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ICM0201, a New Inhibitor of Osteoclastogenesis from *Cunninghamella* sp. F-1490

I. Taxonomy, Fermentation, Isolation and Biological Activities

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In the course of screening for inhibitors of osteoclastogenesis, a new substance designated as ICM0201 was isolated from a fermentation broth of *Cunninghamella* sp. F-1490. ICM0201 inhibited the formation of osteoclasts in mouse bone marrow cells with an IC_{50} value of 0.78 μg/ml and showed weak cytotoxicity against bone marrow cells.

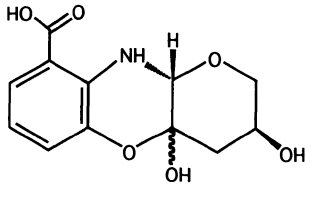
Osteoclasts are large multinucleated cells that are induced from osteoclasts precursors and play a significant role for resorption of bone[1~3]. These cells are involved in pathogenic destruction of bone such as osteoporosis, Paget's disease, rheumatoid arthritis and humoral hyper calcemia of malignancy (HHM)[4~7]. Most of these diseases are thought to be due to an imbalance between bone resorption and formation. Because the inhibition of bone resorption is a primary therapeutic objective, we have established *in vitro* screening system for natural products that inhibit osteoclastogenesis. There are many osteoclastogenic factors including 1,25-dihydroxyvitamin D_3[8,9], prostaglandin E_2 (PGE_2)[10,11], parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP)[12,13]. Osteoclasts have various characteristics including tartrate-resistant acid phosphatase (TRAP) activity and pit-forming activity on bone slices[14,15]. Thus, we have searched for substances that inhibit osteoclastogenesis in mouse bone marrow cell cultures using PTHrP as inducer of osteoclastogenesis and TRAP activity expression as an index of osteoclast formation. As a result, we have isolated a new substance, ICM0201 (Fig. 1), from a culture filtrate of a fungal strain F-1490. In this paper, we describe the taxonomy of producing strain, fermentation, isolation and biological activities of ICM0201.

Materials and Methods

Taxonomic Studies

The producing microorganism, strain F-1490, was isolated from a soil sample collected at Tsukuba City, Ibaraki Prefecture, Japan. The taxonomic studies of the strain-F-1490 were done according to the method of VON ARX[16]. The color guide of KORNERUP and WANSCHER[17] was

Fig. 1. Structure of ICM0201.



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used in determining and standardizing colors.

Fermentation

A slant culture of strain F-1490 was inoculated into 500 ml flasks containing 60 ml of seed medium consisting of potato starch 2%, glycerin 1%, soy bean meal 2%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and five glass beads, and cultured at 25°C for 3 days on a rotary shaker (225 rpm). One ml of the seed culture was transferred into 500 ml flasks containing 100 ml of a culture medium consisting of starch syrup 4%, peptone 2%, yeast extract 1%, $(NH_4)_2SO_4$ 0.5%, KH_2PO_4 0.9%. (pH 6.5 before sterilization). The fermentation was carried out at 25°C for 5 days on a rotary shaker (225 rpm).

Osteoclastogenesis of Bone Marrow Cells

Bone marrow was obtained from tibias of 6 to 8 weeks old male C57BL/6 mice (Charles River Japan Inc. Yokohama, Japan). Bone marrow cells were prepared at 1.5×10^6 cells/ml with RPMI1640 medium (GIBCO BRL, LIFE TECHNOLOGIES, Tokyo, Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, USA), 50 μg/ml of L-ascorbic acid (Kokusan Chemical, Tokyo, Japan) and 200 ng/ml of PTHrP (Human, 1-34 amide, Peptide Inst. Inc., Osaka, Japan). One ml of the cells was plated on each well of a 24-well plate and cultured with test sample for 8 days at 37°C in 5% CO_2 - air. On day 3 or 4 of culture, a half of the medium was replaced with freshly prepared medium containing the same concentration of test sample. Cell viability was determined by trypan blue exclusion assay.

Assay of TRAP-positive Multinucleated Cells Formation

On day 8 of culture, cells were fixed with methanol/acetone (1 : 1) for 1 minute at room temperature. After drying, the cells were incubated for 1 hour at 37°C in an acetate buffer (0.1 M sodium acetate, pH 5.0) containing 0.01% naphthol AS-MS phosphate (Sigma Chemical Co. St. Louis, MO, USA) as a substrate and 0.03% fast red violet LB salt (Sigma Chemical Co.) as a staining reagent dissolved in 50 mM sodium tartrate[18]. TRAP-positive cells containing more than three nuclei were counted as osteoclast-like multinucleated cells (OCLs).

Pit Formation Assay

Bone marrow cells were prepared at 1.5×10^6 cells/ml with RPMI1640 medium supplemented with 10% FBS, 50 μg/ml of L-ascorbic acid and 200 ng/ml of PTHrP. One ml of the cells was seeded onto the ceramic calcium phosphate-coated discs (BD Bio Coat Osteologic Bone cell Culture System, Nippon BD, Tokyo, Japan) in a 24-well plate and was cultured for 14 days at 37°C in 5% CO_2 - air. The discs were washed with aqueous solution containing 6% NaOCl and 5.2% NaCl for removal of the cells to observe the resorption pits more clearly[19]. The area of resorption pit on each disc was analyzed by NIH image. Cell viability was determined by alamar blue method[20].

Cytotoxicity

L-1210 (mouse leukemia), Colon 26 (mouse colon cancer) and B16BL6 (mouse melanoma) cells were cultured in RPMI1640 medium supplemented with 10% FBS for 48 or 72 hours in the presence of various concentration of test samples at 37°C under 5% CO_2 - air. After the end of culture, cell growth was measured at 570 nm by MTT method. Inhibitory activities of ICM0201 on various assays are expressed as concentration required for 50% growth inhibition (IC_{50}) values.

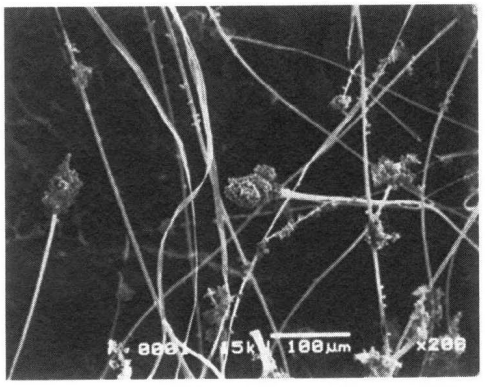
Results and Discussion

Taxonomy of the Strain F-1490

The producing strain F-1490 grew extremely fast on potato dextrose agar, malt extract agar and oatmeal agar plates at 25°C. The surface of colonies was white (A1) at first and turned yellowish white (4A2), and afterwards cream (4A3). Exudates and soluble pigment were not shown. Sporangia were observed after 2 days of culture. The color of sporangia was white (A1) at first and turned brownish gray-grayish brown (8F2-3) gradually.

The sporangiophores were erect and the stripes were smooth-walled. The sporangiophores were simple or branched with verticillate or sympodial branches in a few positions, terminated in vesicles with stergmata and spores (sporangispores), with stolons and rhizoids basally. The vesicles were subglobose or egg-shaped. The vesicles on main sporangiophores were 30 μm in diameter, and the vesicles on branches 10~20 μm in diameter. The spores were brown, 6~9 μm in diameter, globose or ellipsoidal, 1-celled, short-echinulate. Zygote was not found. These cultural and morphological properties (Fig. 2) suggested that the strain should be included in the genus *Cunninghamella*. However, the above properties did not agree with those of any known species in the genus. It was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as *Cunninghamella* sp. F-1490 with the accession number FERM-p18548.

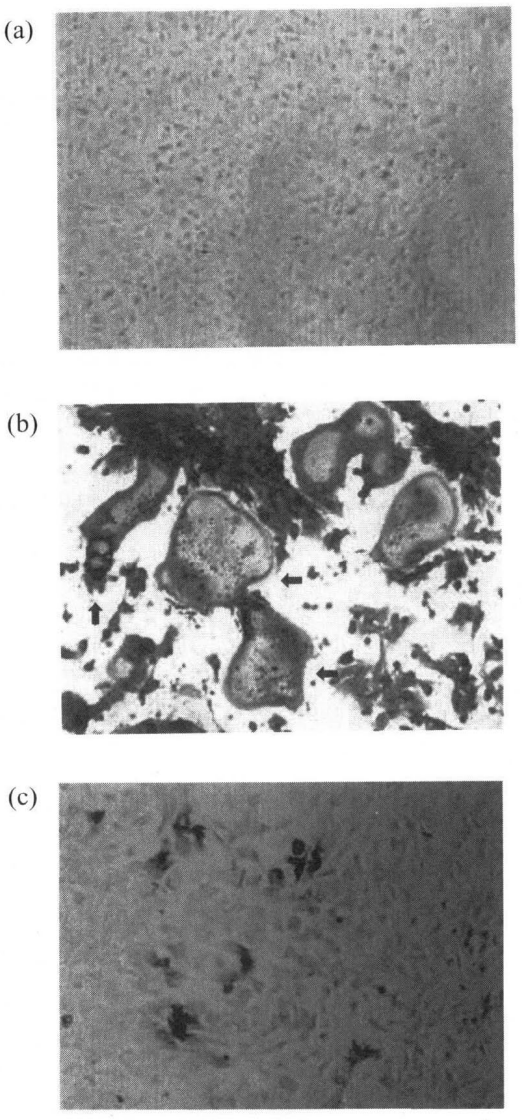
Fig. 2. Scanning electron micrograph of ICM0201 producing strain.



Isolation

The fermentation broth (17 liters) was adjusted to pH 2.5 with 6 N-HCl. After filtration, the filtrate was applied to a Diaion HP-20 (Mitsubishi Chemical) column (5 liters) equibrated with water. The column was washed with water, 20% aqueous methanol and eluted with methanol. The eluate was concentrated *in vacuo* and thus acquired aqueous layer was washed with ethyl acetate (3 liters) after addition of 3 liters of 4% $NaHCO_3$. The aqueous solution was adjusted to pH 2.5 with 1 N-HCl and extracted twice with equal volume of ethyl acetate. The combined organic layer was washed with water saturated with NaCl and dried over Na_2SO_4. After filtration, the organic layer was evaporated to afford an oily residue. The residue was subjected to a silica gel columns chromatography and developed with toluene : acetone = 2 : 1. The active fractions were concentrated *in vacuo* and applied on a Sephadex LH-20 (Amersham Bioscience) column with methanol as eluant. The active fractions were evaporated to give 47 mg of ICM0201 (Fig. 1) as a pale yellow powder. The studies on structure determination of ICM0201 are reported in the accompanying paper[21].

Fig. 3. Effect of ICM0201 on TRAP-positive multinucleated cell formation in mouse bone marrow culture.



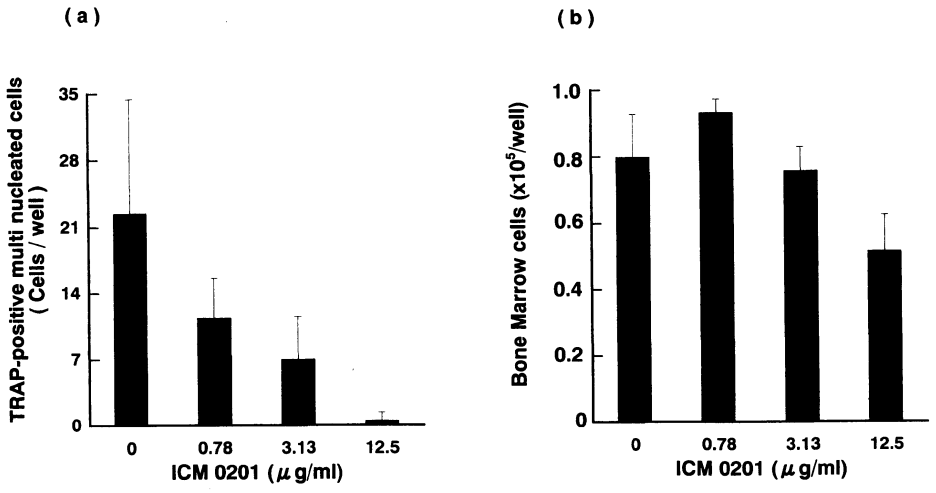
Mouse bone marrow cells were incubated without (a) or with 200 ng/ml of PTHrP (b) and 12.5 μg/ml of ICM0201 with 200 ng/ml of PTHrP (c). Arrows in (b) indicate TRAP positive multinucleated cells. (magnification ×100)

Biological Activities

PTHrP stimulated the formation of OCLs in mouse bone marrow culture (Fig. 3-b). Under the culture conditions without PTHrP, the formation of OCLs was not observed at all microscopically (Fig. 3-a). ICM0201 decreased significantly the number of TRAP-positive multinucleated cells (Fig. 3-c) in a dose dependent manner and the IC_{50} value was 0.78 μg/ml (Fig. 4-a). On the other hand, ICM0201 did not show cytotoxicity against bone marrow cells determined by trypan blue exclusion assay (Fig. 4-b) at the same concentration.

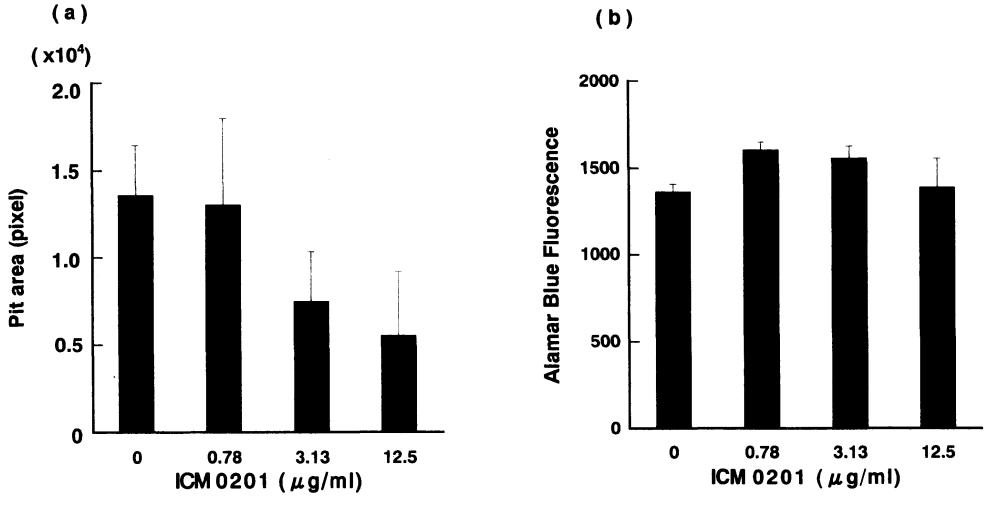
As shown in Fig. 5, PTHrP-induced osteoclasts revealed the ability to form resorption pits. ICM0201 inhibited the pit-formation at 3.13 and 12.5 μg/ml on the osteologic discs

Fig. 4. Effect of ICM0201 on the osteoclastogenesis of mouse bone marrow cells.



(a) TRAP-positive multinucleated cells were counted as osteoclast-like multinucleated cells. (b) Viability of mouse bone marrow cells was assessed by trypan blue staining. Each error bar indicates S.D. The experiments were performed at least in triplicate.

Fig. 5. Effect of ICM0201 on pit-formation.



(a) Mouse bone marrow cells were cultured on a ceramic calcium phosphate-coated disc for 14 days with ICM0201 at the indicated doses. (b) Viability of mouse bone marrow cells was assessed by alamar blue staining. Each error bar indicates S.D. The experiments were performed at least in triplicate.

(Fig. 5-a). On the other hand, ICM0201 showed no effect on the viability of the bone marrow cells determined by alamar blue assay (Fig. 5-b).

In the course of screening for inhibitors of osteoclastogenesis of bone marrow cells, we found ICM0201. ICM0201 inhibited the pit-formation induced by PTHrP at 3.13 μg/ml. As shown in Fig. 5b, ICM0201 up to 12.5 μg/ml did not significantly change alamar blue

fluorescence values. In addition, ICM0201 showed no cytotoxicity against mouse tumor cell lines such as L-1210, colon 26 and B16BL6 showing IC_{50} value over 100 μg/ml (data not shown). These results suggest that the inhibition of pit formation by ICM0201 was not mediated by its cytotoxicity. On the other hand, ICM0201 also inhibited the differentiation into osteoclastic cells induced by PGE_2 (data not shown), one of osteotropic factors, at the same concentration. Osteoclastogenesis consists of multiple steps such as differentiation of osteoblast/stromal cells/osteoclast progenitors and is regulated by many factors. Because our data are limited, the mechanisms by which ICM0201 inhibits osteoclastic bone resorption are not clear. However, ICM0201 may be useful for therapy and prevention of diseases affected by osteoclast such as rheumatoid arthritis due to inhibition of osteoclastogenesis. Further studies are necessary to elucidate the inhibitory mechanism of ICM0201 on osteoclastogenesis.

Acknowledgments

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