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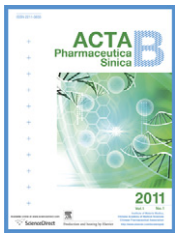

ORIGINAL ARTICLE

Fungal pyrrolidine-containing metabolites inhibit alkaline phosphatase activity in bone morphogenetic protein-stimulated myoblastoma cells

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KEY WORDS

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Abstract Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant congenital disorder characterized by progressive heterotopic ossification in muscle tissues. A constitutively activated mutation of a bone morphogenetic protein (BMP) receptor, ALK2, has been identified in patients with FOP. We report here that four structurally related compounds, lucilactaene, hydroxylucilactaene, NG-391 and NG-393, produced by fungal strain *Fusarium* sp. B88, inhibit BMP signaling *in vitro*. Alkaline phosphatase activity, a marker enzyme of osteoblastic differentiation, was decreased in C2C12 myoblasts stably expressing mutant ALK2 by treatment with those compounds with IC_{50} values of 5.7, 6.8, 6.9 and 6.1 μM, respectively. Furthermore, NG-391 and NG-393 inhibited BMP-specific luciferase reporter activity, which is directly regulated by transcription factor Smads, with IC_{50} values of 1.4 and 2.1 μM, respectively. These findings suggest that these fungal metabolites may provide a new direction in the development of FOP therapeutics.

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1. Introduction

Bone is a connective tissue that guarantees the protection and support of organ function. Contrary to the common view, bone is a dynamic tissue that constantly undergoes turnover in order to maintain stability and remodeling. Bone remodeling is a process coupled with bone resorption and bone formation that determines bone structure and quality during adult life. Osteoblasts, bone-forming cells, are derived from the embryonic mesoderm. During the early stages of osteoblast differentiation, several molecules such as bone morphogenetic proteins (BMPs), transforming growth factor-βs (TGF-βs), leukemia inhibitor factor (LIF), fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs), drive the differentiation of stem cells to inducible osteoprogenitors (stromal mesenchymal stem cells) and then to determined osteoprogenitors[1]. Among these molecules, BMPs are reported as unique factors, which can induce ectopic bone formation in muscle[2].

The BMP signaling pathway is one of the most highly conserved signaling pathways among the bone remodeling systems[3,4]. The BMP signal starts with binding to heterotetrameric transmembrane complexes of type I and type II BMP receptors. Four type I receptors, ALK1, ALK2, ALK3/BMPR-IA and ALK6/BMPR-IB, and three type II receptors, BMPR-II, ActR-II and ActR-IIB, have been reported[5]. Following ligand binding, serines and threonines in the glycine/serine (GS) domain of type I receptor are phosphorylated by the constitutively activated type II receptor. Thus, the BMP type I receptor is activated by the phosphorylation event, which transmits downstream to BMP pathway-specific Smad1/5/8 and p38 MAPK[6]. Furthermore, phosphorylated Smad1/5/8 forms complexes with Smad4, and they move to the nucleus and work as transcription factors to express early responsive genes such as Id1 (an inhibitory protein for myogenesis). Smads bind to a GC-rich BMP responsive element (BRE) in the early responsive genes[6]. The cells activated via BMP signaling differentiate to osteoblastic cells, which express typical differentiation markers such as alkaline phosphatase (ALP), osteocalcin and osteopontin.

Fibrodysplasia ossificans progressiva (FOP) is a congenital disorder of progressive and widespread postnatal ossification of soft tissues[7]. Ectopic bone formation in FOP occurs through an endochondral pathway in which cartilage is formed initially at the site and is subsequently replaced by bone[8,9]. FOP results in severe debilitation and reduces the life span due to joint fusion and restrictive lung disease with thoracic involvement. The median age of survival is approximately 41 years[10]. Surgical attempts to operatively remove heterotopic bone have commonly led to episodes of explosive and painful new bone growth called "flare-ups"[11]. Shore et al.[12] found a recurrent heterozygous mutation, c.617G→A, in the *ACVR1*/*ALK2* gene in both familial and sporadic patients with FOP that causes an amino acid substitution of Arg to His at codon 206 (R206H) of ALK2. Since this mutation has been shown to constitutively activate ALK2, specific inhibitors of ALK2 could offer therapeutic benefit for FOP. Consequently, the quest for novel pharmacological agents that target specific steps of FOP has significantly intensified. As a result, synthetic dorsomorphin[13,14], originally discovered as an AMP kinase inhibitor, was found to selectively inhibit BMP signaling induced by type I receptors such as ALK2, ALK3 and ALK6[13]. Cuny et al.[15] improved dorsomorphin to successfully obtain more potent derivative LDN-193189, which prevented ectopic bone formation in mice carrying an active mutant ALK2 and attenuated lesions in the remainder[16]. C2C12 myoblasts, derived from murine thigh muscle, inhibit myogenesis and express osteoblastic phenotypes by treatment with BMPs or over-expression of constitutively activated BMP type I receptors[6]. C2C12 cells have been widely used for studies of osteoblast differentiation induced by BMP signaling *in vitro*[17–19].

In this study, we screened for potent inhibitors of osteoblastic differentiation induced by BMP signaling using a stable ALK2(R206H)-expressing C2C12 cell line (abbreviated as C2C12(R206H) cells), which exhibited ALP activity more quickly and more strongly than original C2C12 cell line, to develop FOP chemotherapy[20]. After testing the natural product library (217 compounds) and the actinomycetal and fungal culture broths (9831 samples) in this screening, we found four structurally related fungal metabolites, lucilactaene[21], hydroxylucilactaene[22], NG compounds (NG-391 and NG-393)[23,24], from the culture broth of *Fusarium* sp. B88. Moreover, NG-391 and NG-393 inhibited a BMP-specific luciferase reporter activity, which is directly regulated by transcription factor Smads. These findings suggest that these fungal metabolites may provide a new direction in the development of FOP therapeutics.

2. Materials and methods

2.1. Materials

Fusarium sp. B88 was isolated from the body of a grasshopper collected in 2006 on Ishigakijima where is a small southern island belonging to Okinawa in Japan. This fungus was incubated in BYK-1 broth (25 g RISO VIALONE NANO RICE (Japan Europe Trading Co., Ltd., Tokyo, Japan) and 0.6 g Difco™ Potato Dextrose Broth (Becton, Dickinson Company, France) in 25 mL H_2O) at 27 °C for 14 day under static conditions. The culture broth (1 kg) was extracted with acetone. After the acetone extracts was concentrated, the resulting aqueous solution was extracted with EtOAc. The EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield 2.3 g of solid material. The material was dissolved in a small volume of $CHCl_3$, applied to a silica gel column (30 g, 70–230 mesh, Merck), and eluted stepwise with 100:0, 100:1, 50:1, 10:1, 1:1 and 0:100 (*v*/*v*) of $CHCl_3$–CH_3OH solvents (300 mL each). The $CHCl_3$:CH_3OH = 10:1 fraction was concentrated *in vacuo* to dryness to give a brown material (66.0 mg). The material was finally purified by preparative HPLC (column, PEGASIL ODS, 20 mm × 250 mm, Senshu Scientific Co.; solvent, 45% CH_3CN; detection, UV at 210 nm; flow rate, 8.0 mL/min). Under these conditions, hydroxylucilactaene, NG-391, NG393 and lucilactaene were eluted as peaks with retention time of 21, 23, 28 and 38 min. The pooled fractions were concentrated *in vacuo* to dryness to give pure hydroxylucilactaene (1.9 mg), NG-391 (4.0 mg), NG393 (2.3 mg) and lucilactaene (1.8 mg), respectively. The MS and ^{1}H NMR spectra of these compounds were identical to those reported previously[21–24]. All these samples (1 mg each) were dissolved in 1 mL CH_3OH and used as assay samples.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) and *p*-nitrophenyl phosphate were purchased from Sigma (St. Louis, MO, USA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, diethnolamine and rhBMP-4 were purchased from Nacalai Tesque (Kyoto, Japan), HyClone (Waltham, MA, USA), Invitrogen (Carlsbad, CA, USA), Wako Pure Chemical Industries (Osaka, Japan) and R&D Systems (Mountain View, CA, USA), respectively.

2.2. Cell culture

C2C12 and C2C12 (R206H) cells[20] were cultured in DMEM supplemented with 15% FBS and 100 units/mL penicillin and 100 μg/mL streptomycin (hereafter referred to as medium A) at 37 °C in 5.0% CO_2. Both cells were subcultured once every 3 day.

2.3. Assay for alkaline phosphatase in BMP-treated C2C12 cells

Alkaline phosphatase (ALP) activity, a typical marker of osteoblastic differentiation, was measured as described previously[19]. In brief, C2C12 (R206H) cells (7.5×10^3 cells/well) in a 96-well plastic plate were cultured at 37 °C in 5.0% CO_2. Following overnight recovery, the culture media were replaced with 100 μL fresh medium A containing rhBMP-4 (10 ng/mL) and a sample (1 μL in CH_3OH solution). After 48 h incubation, the cells were incubated for 60 min with 100 μL substrate solution (100 mM diethanolamine, 0.5 mM $MgCl_2$ and 1.0 mg/mL *p*-nitrophenylphosphate) at room temperature. The reaction was terminated by adding 50 μL 3 M NaOH, and the absorbance at 405 nm was measured with a Power Wave × 340 (BIO-TEK Instruments, Highland Park, IL, USA).

2.4. Cytotoxicity

Cytotoxicity of a compound to C2C12 (R206H) cells was evaluated by the MTT assay[25]. In brief, C2C12 (R206H) cells (7.5×10^3 cells/well) were cultured in 96-well plates in the absence or presence of a compound for 48 h at 37 °C in 5.0% CO_2. After incubation, the cells received 10 μL MTT solution (5.5 mg/mL in phosphate-buffered saline), and were then incubated at 37 °C for 3 h. A 90 μL aliquot of the lysis solution (40% *N,N*-dimethylformamide, 2.0% CH_3COOH, 20% SDS and 0.03 M HCl) was added to each well, and the plates were incubated for 2 h. The absorbance at 550 nm of each well was read with a Power Wave × 340. Inhibition of cell growth is defined as (absorbance-sample/absorbance-control) × 100%. The IC_{50} value is defined as a sample concentration that causes 50% inhibition of cell growth.

2.5. Reporter gene assay for monitoring BMP signaling

The effect of a compound on BMP signaling via Smads was examined using a BMP-specific luciferase reporter, Id1WT4F-luc, which is driven by four tandem copies of BRE in the Id1 gene[6]. In brief, C2C12 cells were inoculated at 1.0×10^4 cells/well in 96-well plates with medium A and incubated for 24 h. The cells were transfected with 200 ng of plasmid DNA (40 ng of Id1WT4F-luc, 10 ng of phRL-SV40 and 150 ng of ALK2 (R206H)) using 0.5 μL of Lipofectamine 2000 (Invitrogen) in OPTI-MEM (GIBCO) according to the manufacture's protocol. After 2.5 h incubation, the culture medium was replaced with 100 μL fresh DMEM containing 2.5% FBS without penicillin and streptomycin. After additional 3 h incubation, a compound (1 μL CH_3OH solution) was added to each well and cultured for 24 h. Both firefly and renilla luciferase activities in the cells were determined using Dual Glo Luciferase assay system (Promega, Madison, WI, USA).

3. Results and discussion

In the present study, we screened microbial culture broths for inhibitors of ALP activity of C2C12 (R206H) cells. As a result, structurally related four compounds, lucilactaene[21], hydroxylucilactaene[22], NG-391[23] and NG-393[23,24] (Fig. 1) were isolated from a fungal strain *Fusarium* sp. B88. In fact, all of the compounds inhibited ALP activity in a dose-dependent manner with analogous IC_{50} values of 5.7, 6.8, 6.9 and 6.1 μM (Fig. 2). Although they were originally reported as a cell cycle inhibitor[21],

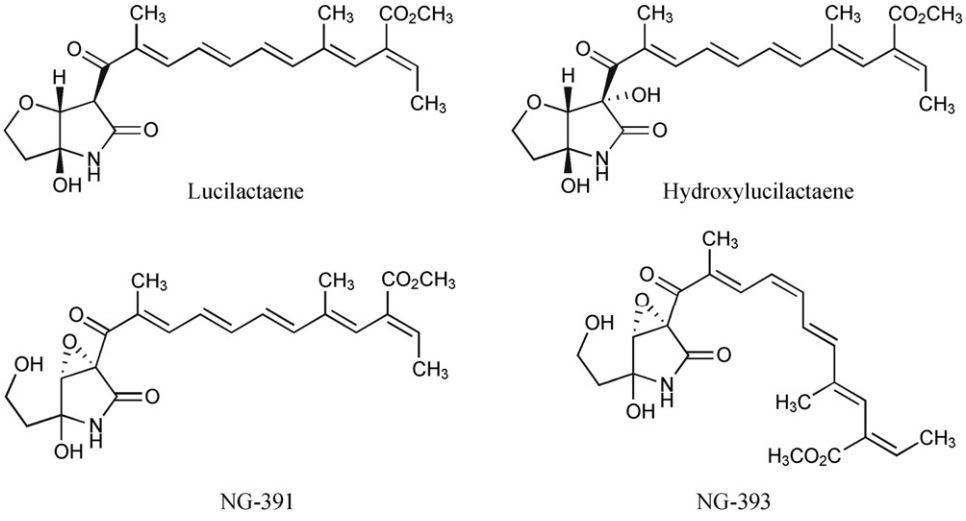

Figure 1 Structures of lucilactaene, hydroxylucilactaene, NG-391 and NG-393.

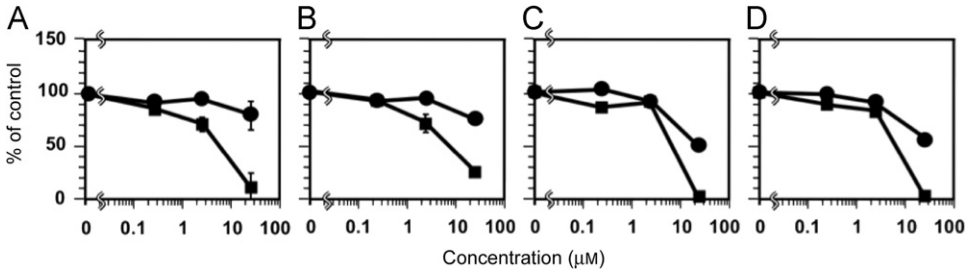

Figure 2 Effect of lucilactaene, hydroxylucilactaene, NG-391 and NG-393 on ALP activity and cytotoxicity of C2C12 (R206H) cells. C2C12 (R206H) cells (7.5×10^3cells/well) were cultured to 70% confluence and then treated with lucilactaene (A), hydroxylucilactaene (B), NG-391 (C) and NG-393 (D) in the presence BMP (10 ng/mL). After 48 h incubation, ALP activity (■) and cytotoxicity (●) were measured as described in Section 2. Values are the mean ± SD of three independent experiments.

an anticancer analog[22], promoters of nerve growth factor production[23] and mutagenic agents against the *Salumonella*[24], we first showed that these compounds also inhibit ALP activity, a key marker of osteoblast differentiation of C2C12 (R206H) cells. No marked or very weak cytotoxic effects on C2C12 (R206H) cells were observed at the highest dose (30 μM) of each compound in a MTT assay (Fig. 2).

The ALP activity is one of the markers of osteoblast differentiation *in vitro* and *in vivo*. The induction of ALP activity in C2C12 cells is an output through the multiple intracellular events initiated by the activation of BMP receptors. To examine direct effect of those compounds on BMP signaling, we determined Id1WT4F-luc activity in C2C12 cells. The luciferase activity in Id1WT4F-luc is driven by BRE in the Id1 gene, which is recognized by a complex of phosphorylated Smad1/5/8 and Smad4. Since Smad1/5/8 is phosphorylated by BMP type I receptors including ALK2, the formation of Smad complexes on BRE and the induction of reporter activity is highly specific for BMP signal transduction. As shown in Fig. 3, NG-391 and NG-393 inhibited luciferase activity of Id1WT4F-luc in C2C12 cells with IC_{50} values of 1.4 and 2.1 μM, respectively. These concentrations were much lower than those of cytotoxic effects. Lucilactaene and hydroxylucilactaene showed no effect on luciferase activity at 2.5 μM (Fig. 3), and inhibited the activity at 20 μM probably due to cytotoxic effects.

Our findings suggest that NG-391 and NG-393 may inhibit an early event(s) in BMP signal transduction because they inhibited the BMP-specific luciferase reporter activity, which expression is directly regulated by Smads. On the other hand, lucilactaene and hydroxylucilactaene may inhibit a different event(s) from that NG compounds. To the best of our knowledge, this is the first report of BMP signal inhibitors of natural origin. BMP activity is controlled at multiple steps in signal transduction. It was reported that BMP antagonists such as noggin and follistatin inhibit a binding step of BMPs to the receptors in the extracellular space[26,27] and that an intracellular domain-truncated type I receptor, BAMBI, inhibits BMP signaling by acting as a dominant negative receptor on the cell membrane[28]. In contrast, several types of BMP inhibitors act in the intracellular spaces; FKBP-12 and I-Smads, such as Smad6 and Smad7, bind to the GS domain and kinase domain of the type I receptors, respectively, and block the kinase activity[29]. The chemical BMP inhibitors, dorsomorphin[13,14] and LDN-193189[15], have been shown to bind to the ATP-binding pocket of the type I receptors to

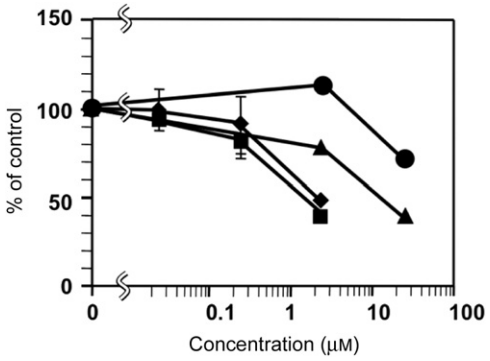

Figure 3 Effect of lucilactaene, hydroxylucilactaene, NG-391 and NG-393 on BMP signaling in C2C12 (Id1-BRE) cells. C2C12 cells (1.0×10^4 cells/well) were cultured for 24 h and then Id1-WT4F-luc, phRL-SV40 and ALK2 (R206H) were co-transfected to construct C2C12 (Id1-BRE) cells. After 2.5 h incubation, the culture media were replaced with DEME containing 2.5% FBS without penicillin and streptomycin. After another 3 h incubation, the cells were treated with lucilactaene (●), hydroxylucilactaene (▲), NG-391 (■) and NG-393 (♦). Luciferase activities in the cell extracts were determined using the Dual Glo Luciferase assay system as described in Section 2. Values are the mean ± SD of three independent experiments.

prevent phosphorylation of Smads[30]. Two types of protein phosphatases, protein phosphatase Mg^{2+}-dependent 1A and small C-terminal protein phosphatases, inhibits Smads and further downstream effectors in BMP signaling[31]. Since NG-391 and NG-393 inhibited BMP-induced ALP activity and the luciferase reporter activity, these compounds may target a step(s) between BMP binding to the receptor and activation of Smads on the BRE. Further studies will be needed to elucidate molecular mechanisms of the inhibition of BMP signaling by the compounds found in this study. We expect that these compounds will provide a new direction in the development of FOP therapeutics.

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