Jpn. J. Pharmacol. **86**, 86 – 96 (2001)

Incadronate and Etidronate Accelerate Phosphate-Primed Mineralization of MC4 Cells via ERK1/2-Cbfa1 Signaling Pathway in a Ras-Independent Manner: Further Involvement of Mevalonate-Pathway Blockade for Incadronate

Takashi Fujita, Nobuo Izumo, Ryo Fukuyama, Toru Meguro, Chigusa Yasutomi, Hiromichi Nakamuta and Masao Koida*

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata 573-010, Japan

Received October 26, 2000 Accepted February 22, 2001

ABSTRACT—Two types of bisphosphonates (BPs), incadronate (INC) and etidronate (ETI) accelerated phosphate (Pi)-primed mineralization of MC4 cells in a subnanomolar dose range. Intracellular signaling pathways involved were examined. 1) The effect of INC but not ETI was partially suppressed by two mevalonate (MVA) pathway metabolites, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). 2) The BP-like accelerating effect was produced by statins and also by Toxin B, a Rho GTPases-specific inhibitor. 3) INC induced Cbfa1-nuclear localization within hours; and in an in vivo experiment using ovariectomized mice, its 3 weeks dosing exhibited the same effect in tibial extracts. 4) BPs promoted luciferase expression in murine p1.3-osteocalcin gene 2-luc and p6-osteoblast specific element 2-luc transfected cells, just as MVA, FPP and GGPP did independently and additively to INC. 5) BPs activated extracellular signal-regulated kinase (ERK1/2) in a Ras-independent manner within 5 min, and Pi was found to sensitize MC4 cells to BPs. MVA and its metabolites also activated ERKs but in a Ras-dependent manner and additively to INC. Ras dependency was determined using N17Ras-transfected cells. A MEK (MAP kinase-ERK kinase)-specific inhibitor PD98059 alone partly and with FPP completely blocked INC-induced mineralization. The results suggest that BPs act on Pi-sensitized MC4 cells to accelerate mineralization via nonRas-MEK-ERK1/2-Cbfa1 transactivation pathway and INC additionally acts by inhibiting the MVA pathway.

Keywords: Bisphosphonate, Extracellular signal-regulated kinase, Runx2/Cbfa1, Ras, Statin

Bisphosphonates (BPs) are a major class of drugs for the treatment of bone diseases in which excessive osteoclastic activity is a prominent pathological feature, with a pharmacological profile that they are preferentially taken up by osteoclasts and directly inhibit osteoclast recruitment, differentiation and resorptive activity (see ref. 1). Recent biochemical studies on the mechanism of action have classified BPs into two groups: 1) nitrogen (N)-containing BPs that act by inhibiting farnesyl diphosphate synthase in the mevalonate (MVA) pathway which is essential for prenylation of the osteoclastic Ras family of small GTPases (2 – 9) and 2) non-N-containing BPs that are metabolically transformed into ATP analogs cytotoxic to osteoclasts (10 – 13).

*Corresponding author. FAX: +81-72-866-3108
E-mail: koida@pharm.setsunan.ac.jp

So far, clinical trials mainly on a N-containing BP, alendronate, have accumulated evidence showing that BPs almost freeze bone remodeling by its anti-osteoclastic action while keeping osteoblastic mineralization rate normal (14 – 20), but no convincing explanation appears to have been presented to explain for "up to 50% drop in bone fracture rate from several % increase in bone mineral density (BMD)" (21). This has led us to assume that under the influences of BPs, microarchitectually tougher bones may be built by BP-affected osteoblastic lineage of cells. However, we have only a limited number of studies about BP actions on the osteoblastic lineage. Tsuchimoto et al. (22) first noted that alendronate potentiated calcitriol-stimulated mineralization of human osteoblastic cells in subnanomolar concentrations. Giuliani et al. (23) reported that etidronate (ETI) and alendronate stimulated the formation of colony-forming units for fibroblasts and colony-forming units of

osteoblasts in murine and human bone marrow cultures. Lately Plotkin et al. (24) reported that BPs constantly prolonged the life spans of osteocytes and osteoblasts by protecting them from apoptosis, suggesting that such protection would lead to the growth of good quality of bones. Biochemically, however, we know too little about how BPs, if any do, exert the osteogenic action; such information would be essential for understanding the events that lead to such clinical improvements in fracture rate as the final goal.

In order to define the mechanism(s) underlying the osteogenic action of BPs, we developed an in vitro quick assay system of osteoblastic mineralization activitiy using MC4 cells, a subclone of MC3T3-E1 cells which rapidly differentiates and mineralizes (25), and found that when sensitized with inorganic phosphate ion (Pi), the clone quickly and sensitively responded to both incadronate (INC), a N-containing BP, and ET, a classic non-N-containing BP in subnanomolar concentrations, resulting in accelerated mineralization. As intracellular signaling pathways that may amplify the BP stimulus to the acceleration, a) the MVA pathway and b) extracellular signal-regulated kinase (ERK1/2) pathway were selected, since it is now known that statins are endowed with an intrinsic osteogenic property at least as their end effect (26) and also that Runx2 /Cbfa1, a transcription factor essential for osteoblastic bone formation (27, 28), is activated by ERK1/2 (29). The results obtained in vitro and some by in vivo experiments indicate that INC can be osteogenic by acting on both pathways but ETI only on the ERK1/2 pathway.

MATERIALS AND METHODS

Materials

The subclone (MC4) of murine calvaria derived osteoblastic cell line MC3T3-E1 was a gift of Dr. Renny T. Franceschi of University of Michigan School of Medicine (Ann Arbor, MI, USA). Female ddY mice (12-week-old) were obtained from Shimizu Experimental Supplies (Kyoto). ETI was supplied by Sumitomo Pharm. Co., Ltd. (Osaka); INC was from Yamanouchi Pharm. Co., Ltd. (Tokyo) and used as a stock solution in phosphate-buffered saline (PBS), which was adjusted in pH to 7.4 with NaOH solution and filter-sterilized (0.22 μm filter); fluvastatin was from Novartis Pharma Co., Ltd. (Tokyo), and it was converted to its active form by dissolving 5 mg in a mixture of 100 μl ethanol and 100 μl of 1 N NaOH, then mixed with 1 ml of PBS, and adjusted to pH 8.0 using 1 M HCl before filter sterilization. A stock solution of 10 mM mevalonic acid lactone was prepared by directly dissolving the solid (Sigma Chemicals Co., Poole, UK) in dry ethanol. Farnesyl pyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) in solution from Sigma were freed of solvent and used by directly suspending it in culture media immediately before use. A set of pBluescript KS(−) containing murine osteocalcin gene 2 (OG2) promoter region, p6 osteoblast-specific element 2 (OSE2)-luc and p6OSE2-mut-luc vectors (28), and anti-mouse Cbfa1 antisera (28) were kindly provided by Dr. Gerard Karsenty of Baylor University (Houston, TX, USA). Ras dominat negative form N17 Ras was a gift from Dr. Daniel Altschuler of University of Pittsburgh (Pittsburgh, PA, USA). Y27632 ((+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride, monohydrate), a Rho-dependent kinase inhibitor (30), was supplied by Welfide (Osaka); and the Rho GTPases-specific inhibitor, Toxin B, from *Clostridium difficle* (31) was a gift of Dr. Holger Barth of Albert-Ludwigs University (Freiburg, Germany). The MEK (MAP kinase-ERK kinase)-specific inhibitor PD98059 (2'-amino-3-methoxyflavone) (32) was obtained from Promega (Madison, WI, USA). Unless otherwise described, all the other reagents were purchased from either Nacalai Tesque (Kyoto) or Wako Pure Chemicals (Osaka).

Cell culture

Unless otherwise specified, MC4 cells were cultured in α-minimum Eagle's medium (α-MEM) (Gibco BRL, Rockville, NY, USA) supplimented with 10% fetal calf serum (FCS) (Gibco BRL) and a mixture of antibiotics (Gibco BRL) in tissue culture plates (6-well for protein analyses or 48-well for assays of calcium deposition and alkaline phosphatase (ALP); Iwaki, Tokyo) at a density of 5×10^4 cells/ml (medium volume/well: 2 ml for 6-well and 0.25 ml for 48-well) at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. The medium was renewed every other day. Subculturing was performed until passage 15. After confluence, cells were pre-cultured in the media containing 50 μg/ml ascorbic acid (AA) for 8 days and used for experiments.

Measurements of calcium deposition and ALP activity

Extracellular deposits in wells were decalcified with 0.6 M HCl for 24 h and calcium contents in the supernatants were estimated colorimetrically by *o*-cresolphthalein complexion method (Calcium C-test, Wako). For the measurement of ALP activity, cells were sonicated in 0.1 M Tris-Cl buffer (pH 7.2) containing 0.1% Triton X-100, and the enzyme activity was determined using *p*-nitrophenylphosphate as a substrate in 50 mM 2-amino-2-methyl propanol and 2 mM $MgCl_2$ (pH 10.5) from absorbancy of released *p*-nitrophenol at 410 nm. Protein concentration was quantified by the BCA protein assay kit (Pierce Chemicals Co., Rockford, IL, USA).

Preparation of nuclear extracts and gel retardation assays

Nuclear extracts were prepared and gel retardation

assays conducted as described by Dignam et al. (33) with minor modification. Cells were homogenized using a Dounce homogenizer with a B-type pestle in 4 vol of buffer A (10 mM HEPES-NaOH (pH 7.9) containing 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 μg/ml each of the protease inhibitors (*p*-amidinophenyl methanesulfonyl fluoride, leupeptin, pepstatin A and aprotinin)) and were mixed with NP-40 to 0.6%. After 5 min standing on ice, the homogenates were centrifuged at $20,000 \times g$ for 5 min. The supernatants were adjusted to buffer B by adding a stock solution of buffer B and stored as the cytosolic fractions at −80°C for analysis. The pellets were resuspended with 4 vol of buffer B (50 mM Tris-HCl buffer of pH 7.5 containing 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol and protease inhibitors). After 30 min standing on ice, the nuclear extracts were separated by centrifugation at $20,000 \times g$ for 20 min and stored at −80°C. All procedures were performed at 4°C. The nuclear and cytosolic extracts of mice tibiae samples were similarly prepared from the homogenates of one fourth from the proximal end. Protein concentration was determined as described above. Double stranded oligonucleotides were individually labelled by [α-^{32}P]-dATP using the klenow fragment and purified by a Nick column. Each DNA binding reaction mixture contained poly(d[I-C]) (Amersham Pharmacia Boitech, Buckinghamshire, UK), 3000 – 6000 cpm of labelled DNA fragments, 5 μg of nuclear extracts, and a buffer composed of 10 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1 mM EGTA and 10% glycerol. For supershift assays, cellular extracts were incubated with anti-mouse Cbfa1 antisera at 4°C for 30 min prior to the addition of labeled probe.

Production of ovariectomized osteopenic mice, INC treatment and dual-energy X-ray absorptiometry

After a 1-week acclimation to feeding conditions, 15 female ddY mice (13-week-old) were divided into three groups (n = 5/group), sham-operated (SHAM), ovariectomized (OVX), and OVX + INC, to receive sham operation or ovariectomy as described previously for rats (34, 35). Starting 2 weeks later, the OVX group was s.c. treated with vehicle alone and the OVX + INC group with INC (0.1 mg/kg), every other day for 3 weeks. Mice were weighed weekly and the dose was adjusted to the actual body weight. Mice were killed by cervical dislocation for bone sampling. Bone mineral content (BMC) and BMD of a whole bone were determined by dual-energy X-ray absorptiometry (DXA). Bones were placed and scanned by a line spacing of 0.508 mm, a point resolution of 0.005 mm, and analyzed by the regional high resolution analysis program for small animals (DCS-600; Aloka, Tokyo).

DNA construction, transfection and luciferase assay

The pBluescript KS(−) containing mOG2 promoter region was double-digested by KpnI and HindIII and subcloned in the pGL3 promoterless luciferase expression vector. The day before transfection, cells were plated on 35-mm dishes at a density of 10^5 cells/ml and then transfected by FuGENE 6 (Roche, Mannheim, Germany) according to the manufacturer's protocol, using 10 μg of reporter plasmid constructs and 0.1 μg of pRL-TK vector, which was used to normalize the transfection efficiency between different experiments. Stable transformants were prepared by multiple transfections using the vector containing both green fluorescent protein and neomycin resistant gene, pEGFP-N1 (Clontech Lab., Inc., Valencia, CA, USA), and luciferase vector or N17Ras mammalian expression vector. Subconfluent cells were trypsinized and plated at low density before selection, which was performed by culturing them in the presence of 400 μg/ml of neomycin (G418) for 3 weeks. Before use, transformants were precultured in AA containing medium for 8 days and then incubated in AA plus Pi (3 mM) for a certain period. Cells were harvested by scraping them into 0.5 ml of Passive Lysis Buffer (Promega) and lysed by three cycles of freezing-thawing. Luciferase activities were assayed by using Luciferase Reporter Assay System (Promega) and a model TD20/20 luminometer (Promega), as described (36).

Immunoblotting of activated ERK1/2

Cells were solubilized in Tris-HCl buffer (pH 6.8) containing 3% sodium dodecylsulfate (SDS) and 10% glycerol, and the protein contents were estimated as described above. The sample was mixed with 0.1% bromphenol blue and 0.05% 2-mercaptoethanol, boiled for 5 min and then loaded (equal amount of protein/lane) on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane for immunoblotting, using antibodies toward phospho-p44/42 ERK1/2, phospho JNK, phospho p38 and horseradish peroxidase-conjugated anti-rabbit antibody (New England Biolab, Beverly, MA, USA), as described previously (37).

Statistical analyses

Unless otherwise described, statistical analyses were performed using Student's *t*-test. At $P<0.05$, the difference was considered to be significant.

RESULTS

Pi-primed mineralization of MC4 cells and accelerating effects of BPs

During 8 days preculture in the presence of AA (50 μg/ml) and 1 mM Pi, no nodule formation occurred and ALP activity remained low, but thereafter by increasing Pi con-

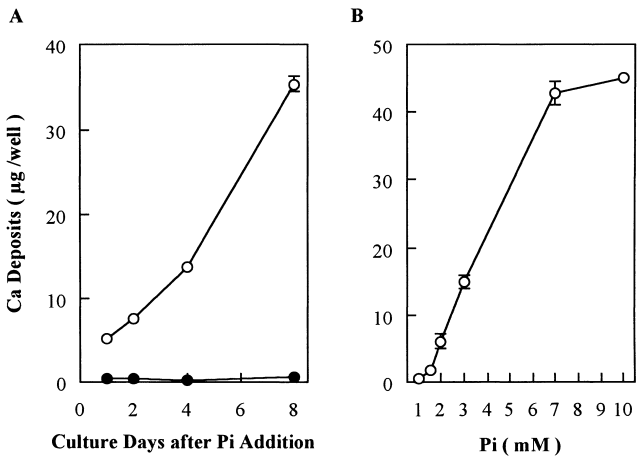


Fig. 1. Pi-induced mineralization of MC4: time course and Pi-dose-dependency. After 8 days preculture in a conventional medium that contained 1 mM Pi, MC4 cells were cultured in panel A, 1 mM (closed circles) or 3 mM (open circles) Pi-containing medium for 1 to 8 days, and in panel B, from 3 to 10 mM (open circles) Pi-supplemented medium for 4 days. Ca deposition was monitored by measuring total Ca content per well (48-well plate). AA (50 μg/ml) was present throughout preculture to Ca measurements. Panel A shows culture time-dependent increase of Pi-induced mineralization and panel B shows Pi dose-dependent increase, which plateaued with 7 mM. Results are means ± S.E.M. of 6 – 12 wells obtained from 3 – 5 separate experiments.

centration to 3 mM, cells initiated mineralization within 24 h which further increased over time (Fig. 1A). Pi effect developed concentration-dependently up to 7 mM (Fig. 1B). ALP activity was also enhanced similarly (data not shown).

When assayed on a 4 day-culture system in the presence of AA and 3 mM Pi, both INC and ETI in the same dose range (10^{-11} to 10^{-7} M) dose-dependently accelerated Pi-primed mineralization (Fig. 2A) and ALP expression (data not shown). Without Pi increase, neither mineralization nor drug effect have ever developed within this period (data not shown). To be noted is a small difference between INC and ETI in the height of response (Fig. 2A, $P<0.05$ at 1 and 100 nM).

The results in Fig. 2, B and C, show that the effect of INC (1 nM) but not ETI (1 nM) was partially suppressed by the downstream metabolites of the MVA-pathway, FPP and GGPP, indicating that INC would be osteogenic at least in part by down-regulating the MVA pathway, and the lack of this action would have given the lower dose-response curve for ETI in Fig. 2A. The past biochemical studies of the BP effects on osteoclastic cells (refer to ref. 1) have identified that the N-containing BPs inhibit the MVA pathway at its entrance, limiting the supply of FPP and GGPP, which is indispensable for prenylation of Rho GTPases participating in intracellular signal transduction. In contrast, so far, no biochemical study on BP-effects on osteoblastic cells has been reported. Thus, first of all, we attempted to pharmacologically characterize the effect of BPs on Pi-primed mineralizaiton using specific inhibitors of intracellular signaling pathways.

Figure 3 shows that similar enhancement of BP-induced mineralization was produced by a Rho-specific inhibitor Toxin B (200 pg/ml), mevastatin (10 μM) and fluvastatin (10 μM), but not by a MEK-specific inhibitor PD98059 (50 μM) and a Rho-dependent protein kinase inhibitor Y27632 (50 μM), implying that INC may down-regulate the activity of Rho GTPases by limiting FPP and/or GGPP supply for prenylation, just as statins that were also found to accelerate Pi-primed mineralization.

Stimulation by INC of Cbfa1 nuclear localization in vitro and in vivo

Cbfa1 is a transcription factor essential for osteoblastic bone formation (27, 41) and we examined how Pi and/or INC would affect the nuclear translocation of Cbfa1. As seen in Fig. 4A and reported separately (39), on 4-day culture in the presence of AA and 3 mM Pi, Pi quickly drove the leptomycin-sensitive nuclear export of Cbfa1, which thereafter kept the cell nuclei free of the transcription factor and INC potentially reversed the effect of Pi.

A similar observation was also made in an in vivo experiment. When dosed to OVX mice starting 2 weeks after operation for 3 weeks, as summarized in Table 1, INC efficiently protected the animals from developing osteopenia, in terms of retaining BMD which otherwise fell. Though the data is not shown, the in vivo effectiveness of the drug has been estimated to be protective in a dose range of 0.1 mg/kg per 2 days, s.c. or higher. When nuclear Cbfa1 binding was compared among 3 groups in Table 1, the results in Fig. 4B were obtained indicating that INC up-regulated Cbfa1 nuclear binding in vivo as it did in vitro.

Stimulation of p1.3mOG2-luc and p6OSE2-luc expression by INC and/or MVA-pathway metabolites

Next, it was examined if nuclear Cbfa1 increased by BPs would have been transactivated or not by measuring the expression level of osteocalcin gene, which is directly controlled by Cbfa1 (28). Such transfected cells were found to respond well to either BP and/or MVA-pathway metabolites (Fig. 5). In this reporter assay, INC and ETI were equipotent. All the metabolites of the MVA pathway were also active, and those effects appeared to be additive to that of INC or ETI (Fig. 5B). In addition, it was found that PD98059, a MEK-specific inhibitor, was able to completely inhibit the effect of both INC and ETI, suggesting that BPs would first activate ERK1/2, which then would effect the transactivation of Cbfa1. Thus, as the next step, we attempted to see directly how Pi, BPs and the MVA metabolites would activate ERK1/2.

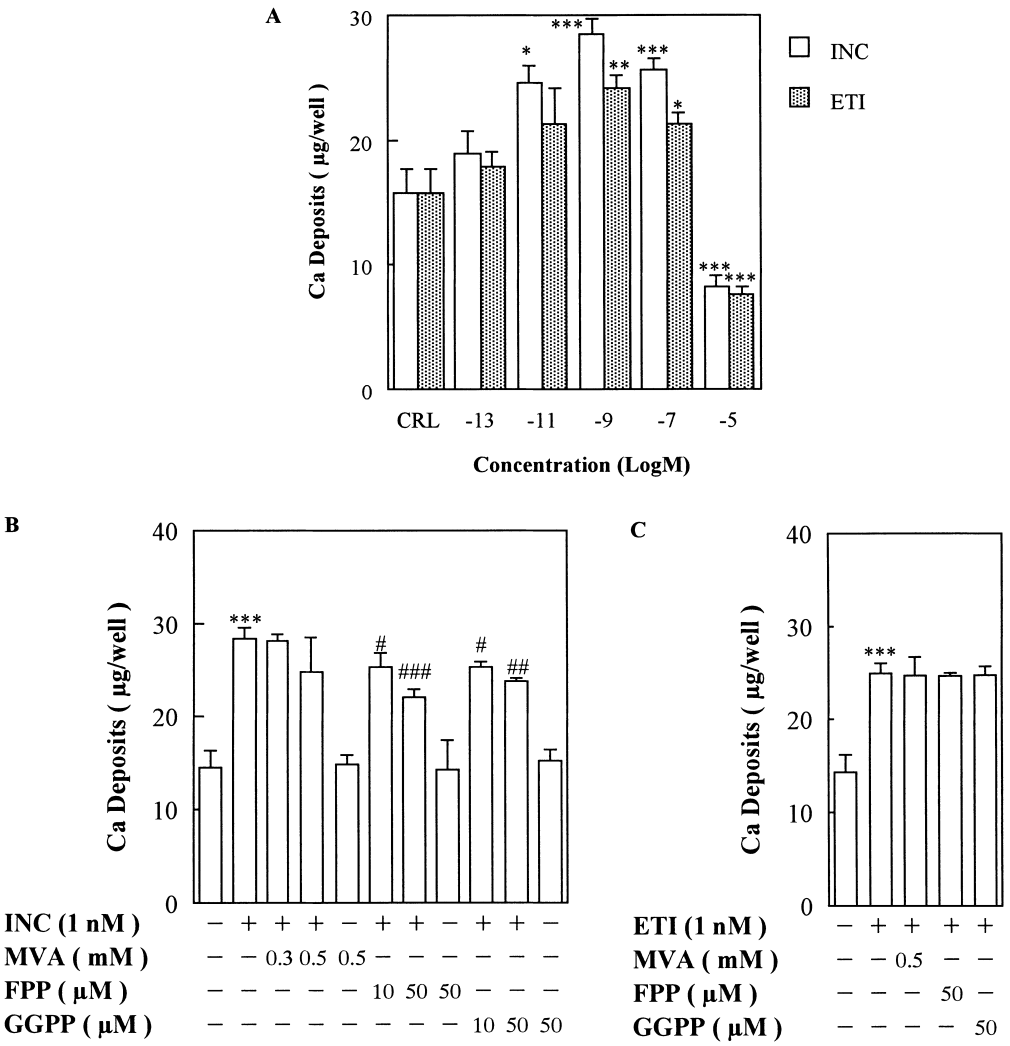


Fig. 2. Promoting effect of INC and ETI on Pi-primed mineralization and influences of the MVA pathway metabolites. In panel A, MC4 cells were cultured with INC or ETI (0.1 pM to 10 μM) in 3 mM Pi and AA (50 μg/ml) containing medium for 4 days and Ca deposits were measured. Both drugs dose-dependently (up to 1 nM) accelerated Pi-primed mineralization, but INC was more potent than ETI in the effects by 1 or 100 nM (P<0.05). Significantly different from the control (CRL): *P<0.05, **P<0.01 and ***P<0.001. In panels B and C, the effect of INC but not ETI, was partially suppressed by either FPP or GGPP. Control mineralization (INC: –) was not affected by any of the MVA pathway metabolites. For other experimental details, refer to Fig. 1 and Materials and Methods. Significantly different from INC (–) or ETI (–): ***P<0.005; significantly different from 1 nM INC alone: $^{\#}P$<0.05, $^{\#\#}P$<0.01 and $^{\#\#\#}P$<0.005. n = 5 – 10.

Activation of ERK1/2 by BPs and/or MVA-pathway metabolites

After 8 days preculture under the standard condition, MC4 cells were transferred in FCS-free medium in order to avoid the influences of serum factors including the MVA pathway metabolites, and the effects of BPs and/or the MVA-pathway metabolites on ERK1/2 activation were examined. Both INC and ETI rapidly stimulated ERK1/2 activation (Fig. 6A), without affecting JNK and p38 activities (Fig. 6B), giving mutually indistinguishable time courses and dose-dependent responses (Fig. 6C) (the data of dose-dependency of ETI effect not given). Each MVA pathway metabolite was found to be the intrinsic activator of ERK1/2 and their effects developed additively to that of BPs (Fig. 6D). In contrast, but as expected, mevastatin was an inhibitor to lower the basal activity of ERK1/2 of MC4 cells, which now would have lost the supply of the MVA pathway metabolites from FCS. A novel finding was that 3 mM Pi alone had no effect on ERK1/2 activity of MC4 cells but sensitized cells to the ERK1/2 activating effect of BPs (Fig. 6A).

As shown in Fig. 6D, the effect of INC to stimulate ERK1/2 activation was found to be PD98059-sensitive and then the effect of the inhibitor on BPs-accelerated

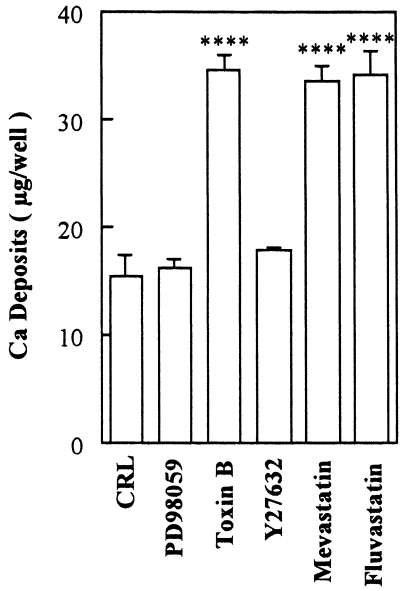


Fig. 3. Effects of various enzyme inhibitors on 3 mM Pi-primed mineralization. After 8 days preculture, MC4 cells were cultured with PD98059 (50 μM), Toxin B (200 μg/ml), Y27632 (50 μM), mevastatin (10 μM) or fluvastatin (10 μM) in AA plus 3 mM Pi-containing medium for 4 days before measurement of Ca deposits. For other experimental details, refer to Fig. 2 and methods. n = 5 × (6 – 12 wells). Significantly different from CRL (control): ****P<0.001.

mineralization was examined as described in Fig. 3. The action of INC was largely but not completely inhibited by PD98059 (Fig. 6E) and the inhibition appeared to be completed by FPP (Fig. 6E), while the inhibitor alone was able to completely suppress the effect of ETI.

Dominant negative study on Ras-dependency of ERK1/2 activation by BPs and MVA pathway metabolites

Finally, we prepared N17Ras-transfected cells and attempted to determine whether or not Ras would be involved in the upstream of the MEK-ERK1/2 route. Both BPs were found to activate ERK1/2 phosphorylation in N17Ras-transfected cells as they did in the original cells, while the effect of the MVA pathway metabolites (only the data of GGPP presented) was suppressed (Fig. 7A) by the transfection, demonstrating that BPs activate the MEK-ERK1/2 route in a Ras-independent manner, while the MVA pathway metabolites activate it in a Ras-dependent manner.

DISCUSSION

As compared in Fig. 2A, both ETI and INC gave dose-response curves that mutually overlap in the effective dose range but appear to differ in height. The minimum effective concentration (MEC) was the same for both BPs, 0.01 nM, which is close to the ones noted with alendronate on dexamethasone-induced mineralization of human bone marrow osteoblasts (23) and on 1,25$(OH)_2D_3$-induced mineralization of femur marrow osteoblasts (22). Such overlapping in the dose-response curves between the N-containing and the Non-N-containing BPs has been reported for their anti-apoptotic effect on MLO-Y4 cells (24). These results taken together indicate that both BPs would exert an osteogenic effect in a subnanomolar concentration range on the osteoblastic lineage of cells (osteoblasts and osteocytes), which would be clinically attainable by therapeutic doses. In so far as this effect is concerned, the two types of BPs do not practically differ, although it has been well documented

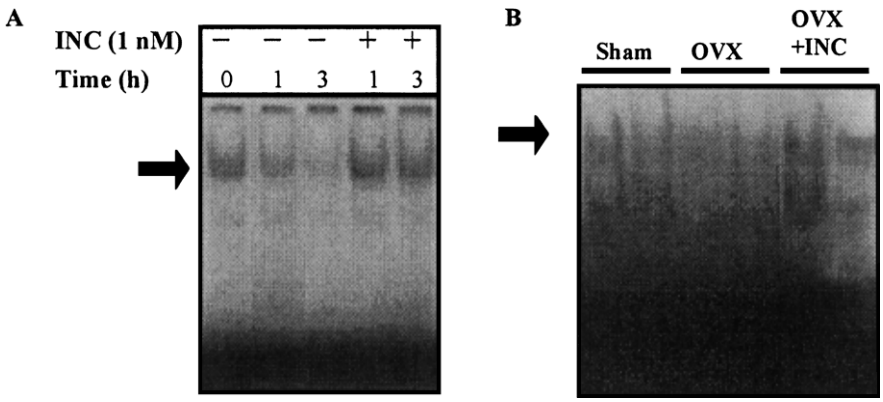


Fig. 4. INC promotes Cbfa1 nuclear localization in vitro and in vivo. In panel A, MC4 cells after 8 days preculture were exposed to AA and 3 mM Pi with or without INC (1 nM), and changes in nuclear Cbfa1/OSE2 binding were monitored by gel retardation assays. In the absence of INC, the nuclear level of Cbfa1 at time 0 (lane 1) decreased with time (lane 2: 1 h and lane 3: 3 h), while INC increased the level (lane 4: 1 h and lane 5: 3 h). A similar result was obtained with 100 nM INC. In panel B, nuclear Cbfa1 binding in the proximal tibiae from Sham or OVX + Vehicle groups in Table 1 was detectable as weak signals and the binding was up-regulated in the OVX + INC group. n = 5. Throughout this experiment, 5 μg as protein of a sample was applied per lane for electrophoretic analysis.

Table 1. Protecting effect of INC dosing (0.1 mg/kg per 2 days, s.c., 3 weeks) on OVX-induced osteopenia in mice

Treatment	Tibial BMC (mg)	Femoral BMC (mg)
Sham + Vehicle	26.33 ± 0.51	31.74 ± 1.16
OVX + Vehicle	23.90 ± 1.24	26.57 ± 1.36††
OVX + INC (0.1 mg/kg per 2 days)	27.97 ± 0.87**	34.78 ± 1.27**

Treatments with vehicle or INC started 2 weeks after operations. Each value represents the mean ± S.E.M. (n = 5). $^{\dagger\dagger}P<0.01$ vs Sham + Vehicle; $^{**}P<0.01$ vs OVX + Vehicle, as analyzed by Fischer's least significant test.

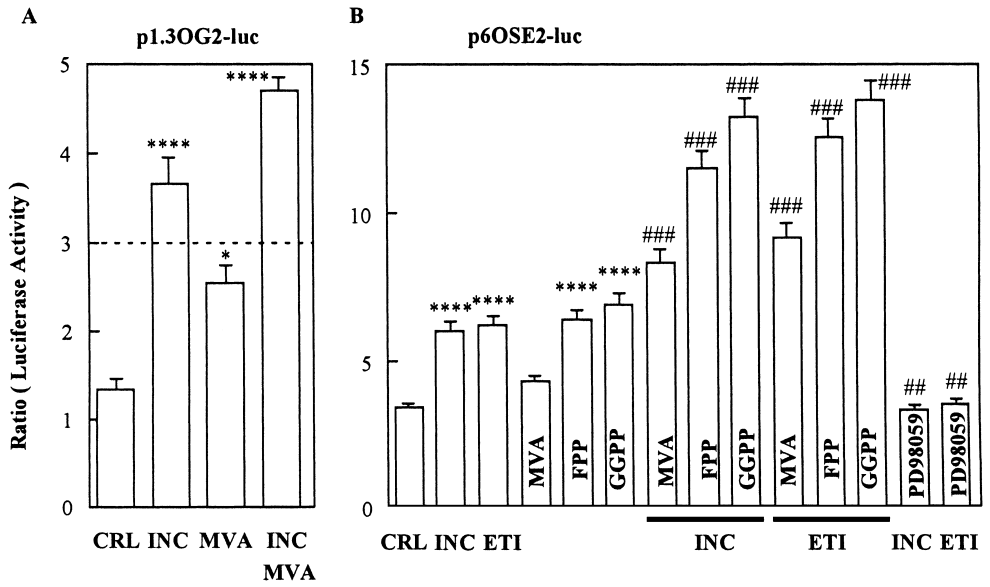


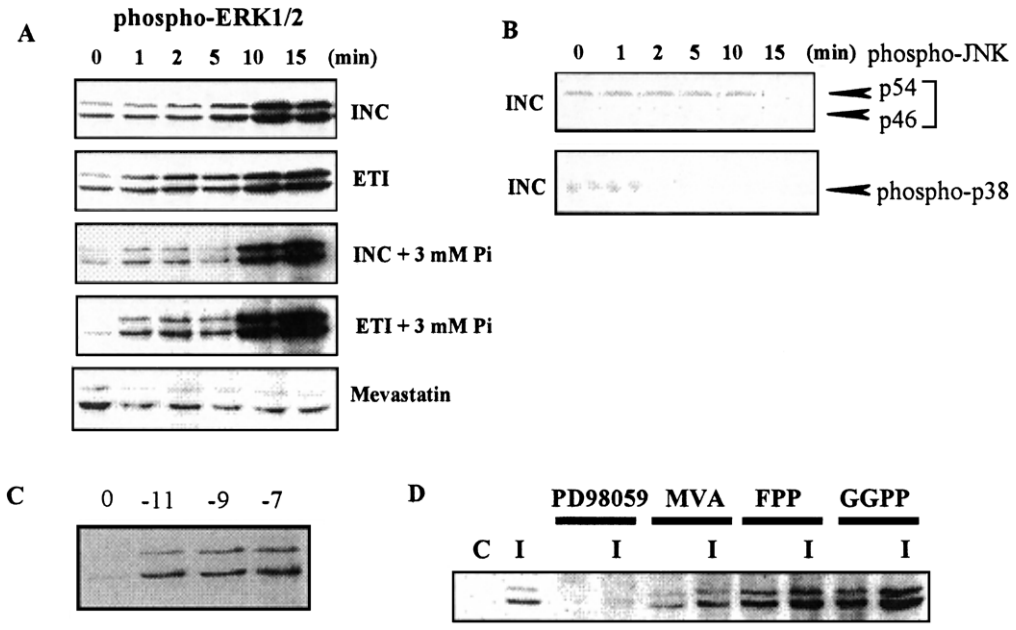
Fig. 5. INC, ETI and MVA pathway metabolites accelerate the luciferase expression of p1.3OG2-luc and p6OSE2-luc transfected MC4 cells. MC4 cells stably transfected with p1.3OG2-luc, p6OSE2-luc or p6OSE2mut-luc retained the ability to exhibit a low but detectable level of constitutive luciferase activity even after 8 days preculture, although the initial levels (the dotted lines) decreased to the levels of each CRL (control) during preculture. Cells were then cultured with INC (1 nM), ETI (1 nM), MVA (0.5 mM), FPP (50 μM) and GGPP (50 μM) separately or additively for 4 days and then the luciferase expression was compared. The expression of the genes except p6OSE2mut-luc were found to be stimulated with either INC, ETI or each metabolite, and the effect of each metabolite appeared to be additive to that of INC or ETI. Either INC or ETI action was completely blocked by PD98059. Results are means ± S.E.M. of triplicate assays obtained in 3 independent transfection experiments. Significantly different from CRL: $^{*}P<0.05$, $^{****}P<0.001$; from INC or ETI alone: $^{\#\#}P<0.01$, $^{\#\#\#}P<0.005$.

for their anti-osteoclastic properties that they significantly differ not only in the anti-osteoclastic potency but also in the action mechanism (1).

To be noted is the suppressive effect on mineralization of both BPs in their micromolar dose ranges. The same effect has been observed in vitro with mouse bone calvaria-derived osteoblasts and also with mouse femoral marrow-derived osteoblasts (data not shown). This effect may be related with clinical findings (14) obtained by using the highest dosage of alendronate in which the N-containing BP appears to lack a consistent dose-dependency and to depress osteoblastic osteogenesis, as has been observed in vitro (22, 23). Future detailed studies in vivo to find an osteogenic-active dose range of BPs may clarify the nature of the osteostatic effect that may result from the anti-apoptotic effect on the osteoblastic lineage of cells (24).

To our knowledge, so far, almost nothing is known about the mechanism by which BPs, if any, exert direct osteogenic action on osteoblastic cells not only in vivo but also in vitro. The biochemical study described herein is the first report on the signaling pathway that would be involved in such osteogenic action of BPs, especially since no concerted study on the action in vivo and in vitro has ever been reported.

First of all, the studies in Figs. 2 and 3 provided a series of evidence suggesting that the osteogenic action of INC, though in a minor part, does derive from its inhibitory effect on the MVA pathway, which was absent in the action of ETI; and as has been observed with osteoclasts, the inhibition may indirectly lead to the blockade of the intracellular signaling pathway by limiting the supply of FPP and/or GGPP (e.g., Rho small GTPases functionally need



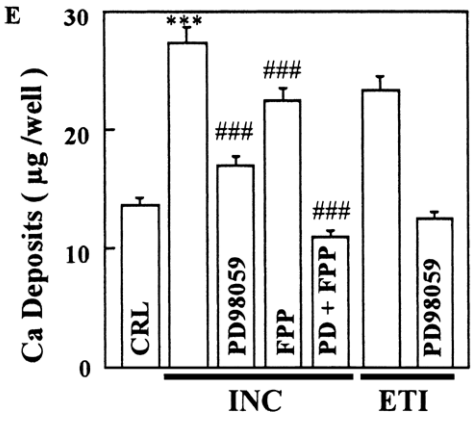


Fig. 6. BPs equally stimulate PD98059-sensitive activation of ERK1/2 (but not JNK or p38), time- or dose-dependently and additively to each MVA metabolite, although their effects on mineralization differ in PD98059 sensitivity. In panels A to D, after 8 days preculture, MC4 cells were transferred in FCS-free medium and then used for this experiment 24 h later. Unless otherwise indicated, the FCS-free medium contained AA plus 1 mM Pi. In panel A, cells were incubated with INC (1 nM), ETI (1 nM) or mevastatin (10 μM) for 0 to 15 min and the phosphorylation was monitored using phospho-specific antibody. INC and ETI stimulated phosphorylation time-dependently and the stimulation was potentiated by 3 mM Pi, although 3 mM Pi alone had no effect on phosphorylation (data not shown). In contrast, mevastatin inhibited phosphorylation. In panel B, both INC (1 nM) and ETI (1 nM) failed to affect phosphorylation of either JNK or p38 (the data of ETI not presented). In panel C, the dose-dependency of the INC effect was monitored and ETI gave a comparable pattern (data not shown). Panel D compares the individual or INC-additive effect of MVA (0.5 mM), FPP (50 μM) and GGPP (50 μM), which was measured after 5-min incubation. The effect of INC (I: 1 nM) appeared to be additive to that of each MVA-pathway metabolite, which was also intrinsically active. Panel C is the control. PD98059 completely inhibited the effect of BPs (data of ETI was not shown). For panels A to D, each lane contained a sample of 50-μg protein for the electrophoretic analysis. Panel E shows that the effect of INC (1 nM) on mineralization was partially inhibited by FPP (50 μM) or PD98059 (50 μM) and completely by both, but that of ETI (1 nM) was completely inhibited by PD98059 alone (refer to Fig. 3 for experimental conditions). Significantly different from CRL: $^{**}P<0.01$, $^{***}P<0.005$; from INC or ETI alone: $^{\#\#\#}P<0.005$.

GGPP for its geranylgeranylation). In fact, statins may be osteogenic because they inhibit the MVA pathway to supply GGPP, while Toxin B was osteogenic through direct inhibition of Rho small GTPases.

Second, the studies in Figs. 4 and 5 revealed that, among the fractions consisting of the osteogenic action of BPs, the main fraction equally shared by INC and ETI would be exerted by the transactivation of Cbfa1, which should be effected exclusively via the activation of MEK, since a MEK-specific inhibitor, PD98059, completely blocked BPs-induced Cbfa1 transactivation.

Third, the results summarized in Fig. 6 were able to identify ERK1/2 as the main entrance where the signals flow in from the PD98059-sensitive MEK route activated by either BPs or the MVA metabolites, although the activation mechanisms of MEK was not be the same as later observed.

On the other hand, either JNK or p38 was not involved in the MEK-ERK1/2 route (Fig. 6B). Mevastatin inhibited the basal level of ERK1/2 phosphorylation possibly by completely stopping the supply of FPP and GGPP under the serum -free culture condition, indicating that statins and BPs exert their osteogenic action through different signaling pathway (Fig. 6A). The results in Fig. 6D appear to

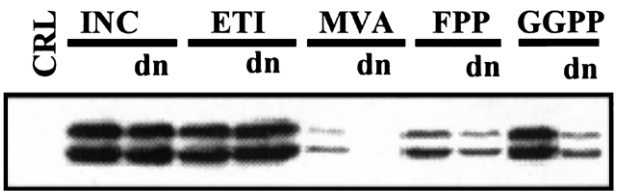


Fig. 7. INC and ETI activate ERK1/2 in a Ras-independent manner but activate the MVA pathway metabolites in a Ras-dependent manner: a study using N17Ras-transduced MC4 cells. N17Ras-transduced MC4 cells were produced as described in Materials and Methods, and the subsequent procedures were the same as those for Fig. 6A. Either INC (1 nM) or ETI (1 nM) stimulated ERK1/2 phosphorylation in N17Ras-transduced cells (dn), as they did in the original cells, while the effect of each MVA pathway metabolite diminished in dn. Throughout this experiment, each lane contained a sample of 50-μg protein for the electrophoretic analysis.

reflect that at least two different signal transduction routes are involved in the osteogenic effect of INC; the one via MEK was inhibited by PD98059 and the rest via the inhibition of the MVA pathway, which was reversed by FPP.

Fourth, a solid information of the confluence in the upstream of MEK came from a study in Fig. 7 using N17 Ras transfected cells, indicating that the signal of BPs mainly takes a Ras-independent route in the upstream of MEK-ERK1/2, while the signal of the MVA pathway metabolites would go through a Ras-dependent route, although both signals would interflow in MEK in the downstream to inducing Cbfa1 transactivation, which led to accelerated mineralization as the final effect of BPs but not of the MVA pathway metabolites.

Another novel finding to be commented on would be that Pi sensitized MC4 cells toward ERK1/2 activation by both BPs (Fig. 6A). For long, it is known that supplementation of culture medium with β-glycerophosphate or directly with Pi would be helpful in inducing early calcification of bone cells within a practical culture time. Lately, Beck et al. (38) reported that Pi acts as an extracellular signal to express osteopontin gene, although the signal transduction system of the Pi effect has not been identified yet and we do not know yet how such sensitization would be related with activation of specific genes. In addition, we have observed as reported more recently (39) that in a variety of clonal bone cells (osteoblastic MC3T3-E1, chondrocytic ATDC5 and osteocytic MLO-Y4), 3 mM Pi as an extracellular signal acts as a speedy driver of the nuclear export (not import) of Cbfa1 completing within an hour in a Leptomycin B-sensitive manner (refer to ref. 40); and a day later, Pi-accelerated mineralization takes place in the osteogenic cells, which keeps Cbfa1-free nuclei. Some experimental attempts to explore how such Cbfa1-free mineralization could occur and how Pi-induced sensitization of ERK1/2 activation by BP observed herein would be related with such typical nuclear export are now underway.

Although we have not yet succeeded in characterizing the BPs-specific receptor in bone cells, the first biochemical study described herein revealed that the main osteogenic signal triggered commonly by both BPs would be transduced in the following order: 1) entering the Pi-sensitized cells via a Ras-independent pathway to activate the MEK $\rightarrow$ ERK1/2 route within minutes, 2) enhancing Cbfa1 transactivation within hours, 3) initiating Cbfa1-dependent accelerated mineralization within a day. The signal by the MVA pathway metabolites also enters the same sequence of signal transduction, but via a Ras-dependent route.

The osteogenic property of statins was first introduced by Mundy et al. (26) based on their activity to induce the expression and autocrine of bone morphogenetic protein-2 (BMP-2) in an osteoblastic cell line (hMG-63). Lately *Runx2* has been identified as the major target gene shared by the TGF-β and BMP-2 signaling pathway and the coordinated action of Cbfa1 and BMP-activated Smads has been suggested to induce the osteoblastic differentiation (41, 42). In this experiment, the effect of statins was found to be biphasic: additive to the osteogenic effect of INC alone as seen in Fig. 3, possibly by limiting the supply of the MVA pathway metabolites, but inhibitory on the signal transduction for the osteogenic effect shared by INC and ETI as seen in Fig. 6. It is possible that BMP-2 would be involved somehow in the osteogenic action of BPs, but at present, it looks premature to discuss about the involvement of BMP-2 in the action of BPs, especially since we have not yet systematically explored such a possibility.

Figure 8 summarizes the signal transduction route for the two BPs identified in this experiment. In route A, INC inhibits the MVA pathway, which could then decelerate protein modification by the MVA pathway metabolites (e.g., geranylgeranylation of Rho small GTPases); and in

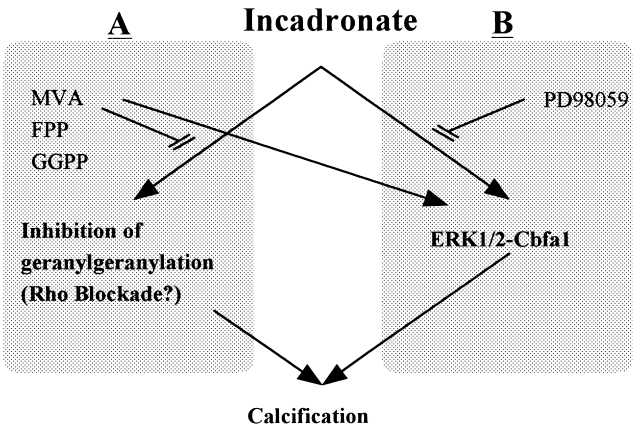


Fig. 8. A schematic presentation of two signal transduction routes by which INC accelerates mineralization of Pi-sensitized MC4 cells.

route B, both INC and ETI commonly activate ERK1/2 via a Ras-independent route in Pi-sensitized MC4 cells, which then transactivate Cbfa1. On the other hand, each MVA pathway metabolite tested herein triggers a signal via route B that enters the Ras → MEK → ERKs route, which is linked with Cbfa1 transactivation, but due to the counteracting effect to reverse INC-effect in route A, each metabolite can exhibit an inhibitory action on INC, but not on ETI-accelerated osteogenesis.

REFERENCES

1 Russell RG, Rogers MJ, Frith JC, Luckman SP, Coxon FP, Benford HL, Croucher PI, Shipman C and Fleisch HA: The pharmacology of bisphosphonates and new insights into their mechanisms of action. J Bone Miner Res **14**, Suppl **2**, 53 – 65 (1999)

2 Amin D, Cornell SA, Gustafson SK, Needle SJ, Ullrich JW, Bilder GE and Perrone MH: Bisphosphonates used for the treatment of bone disorders inhibit squalene synthase and cholesterol biosynthesis. J Lipid Res **33**, 1657 – 1663 (1992)

3 Luckman SP, Hughes DE, Coxon FP, Graham R, Russell RG and Rogers MJ: Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. J Bone Miner Res **13**, 581 – 589 (1998)

4 Luckman SP, Coxon FP, Ebetino FH, Russell RG and Rogers MJ: Heterocycle-containing bisphosphonates cause apoptosis and inhibit bone resorption by preventing protein prenylation: evidence from structure-activity relationships in J774 macrophages. J Bone Miner Res **13**, 1668 – 1678 (1998)

5 Fisher JE, Rogers MJ, Halasy JM, Luckman SP, Hughes DE, Masarachia PJ, Wesolowski G, Russel RG, Rodan GA and Reszka AA: Alendronate mechanism of action: geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation in vitro. Proc Natl Acad Sci USA **96**, 133 – 138 (1999)

6 van Beek E, Pieterman E, Cohen L, Löwik C and Papapoulos S: Nitrogen-containing bisphosphonates inhibit isopentenyl pyrophosphate isomerase/farnesyl pyrophosphate synthase activity with relative potencies corresponding to their antiresorptive potencies in vitro and in vivo. Biochem Biophys Res Commun **255**, 491 – 494 (1999)

7 Reszka AA, Halasy-Nagy JM, Masarachia PJ and Rodan GA: Bisphosphonates act directly on the osteoclast to induce caspase cleavage of mst1 kinase during apoptosis. A link between inhibition of the mevalonate pathway and regulation of an apoptosis-promoting kinase. J Biol Chem **274**, 34967 – 34973 (1999)

8 Benford HL, Frith JC, Auriola S, Mönkkönen J and Rogers MJ: Farnesol and geranylgeraniol prevent activation of caspases by aminobisphosphonates: Biochemical evidence for two distinct pharmacological classes of bisphosphonate drugs. Mol Pharmacol **56**, 131 – 140 (1999)

9 Bergstrom JD, Bostedor RG, Masarachia PJ, Reszka AA and Rodan G: Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. Arch Biochem Biophys **373**, 231 – 241 (2000)

10 Frith JC, Mönkkönen J, Blackburn GM, Russell RG and Rogers MJ: Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5'-(beta, gamma-dichloromethylene) triphosphate, by mammalian cells in vitro. J Bone Miner Res **12**, 1358 – 1367 (1997)

11 Auriola S, Frith JC, Rogers MJ, Koivuniemi A and Mönkkönen J: Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography-electrospray mass spectrometry. J Chromatogr B Biomed Sci Appl **704**, 187 – 195 (1997)

12 Mönkkönen H, Moilanen P, Mönkkönen J, Frith JC, Rogers MJ and Auriola S: Analysis of an adenine nucleotide-containing metabolite of clodronate using ion pair high-performance liquid chromatography-electrospray ionization mass spectrometry. J Chromatogr B Biomed Sci Appl **738**, 395 – 403 (2000)

13 Rogers MJ, Xiong X, Ji X, Mönkkönen J, Russell RG, Williamson MP, Ebetino FH and Watts DJ: Inhibition of growth of *Dictyostelium discoideum* amoebae by bisphosphonates drugs is dependent on cellular uptake. Pharm Res **14**, 625 – 630 (1997)

14 Liberman UA, Weiss SR, Broll J, Minne HW, Quan H, Bell NH, Rodriguez-Portales J, Downs RW Jr, Dequeker J and Favus M: Effect of oral alendronate on bone mineral density and the incidence of fractures in postmenopausal osteoporosis. N Engl J Med **333**, 1437 – 1443 (1995)

15 Cummings SR, Black DM, Thompson DE, Applegate WB, Barrett-Connor E, Musliner TA, Palermo L, Prineas R, Rubin SM, Scott JC, Vogt T, Wallace R, Yates AJ and LaCroix AZ: Effect of alendronate on risk of fracture in women with low bone density but without vertebral fractures. JAMA **280**, 2077 – 2082 (1998)

16 Black DM, Cummings SR, Karpf DB, Cauley JA, Thompson DE, Nevitt MC, Bauer DC, Genant HK, Haskell WL, Marcus R, Ott SM, Torner JC, Quandt SA, Reiss TF and Ensrud KE: Randomised trials of effect of alendronate on risk of fracture in women with existing vertebral fractures. Lancet **348**, 1535 – 1541 (1996)

17 Chavassieux PM, Arlot ME, Reda C, Wei L, Yates AJ and Meunier PJ: Histomorphometric assessment of the long-term effects of alendronate on bone quality and remodeling in patients with osteoporosis. J Clin Invest **100**, 1475 – 1480 (1997)

18 Nevitt MC, Ross PD, Palermo L, Musliner T, Genant HK and Thompson DE: Association of prevalent vertebral fractures, bone density, and alendronate treatment with incident vertebral fractures: effect of number and spinal location of fractures. Bone **25**, 613 – 619 (1999)

19 Hochberg MC, Ross PD, Black D, Cummings SR, Genant HK, Nevitt MC, Barrett-Connor E, Musliner T and Thompson D: Larger increases in bone mineral density during alendronate therapy are associated with a lower risk of new vertebral fractures in women with postmenopausal osteoporosis. Arthritis Rheum **42**, 1246 – 1254 (1999)

20 Pols HA, Felsenberg D, Hanley DA, Stepan J, Munoz-Torres M, Wilkin TJ, Qin-sheng G, Galich AM, Vandormael K, Yates AJ and Stych B: Multinational, placebo-controlled, randomized trial of the effects of alendronate on bone density and fracture risk in postmenopausal women with low bone mass. Osteoporosis Int **9**, 461 – 468 (1999)

21 Delmas PD: How does antiresorptive therapy decrease the risk of fracture in women with osteoporosis? Bone **27**, 1 – 3 (2000)

22 Tsuchimoto M, Azuma Y, Higuchi O, Sugimoto I, Hirata N,

Kiyoki M and Yamamoto I: Alendronate modulates osteogenesis of human osteoblastic cells in vitro. Jpn J Pharmacol **66**, 25 – 33 (1994)

23 Giuliani N, Pedrazzoni M, Negri G, Passeri G, Impicciatore M and Girasole G: Bisphosphonates stimulate formation of osteoblast precursors and mineralized nodules in murine and human bone marrow cultures in vitro and promote early osteoblastgenesis in young and aged mice in vivo. Bone **22**, 455 – 461 (1998)

24 Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC and Bellido T: Prevention of osteocyte and osteoblast apoptosis by bisphophonates and calcitonin. J Clin Invest **104**, 1363 – 1374 (1999)

25 Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH and Franceschi RT: Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. J Bone Miner Res **14**, 893 – 903 (1999)

26 Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M and Gutierrez G: Stimulation of bone formation in vitro and in rodents by statins. Science **286**, 1946 – 1949 (1999)

27 Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S and Kishimoto T: Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblast. Cell **89**, 755 – 764 (1997)

28 Ducy P, Zhang R, Geoffroy V, Ridall AL and Karsenty G Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell **89**, 747 – 754 (1997)

29 Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G and Franceschi RT: MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem **275**, 4453 – 4459 (2000)

30 Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamaguchi K, Inui J, Maekawa M and Narumiya S: Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature **389**, 990 – 994 (1997)

31 von Eichel-Streiber C, Boquet P, Sauerborn M and Thelestam M: Large clostridial cytotoxins-a family of glycosyltransferases modifying small GTP-binding proteins. Trends Microbiol **4**, 375 – 382 (1996)

32 Alessi DR, Cuenda A, Cohen P, Dudley DT and Saltiel AR: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem **270**, 27489 – 27494 (1995)

33 Dignam JD, Lebovitz RM and Roeder RG: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res **11**, 1475 – 1489 (1983)

34 Fukushima T, Nitta T, Furuichi H, Izumo N, Fukuyama R, Nakamuta H and Koida M: Bone anabolic effects of PTH (1-34) and salmon calcitonin in ovariectomy- and ovariectomy-steroid-induced osteopenic rats: a histomorphometric and biochemical study. Jpn J Pharmacol **82**, 240 – 246 (2000)

35 Furuichi H, Fukuyama R, Izumo N, Fujita T, Kohno T, Nakamuta H and Koida M: Bone-anabolic effect of salmon calcitonin on glucocorticoid-induced osteopenia in rats. Biol Pharm Bull **23**, 946 – 951 (2000)

36 Yamamoto K, Hashimoto H, Hagihara N, Nishino A, Fujita T, Matsuda T and Baba A: Cloning and characterization of the mouse pituitary adenylate cyclase-activating polypeptide (PACAP) gene. Gene **211**, 63 – 69 (1998)

37 Takuma K, Fujita T, Kimura Y, Tanabe M, Yamamuro A, Lee E, Mori K, Koyama Y, Baba A and Matsuda T: T-588 inhibits astrocyte apoptosis via mitogen-activated protein kinase signal pathway. Eur J Pharmacol **399**, 1 – 8 (2000)

38 Beck GR Jr, Zerler B and Moran E: Phosphate is a specific signal for induction of osteopontin gene expression. Proc Natl Acad Sci USA **97**, 8352 – 8357 (2000)

39 Fujita T, Izumo N, Fukuyama R, Meguro T, Nakamuta H, Kohno T and Koida M: Phosphate provides an extracellular signal that drives nuclear export of Runx/Cbfa1 in bone cells. Biochem Biophys Res Commun **280**, 348 – 352 (2001)

40 Nakienly S and Dreyfuss G: Transport of proteins and RNAs in and out of the nucleus. Cell **99**, 677 – 690 (1999)

41 Kobayashi H, Gao YH, Ueta C, Yamaguchi A and Komori T: Multilineage differentiation of Cbfa1-deficient calvarial cells in vitro. Biochem Biophys Res Commun **273**, 630 – 636 (2000)

42 Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM and Bae SC: Runx2 is a common target of transforming growth factor β1 and bone morphogenetic protein 2, and cooperation between runx2 and smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol **20**, 8783 – 8792 (2000)