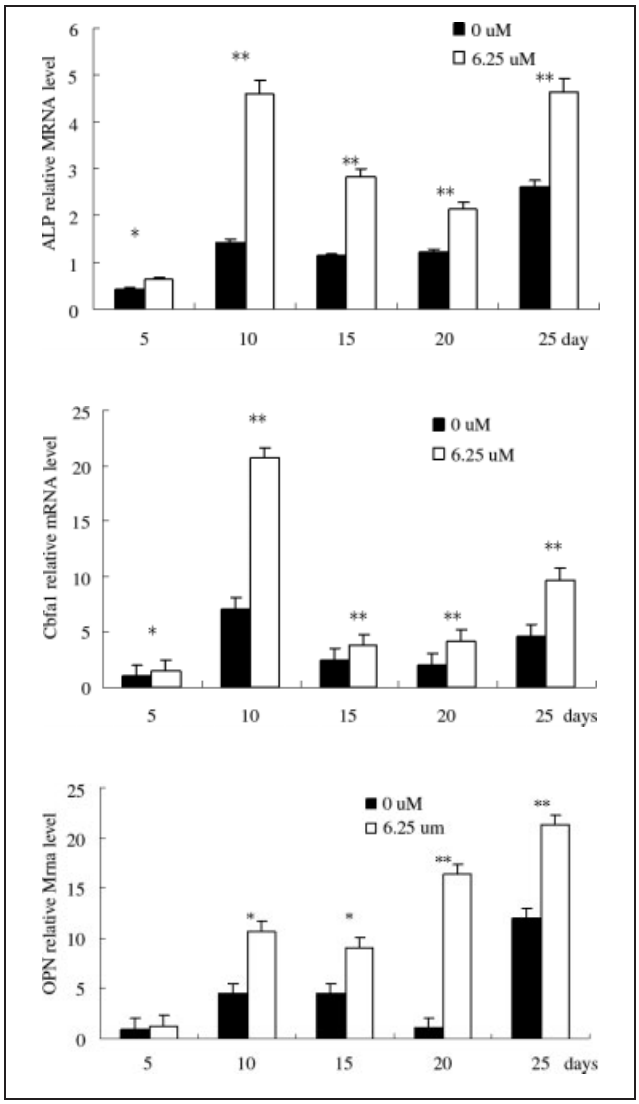


Fig. 2: Bone marker gene expression. To determine the osteogenesis of effect of OIC-A006 in BMBCs, the cells were treated with OIC-A006 for 5, 10, 15, 20, 25 days at optimal concentration of OIC-A006.Relative mRNA levels of three bone marker genes (OPN, Cbfa1, ALP) in bone marrow stem cells were compared. Markers were normalized by mRNA of β-actin in the same sample using the RT-PCR system. By Real-time PCR, we can find that the expression of ALP, OPN, Cbfa1 at concentration of 6.25 μM was all more than the expression at 0 μM for 5, 10, 15, 20, 25 days. Data represent mean ± SD. **: $P < 0.01$, *: $P < 0.05$

level of OPN and Cbfa1 reached at the concentration of 6.25 μM. Based on this observation, we designated the optimal concentration of OIC-A006 at 6.25 μM (Fig. 1A, B). Real-time PCR assay was carried out to evaluate the gene expression of ALP, OPN and Cbfa1 in BMSCs cultured without or with the optimal concentration of OIC-A006. At day 5, 10, 15, 20 and 25, OIC-A006 at 6.25 μM significantly increased osteoblast-related gene expression (Fig. 2).

2.2. OIC-A006 increases OPN and OC protein expression in BMSCs

To confirm that OIC-A006 has the potential to enhance the OPN and OC expression, immunocytochemistry and radioimmunity assays were used to detect the OPN and OC protein expression in BMSCs cultured with OIC-A006 at 6.25 μM for 20 days. The expression of OPN as well as OC in BMSCs cultured with OIC-A006 at 6.25 μM was significantly higher than that in cells cultured without OIC-A006 (Figs. 3, 4).

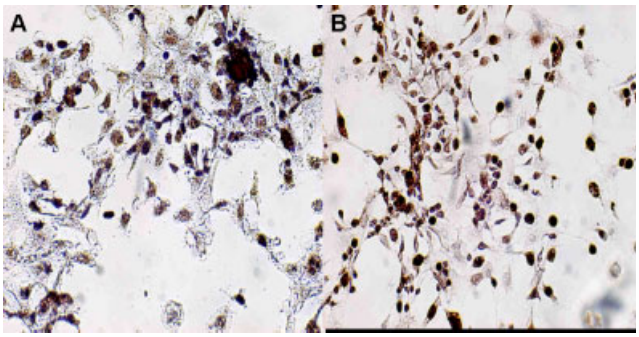


Fig. 3: OIC-A006 enhances the expression of OPN by immunocytochemistry. The expression of OPN can be one marker gene expression in bone formation. To confirm OIC-A006 enhance the OPN expression, we assessed the effect of OIC-A006.The expression of OPN at the best concentration of 6.25 μM was more than cultured at 0 μM by immunocytochemistry when bone marrow stem cells were cultured for 20 days. Data represent mean ± SD. (n = 10). * $P < 0.05$

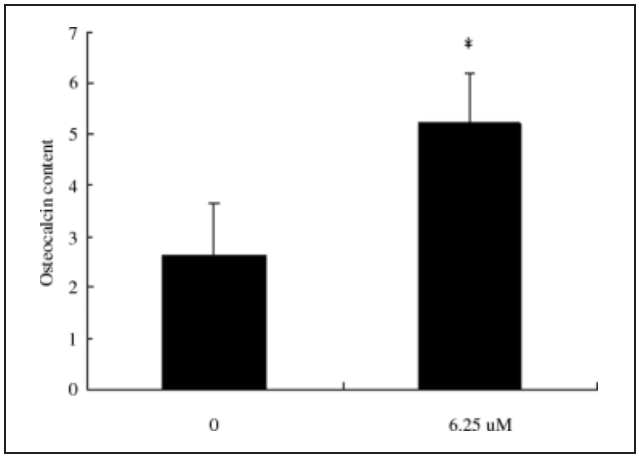


Fig. 4: OIC-A006 enhances the expression of OC by radioimmunity. The expression of OC can be one marker gene expression in bone formation. In our study, To confirm OIC-A006 enhance the OC expression, we assessed the effect of OIC-A006.The expression of OC at concentration of 6.25 μM was more than cultured at 0 μM by radioimmunity technology when bone marrow stem cells were cultured for 20 days. Data represent mean ± SD. (n = 10) * $P < 0.05$

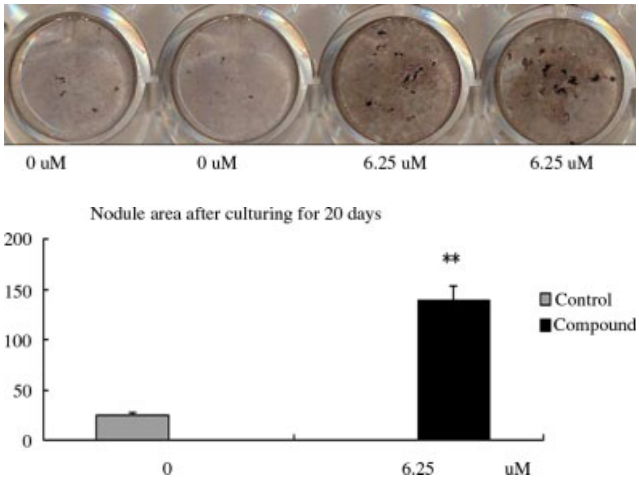


Fig. 5: OIC-A006 enhances Mineralized Bone Matrix Formation A, Black mineralized nodule was observed with von Kossa stain. Mineralized nodule at concentration of 6.25 μM was more than at 0 μM apparently when cells cultured for 20 days. B, Dose-dependent effect of OIC-A006 on mineralized nodule formation in mouse bone marrow stromal cell culture. Cells were cultured in medium with or without concentrations of OIC-A006 for 20 days. Mean ± S.E. (n = 5). ** $p < 0.01$ versus control. Dunnett's multiple comparison

2.3. OIC-A006 enhances bone matrix mineralization

Matrix mineralization is the late stage of bone formation. Osteogenesis of BMSCs is usually accompanied by the formation of bone-like nodules. Mineralized nodule was observed with Von Kossa Staining by microscopy and Von Kossa Staining nodule area was measured. Apparently, Mineralized nodule in BMSCs cultured with OIC-A006 at the concentration of 6.25 μM was more obvious than that in cells cultured without OIC-A006 for 20 days (Fig. 5A, B).

2.4. OIC-A006 enhances murine calvarial and metatarsal bone formation

Previous studies have suggested that osteoinductive factors can induce bone formation in bone tissue culture (Heckmann et al. 1999; Notoya et al. 1994; 1999). In our study, we chose murine calvarial and metatarsal bone as the bone tissue culture system. The new bone formation was assessed by H&E staining and tetracycline fluorescence labeling, and they also can be measured through new bone width and area, which demonstrated that the compound at the concentration of 6.25 μM could apparently increase the new bone formation this tissue culture system (Fig. 6A–D).

2.5. Skull defect model in rabbits

To elucidate the *in vivo* osteogenic potential of OIC-A006, we used a rabbit skull defect model treated with sustained release microcapsules (OIC-A006/PLGA-MC) injected s.c. adjacent to the defect. The 1-cm trephine defects made after stripping off the pericrania did not completely heal within 12 weeks and the spontaneous new bone area that formed in the defect by 4 weeks was less than 20 to 30% of the defect area). Four weeks after the operation, treatment with a single local application of OIC-A006/PLGA-MC (4–6 mg/site) resulted in a dose-dependent increase in the radio-opaque area formed in the defect (Fig. 7B). Histological studies showed that the defect area was occupied by a bony bridge (Fig. 7D) and the newly-formed radio-

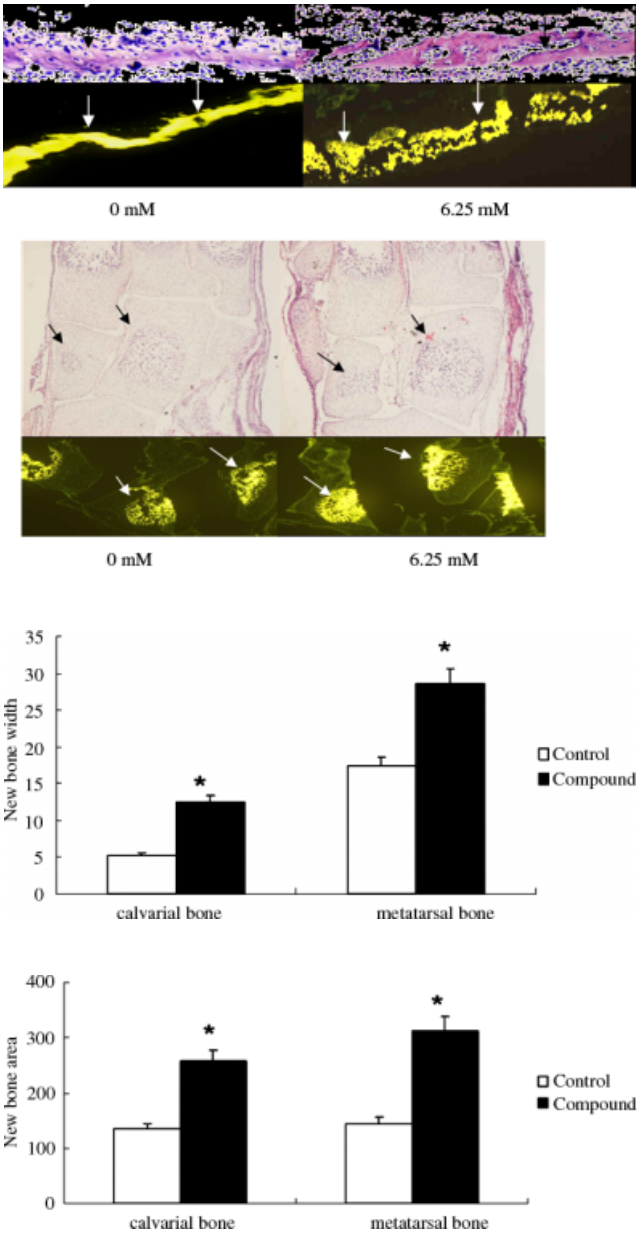


Fig. 6: OIC-A006 enhances Murine Calvarial and Metatarsal Bone Formation Studies have revealed mineralized bone matrix formation had an apparent effect in osteogenesis. In our research, Osteogenesis of bone marrow stem cells were accompanied by the initiation of nodule formation. OIC-A006 increase bone formation during bone tissue culture. Compared with 0 μM, The concentration of 6.25 μM can apparently increase the new bone formation can be observed by Hematoxylin and eosin stain and tetracycline fluorescence labelling technology. A, The calvarial bones. B, The metatarsal bones. C, D, The apparent effect of OIC-A006 on new bone formation in bone tissue culture. Mean ± S.E. (n = 5). * $p < 0.05$, ** $p < 0.01$ versus control. Dunnett's multiple comparison

opaque area corresponded to a calcified bone containing bone marrow cavities surrounded by thick osteoid seams with cuboidal osteoblasts (Fig. 7F). In contrast, the placebo-treated defects were filled with a dense fibrous connective tissue and an only small amount of bony callus at the periphery (Fig. 7A, C, E). Furthermore, to examine the effect of OIC-A006/PLGA-MC on intact bone, we measured the mineralized bone and osteoid of bilateral parietal bone. There was a significant difference in either of the indices between placebo- or OIC-A006/PLGA-MC treated skulls. These results indicate that OIC-A006/ PLGA-MC stimulate intramembranous ossification in bone defects.

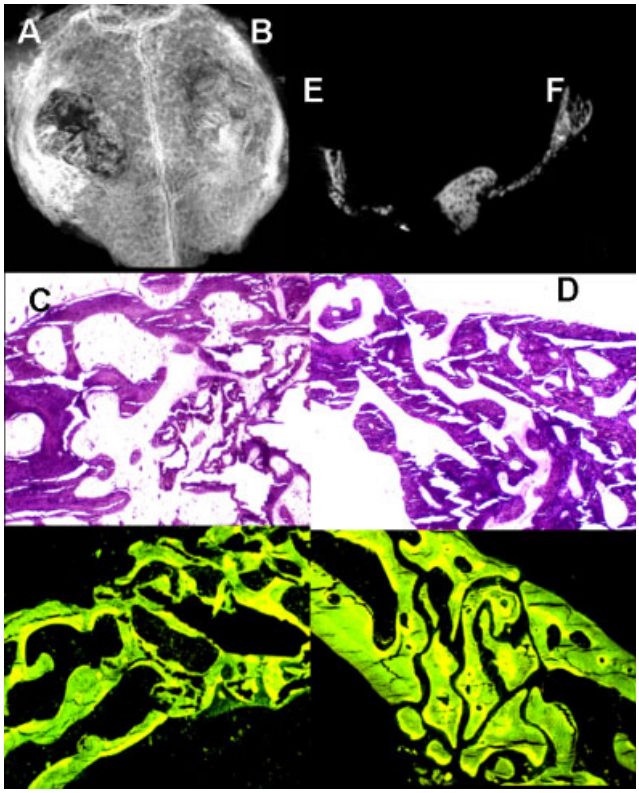


Fig. 7: Skull Defect Model in Rabbits To elucidate the in vivo osteogenic potential of OIC-A006, we used a rabbit skull defect model treated with sustained release microcapsules (OIC-A006/PLGA-MC) injected s.c. adjacent to the defect by Soft X-ray radiographs (A and B; original magnification 10×), Hematoxylin and eosin stain and tetracycline fluorescence labelling technology (C and D;), Micro CT (E and F). The defects treated with placebo (A, C, and E) and OIC-A006/PLGA-MC (1 mg/site; B, D, and F). Rabbit skull defects 4 weeks after the operation. Approximately two thirds of the defects were occupied by a radio-opaque area (B) and a bony bridge across the defect treated with OIC-A006/PLGA-MC (D). Histologic examination revealed the bony bridge consisted of a calcified bone containing bone marrow cavities surrounded by thick osteoid seams with cuboidal osteoblasts (F). In contrast, the placebo treated defect was filled with a dense fibrous connective tissue and an only small amount of bony cal

3. Discussion

This study provided the evidence that OIC-A006 has the potential to promote bone formation *in vitro* and *in vivo*. The process of bone-like nodule formation *in vitro* resembles that of intramedullary bone formation in repair of bone marrow tissue after local injury: osteoprogenitor cells with multi-potent differentiation potential in the the bone marrow proliferate and differentiate into functional osteogenic cells, resulting in the formation of woven bone in the bone marrow (Aubin et al. 1993). Previous studies have demonstrated that some compounds like ipriflavone (Notoya et al. 1994), TAK-778 (Notoya et al. 1999), statins (Mundy et al. 1999) can stimulate the formation of bone-like nodules in bone marrow stromal cells. Hence, the stimulatory effect of OIC-A006 on cell differentiation during bone-like nodule formation *in vitro* may reflect the effect of this compound on cellular events during bone marrow regeneration.

Moreover, OIC-A006 stimulated the osteogenic gene expression in BMSCs, such as ALP, OPN and Cbfa1, demonstrating that OIC-A006 has the potential to promote the osteoblast lineage differentiation of BMSCs. This direct positive effect of this compound on BMSCs may fa-

cilitate primary bone healing, in which osteoblasts directly mediate the osteogenesis process.

In addition to the stimulatory effect on bone formation *in vitro*, OIC-A006 also has the potential to induce the ectopic ossification process, which is evidenced by the existence of chondrocytes and osteoid around the muscle where the compound was injected. Hughes and McCulloch (1991) suggested that differentiation of osteoprogenitor cells might be regulated by the production of autocrine/paracrine factor(s) based on the findings that conditioned media from calvaria-derived cells enhances bone mineralization in rat BMSCs culture.

There are several similarities and differences between the pharmacological profiles of OIC-A006 and endogenous bone-forming agents like BMPs. These agents are also synthesized and localized in the callus during fracture healing (Bolando 1992). Both BMPs and bFGF stimulate bone-like nodule formation *in vitro* (Hughes and McCulloch 1991) and the local administration of these agents enhance callus formation during fracture repair in animal models. Previous studies suggested that BMPs, included in the TGF-β superfamily, have a positive effect on mesenchymal cells and osteoprogenitor cells to promote bone formation (Onishi et al. 1998). The osteoinductive function of BMPs has been proved to be mainly involved in the process of endochondral bone formation. Consistent with BMPs, our data also suggest that OIC-A006 is involved in the process of endochodral bone formation, as demonstrated by the ectopic ossification assay.

During the bone healing process anabolic agents are more desirable than the anti-catabolic agents, due to the fact that the anabolic agents have the potential to enhance bone formation and facilitate bone healing and regeneration. Small molecular weight compounds have been reported to have the capacity to enhance *in vivo* bone formation (Hughes and McCulloch 1991), which is consistent with our findings that the *in vivo* and *in vitro* osteogensis was promoted by the treatment of OIC-A006. Small molecular chemical compounds, with the advantage of availability and cost-effectiveness, may provide an alterative approach to increase bone formation. But since these studies are focused on animal models, studies on humas are necessary to further clarify the potential clinical application of these compounds.

4. Experimental

4.1. Bone marrow stem cells (BMSCs) cultures

BMSCs were obtained from 3-month-old female adult C57BL/6 mice (purchased by Chinese Academy of Sciences, China) by flushing the lumen of the femur shaft with 1.5 ml of Dulbecco's modified Eagle's low glucose medium (DMEM) (Hyclone, USA) supplemented with antibiotics and 10% fetal bovine serum (FBS) (Hyclone, USA). Cells were then cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified incubator supplied with 5% CO_2. After incubation for 4 h, non-adherent cells were removed by replacing the medium. The adherent cells were cultured in DMEM for 72 h. Medium was then changed to osteogenic medium containing dexamethasone (100 nM), L-ascorbic acid-2-phosphate (0.05 mM) and glycerophosphate (10 mM), 2 mg/ml doxycycline (Sigma, USA), DMEM and 10% FBS.

The compound OIC-A006 was produced in the Shanghai Institute of Material Medica, Chinese Academy of Sciences.

The stock solution was dissolved at dimethyl sulfoxide (DMSO) (Sigma, USA) at a concentration of 10 mM and stored in room temperature. OIC-A006 was diluted with culture medium and added to cells cultured at the designated final concentration of 3.1 μM, 6.25 μM, 12.5 μM and 25 μM in the osteogenic medium, respectively. The cells were then exposed to OIC-A006 for 5, 10, 15, 20 and 25 days when they had attached for 72 h, with the medium changed every 3 days. The final concentration of DMSO was less than 1% in culture medium. All procedures involving mice were approved by the Institutional Animal Experiment Committee of Shanghai Jiaotong University School of Medicine and Shanghai Institutional Animal Care and Use Committee.

4.2. RT-PCR and Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to manufacturer's instructions. Reverse Transcription (RT) was used to synthesize cDNA, which was subsequently amplified by PCR and Real-time PCR. Primers were designed to amplify 164, 114, 186, 218 bp sequences within the coding sequences of the selected genes, ALP, Cbfa1, OPN and β-actin respectively.

All RT-PCR reactions were performed using thermocycler (Biometra, Germany). All Real-Time PCR reactions utilized the SYBR® Green RT-PCR Reagents (Biosystems, USA) and were performed using RG-3000 (Corbett Research, Australia). Reaction conditions were optimized for each of the genes by varying the annealing temperature (50–55 °C) and RNA concentration (1–5 ng/reaction). Cycle threshold (Ct) values were measured and calculated by the sequence detector software. Relative amounts of mRNA were normalized to β-actin and calculated with the software program Microsoft Excel. Relative mRNA contents were calculated as $x = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta E - \Delta C$ and $\Delta E = Ct_{sample} - Ct_{\beta\text{-actin}}$ and $\Delta C = Ct_{control} - Ct_{\beta\text{-actin}}$ (Bellows et al. 1986).

4.3. Radioimmunity and immunocytochemistry analysis

Based on the related results above, bone marrow stem cells were cultured for up to 20 days upon the optimization of concentration of OIC-A006 resulted from the RT-PCR and Real-time PCR experiment. To determine the expression of osteocalcin (OC), Total OC concentrations were measured with a radioimmunity kit (lowest detection limit: 5 pg/ml, General Hospital of PLA, China). The values are normalized to the amount of OC extracted from normal cells, which is arbitrarily set equal to 1.00. Cells were processed by ^{124}I-OC and OC antibody in turns. Then precipitation was measured and calculated when the cells placed for 20 min and centrifuged at 3500 rpm for 25 min at 4 °C. All procedures followed the manufacturer's protocols. To determine the expression of OPN, staining cells are measured by immunocytochemistry (Santa Cruz, USA) to perform histomorphometric measurements on the cells.

4.4. Von Kossa staining

Bone cell differentiation was monitored by mineralized bone matrix (Bellows et al. 1986)). BMSCs were plated on 6-well plates with a density of 2×10^6 cells/well and cultured in the osteogenic medium supplemented with the optimal concentration of OIC-A006 for 20 days, which is based on the result of RT-PCR and Real-time PCR experiment mentioned above. Histochemical staining for mineralized bone matrix formation in the culture was determined by von Kossa staining. In brief, cells were fixed with 10% (v/v) formalin and incubated in fresh substrate solution for 30 min at 25 °C. Von Kossa staining was carried out by adding 3% silver nitrate solution to formalin-fixed cells and cells were subjected to UV light. The deposits of calcium were demonstrated by the formation of black nodules. Images of von Kossa staining were obtained with an IX70 Inverted Research Microscope (Olympus America Inc, NY).

4.5. Fetal mouse calvarial and metatarsal bone formation assay

The calvarial and metatarsal bones from 4-day-old pups of mice were excised and dissected free of adjacent connective tissue. The isolated calvaria and metatarsals were cultured in rotary cell culture system (RCCS, Synthecon, USA) in 20 ml tissue culture medium (Sigma, USA) containing OIC-A006 (6.25 μM) for 7 days, with medium replacement every 2 days. Tetracycline (5 mM) (Sigma, USA) was added to the medium freshly each time on medium change. Explants were then divided into two groups randomly. One group was fixed in 10% buffered formalin, decalcified in 8% Na2EDTA, paraffin embedded and stained with H&E using standard method. Another group was fixed in acetone and embedded in methyl methacrylate without decalcification. Four 5-μm thick cross sections were obtained. The new bone formation was assessed by histomorphometry and tetracycline fluorescence labeling, which were visualized by fluorescence microscopy (LEICA, Germany).

4.6. Skull defect model in rabbits

Three-mouths-old female rabbits were used. After achieving suitable anesthesia by i.p. injection (0.1 ml/100 g b.wt.) of pentobarbital sodium (50 mg/ml; Abbott Laboratories, North Chicago, IL), the skin overlying the calvaria was shaved and sterilized. A semilunar incision was made through the tissues of the scalp, and skin was raised to expose the bilateral parietal bone. The pericrania were stripped off the parietal skulls. Under continuous saline irrigation, a trephine defect, 1 cm in diameter, was made in the center of the parietal bone avoiding sutures using a low-speed dental drill. Particular care was taken to preserve the structure and continuity of the underlying midsaggital blood sinus. Two days later, a

suspension of placebo or OIC-A006/PLGA-MC was injected s.c. adjacent to the defect. Four weeks after the craniotomy, the animals were sacrificed by carbon dioxide inhalation and the skulls were excised. The new bone formation was evaluated radiographically and histologically. The newly formed radio-opaque area in the defect was calculated using an image analyzer: [area of the initial defect created by trephination] – [area of the remaining defect].

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