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Effects of calcitriol and its analogs on protein expression in osteoblast-like cell lines: ROS17/2.8, MG-63, and MC3T3-E1.

Yi-Wen Chien
University of Alabama at Birmingham

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EFFECTS OF CALCITRIOL AND ITS ANALOGS ON PROTEIN EXPRESSION IN OSTEOBLAST-LIKE CELL LINES: ROS17/2.8, MG-63, AND MC3T3-E1

by

YI-WEN CHIEN

A DISSERTATION

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metabolic labeling with $^{32}PO_4$ or ^{35}S-methionine showed that calcitriol, BT, and AT increased the level of total phosphorylated protein in ROS 17/2.8 and MC3T3-E1 cells, but vitamin D analogs did not affect the total amount of protein secreted by these two cell lines. Immunoadsorption assays to assess OPN protein expression showed that compared to control cells, calcitriol, and BT but not AT, BO, or AS stimulated the expression of OPN protein. Northern blots demonstrated that only calcitriol and BT increased OPN mRNA expression in both ROS 17/2.8 and MC3T3-E1 cells. Data from transfection assay showed that the induction of OPN expression by calcitriol and BT in both ROS 17/2.8 and MC3T3-E1 cells is likely due to transcriptional regulation. Calcitriol and BT also stimulated osteocalcin (OCN) mRNA expression in ROS 17/2.8 cells. In MG-63 cells, we detected no phosphorylated protein, including OPN, but calcitriol, BT, and AT increased the total secreted protein. Northern blot analyses of total RNA or mRNA detected no secreted OPN mRNA in cells ± treatment with calcitriol or its analogs after treatment of different cell densities or different time courses. Southern blot analysis indicated that the OPN gene was present, and a putative OPN mRNA was detected. Calcitriol, BT, and AT inhibited VDR mRNA expression in MG-63 cells but also stimulated osteocalcin (OCN), alkaline phosphatase (ALP), type I collagen (COLI) protein expression, and COLI mRNA expression in MG-63 cells. These findings suggest that some analogs of calcitriol can stimulate genomic and nongenomic pathways in osteoblast-like cells but that effects vary with cell line. However, MG-63 cells may not be a good model of osteoblast development in one critical aspect, the production of OPN.

DEDICATION

I wish to dedicate this work to my parents, Chang-shou and Li-tzu Shu Chien.

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LIST OF ABBREVIATIONS

ALP	alkaline phosphatase
AT	16-ene-23-yne 1α,25$(OH)_2D_3$
AS	24,25-dihydroxyvitamin D_3
BO	25-hydroxyvitamin D_3
BSA	bovine serum albumin
BSP	bone sialoprotein
BT	calcipotriol
cDNA	complementary deoxyribonucleic acid
COLI	type I collagen
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
FN	fibronectin
kb	kilobase
kDa	kiloDalton
mRNA	messenger ribonucleic acid
OPN	osteopontin

OCN	osteocalcin
ON	osteonectin
PBS	phosphate buffered saline solution
PMSF	phenymethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TSP	thrombospondin
VDR	vitamin D receptor
VDRE	vitamin D responsive element

INTRODUCTION

Vitamin D_3, or cholecalciferol, the form produced by animals, is a steroid prohormone made in the skin by irradiation of 7-dehydrocholesterol. To produce an active metabolite of vitamin D_3 requires metabolism in the liver to 25-hydroxy-vitamin D_3 and then in the kidney to 1,25-dihydroxy-vitamin D_3 (1).

1α,25-dihydroxyvitamin D_3, also called calcitriol, is an active form of vitamin D_3 metabolite. The major physiologic function of calcitriol is regulation of bone mineral homeostasis, principally through actions on calcium and phosphate handling by bone, intestine, and kidney. Several studies showed that calcitriol can regulate cell differentiation and proliferation and, subsequently, modulate tumor growth (2).

Many studies suggest that calcitriol exerts its effect at the genomic level through a receptor-mediated pathway (3), genomic action. However, recent studies also support a nongenomic level of calcitriol regulation. Calcitriol may regulate protein expression by genomic or non-genomic pathways or by both pathways (4,5).

We are interested in the mechanism by which calcitriol regulates protein synthesis in bone cells. One method to distinguish the mechanism of calcitriol action is by the use of calcitriol analogs, which function selectively through genomic or nongenomic pathways to stimulate biological responses. Calcipotriol (BT), a vitamin D analog with high affinity to vitamin D receptor (VDR), has been shown to act by a genomic pathway; 25-OH-16-ene-23-yne-D_3 (AT), which has low affinity to VDR, stimulates biological responses

mediated by nongenomic mechanisms such as calcium flux. Here, we chose three different osteoblastic cell lines, MG-63 (human osteosarcoma cells), ROS17/2.8 (rat osteosarcoma cells), and MC3T3-E1(mouse osteoblast cells), to study the effects of calcitriol and its analogs [BT, AT, 25-$(OH)D_3$ and 24,25$(OH)_2D_3$] on the expression of osteopontin, osteocalcin, type I collagen, and alkaline phosphatase.

REVIEW OF LITERATURE

Vitamin D

Synthesis. The active form of vitamin D actually is a hormone and is essential for life in higher animals. A family of compounds exhibit vitamin D activity. The most important vitamin D compounds are vitamin D_2 and D_3. In animals, 7-dehydrocholesterol (also called provitamin D_3) is photoisomerized into vitamin D_3 when skin is exposed to sunlight. However, vitamin D_2 (also called ergocalciferol) is produced by ultraviolet irradiation of the plant sterol ergosterol. The more important of these is vitamin D_3 or cholecalciferol (6,7).

Absorption and Metabolism. Vitamin D_3 is transported in the blood stream by transcalciferin to the liver and converted to $25(OH)D_3$ (8). Then, it is transported to the kidney, where it is further hydroxylated at the 1 position to produce the functional form of vitamin D, $1,25(OH)_2$ vitamin D_3, or calcitriol (9).

Functions of Calcitriol. The classical action of calcitriol is maintenance of calcium and phosphorus homeostasis. Calcitriol, along with parathyroid hormone (PTH) and calcitonin, tightly regulates blood calcium by actions on kidney, intestine, bone, and parathyroid gland.

osteosarcoma cells (ROS 17/2.8) in a soft agar culture. Evans and his coworkers (16) observed that calcitriol inhibited the proliferation of human osteoblast-like cells in a dose-dependent manner (10^{-10}-10^{-6} M).

Many studies have demonstrated that $1,25(OH)_2D_3$ inhibited the growth of several cells such as ovarian cells in mammals and birds (17), rat and mouse bone cells (18), malignant melanomas (13), breast cancer cells (19), human osteosarcoma cells (20), and human renal carcinoma cells (21). Therefore, calcitriol may be useful in the treatment and prevention of certain types of neoplastic disease. Holick (22) reported that calcitriol inhibited cell proliferation and induced cell differentiation of human keratinocytes in the management of psoriasis. Czarnetzki (23) also found that both topical and oral administration of $1,25\ (OH)_2D_3$ to psoriatic patients improved the symptoms of the patients. Other studies (24) also found the same antiproliferative effect.

Calcitriol also plays a role in modulating tumor promotion. Several studies indicated that calcitriol, like 12-o-tetradecanoylphorbol-13-acetate (TPA), can enhance chemically induced tumorigenic transformation (25-29), whereas others found that calcitriol inhibits tumor formation (30-32). Wood et al. (30) and Chida et al. (31) reported that calcitriol had an inhibitory effect on tumor promotion in mouse skin. Yoneda and his associates showed that calcitriol had suppressed colony formation of clonal rat osteosarcoma cells in semisolid agar (32). The role of calcitriol in modulation of tumor promotion is contradictory; thus, further understanding of the mechanism of action of calcitriol is needed.

Mechanisms of Action. There are two mechanisms of vitamin D action: one is a genomic pathway and the other is a nongenomic pathway.

Genomic Pathway. Vitamin D receptors were first found by Haussler and Norman in 1969 (33). This was followed by numerous reports indicating that calcitriol regulates gene expression (such as osteopontin and osteocalcin) by interacting with vitamin D receptors. Norman et al. observed that calcitriol regulated gene transcription via a nuclear receptor (34,35). Studies found that calcitriol controls the synthesis of numerous peptides by acting on gene expression, such as procollagen α1I, core protein of proteoglycans, osteocalcin, osteopontin, and calbindins (36).

The calcitriol receptor, VDR, is a member of a family of steroid receptors and contains a ligand binding domain and a DNA binding domain. The calcitriol-VDR complex binds to a specific regulatory region (vitamin D responsive element or VDRE) of the DNA upstream of the gene coding region; this binding can up- or down-regulate the expression of target gene.

Nongenomic Pathway. Another possible mechanism of action of calcitriol is nongenomic. Calcitriol may generate biological responses via activation of voltage-dependent calcium channels that are "coupled" via appropriate signal transduction pathways (37-39).

In 1990, Deboland et al. (39) and Norman (40) found that calcitriol activated a second messenger system such as cAMP or protein kinase C to stimulate rapid intestinal calcium transport in a process called transcaltachia. This response is not inhibited by

actinomycin D (41). Other studies also demonstrated that rapid uptake of calcium in skeletal muscle is stimulated by calcitriol (42-44).

Recently, Deboland and Boland (45) reported that calcitriol rapidly activates voltage-dependent Ca^{2+} channels of the L-type in skeletal and cardiac muscle cells by a nongenomic pathway. This mechanism involves guanine nucleotide binding (G) protein-mediated stimulation of the adenylate cyclase cAMP protein kinase. Calcitriol stimulates phospholipase A2, which generates arachidonic acid in the eicosanoid pathway.

Studies have shown calcitriol and its analogs regulate protein synthesis by genomic and/or nongenomic pathways. There are some analogs that can be used to study the mechanism of calcitriol action.

Vitamin D Responsive Elements (VDREs). VDREs are small regions of DNA that represent the binding sites for the VDR. VDREs have been found in the promoter region of vitamin D-dependent genes such as osteopontin, osteocalcin, beta 3 integrin, and vitamin D-24-OHase (46). Binding to VDR results in an enhancement or suppression of target gene transcription (47).

Several VDREs exhibit a common motif consisting of two imperfect direct hexanucleotides with a spacer of three nucleotides. Table 1 shows nucleotide sequences of some VDREs (48).

Analogs. (See Appendix for structures of calcitriol and analogs). Calcipotriol (BT, MC903) is a synthetic side chain analog of calcitriol. It contains a 22-23 double bond and a 24(S)-hydroxyl function, and carbons 25, 26, and 27 are incorporated into a cyclopropane

ring (49). It binds very well to the nuclear receptor, but does not activate calcium channels; it also mediates gene expression by a genomic pathway (4,50,51).

Table 1. VDREs Sequences

Gene	Nucleotide Sequence
Calbindin D-9K	5'-GGGTGG CGG AAGCCC-3'
Rat Osteocalcin	5'-GGGTGA ATG AGGTCA-3'
Mouse OPN	5'-GGTTGA CGA GGTTCA-3'
Human Osteocalcin	5'-GGGTGA AGG AGGTCA-3'
Rat calcitriol 24-hydroxylase	5'-GGGTGA ACG GGGGCA-3'
Consensus	5'-AGGTCA AGG AGGTCA-3'
Rat OPN (proximal)	5'-AGGTCA CAC AGGGCA-3'
Rat OPN (distal)	5'-AGGTCA TAT GGTTCA-3'

Calcipotriol stimulates terminal differentiation and inhibits proliferation of cultured human keratinocytes (49,52,53) and human osteoblast-like cells (16). Subsequent clinical studies have shown that calcitriol and calcipotriol are effective in the treatment of psoriasis (24, 54-57).

Song et al. (58) found that calcitriol and calcipotriol play an important role in regulating cell growth and differentiation of human osteosarcoma cell line HOS-8603.

AT (16-ene-23-yne 25(OH)D_3), a calcitriol analog which binds very poorly to nuclear receptor, activates calcium channels and stimulates calcium transport by a nongenomic pathway (4,59,60).

BO (25-hydroxyvitamin D_3) and AS (24,25-dihydroxyvitamin D_3) are natural vitamin D metabolites. Barnea et al. (61) found that BO could increase intracellular

connective tissue mesenchymal stem cells which proliferate and differentiate into preosteoblasts. Osteoclasts originate from cells of the mononuclear-phagocyte lineage. The activity of osteoblasts and osteoclasts results in bone remodeling--the process by which bone grows and is turned over (66).

Bone Matrix. Bone matrix is composed of collagen fibers (type I, 90% of total proteins) and noncollagenous proteins produced by osteoblasts. Spindle- or plate-shaped crystals of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] are found on the collagen fibers, within them, and in the matrix. Noncollagenous proteins are also found in the bone matrix and include thrombospondin (TSP), fibronectin (FN), biglycan (proteoglycan I), decorin (proteoglycan II), bone sialoprotein (BSP), osteopontin (OPN), osteonectin (ON), matrix gla protein, and osteocalcin (OCN) (67). The role of these proteins is only partially characterized.

Collagen. Many different types of collagen have been identified. The structural roles of collagen are known for type I collagen in tendons, ligaments, skin and bone; for type II in hyaline cartilage; and for type IV collagens in basement membranes (68).

The basic building block of the bone matrix fiber network is the type I collagen molecule, which consists of two α1(I) chains and one α2(I) chain; together, these chains form a triple-helical, supercoilal molecule. The proportion of glycine residues in collagen molecules is nearly one third, which is unusually high for a protein. Proline also is present to a much greater extent in collagen than in most other proteins. The gly-X-Y repeating triplet (where X is often proline) makes the collagen structure unique from other proteins (69,70).

Because type I collagen is the primary organic constituent of bone, many studies have examined the effects of calcitriol on bone cells. Calcitriol inhibits collagen synthesis in different cell types such as fetal rat and neonatal mouse calvaria (71-73), primary cultures of osteoblast-like cells (74), and permanent clonal cell lines displaying an osteoblastic phenotype (75,76). Harrison et al. (77) found that 1,25-dihydroxyvitamin D_3 inhibits transcription of type I collagen genes in the rat osteosarcoma cell line ROS 17/2.8. Beresford and his coworkers (78), on the other hand, reported that calcitriol increased type I collagen expression but that $24,25(OH)_2D_3$ did not.

Besides collagen, there are many noncollagenous proteins in bone matrix. Here are some examples.

Thrombospondin is a 420 kDa adhesive glycoprotein. It is expressed at high levels in developing heart, muscle, bone, and brain of the embryo. Its function is probably associated with cell proliferation (68).

Fibronectin is a high molecular weight glycoprotein. It has been found to promote cell adhesion and affect cell morphology, cell migration, cell differentiation, and cytoskeletal organization (68).

Biglycan is a small proteoglycan associated with the cell surface or pericellular matrix of a variety of cells. Its function is not known.

Decorin is a small secreted proteoglycan with a single chondroitin or dermatan sulphate chain attached to the fourth amino acid of the 38 kDa core protein. It is associated with collagen fibrils in virtually all connective tissue. Some studies have shown thatdecorin can change the kinetics of collagen fibril formation, affect the morphology of the collagen fibrils, and bind to TGF-β (68)

Bone sialoprotein (BSP) is a heavily glycosylated extracellular matrix protein that supports cell attachment through the Arg-Gly-Asp (RGD) cell attachment mechanism. It has many of the properties of the cell attachment domain of fibronectin or vitronectin but is a considerably smaller gene product (68).

Osteonectin is a prominent constituent of bone and is a 32 kDa Ca^{2+}-binding glycoprotein secreted by a variety of cells. It is probably associated with bone formation and tissue differentiation and remodeling (68).

Osteocalcin (OCN, bone Gla protein, BGP). OCN is the major noncollagenous bone protein produced in the process of bone formation, is a marker of bone turnover in normal and disease states, and is considered a specific marker of bone formation. It is abundant in bone and dentin but absent from most other tissues.

Serum osteocalcin correlates with skeletal growth at the time of puberty and is increased in a variety of conditions characterized by increased bone turnover such as primary and secondary hyperparathyroidism, hyperthyroidism, Paget's disease, and acromegaly. It is decreased in hypothyroidism, hypoparathyroidism, and glucocorticoid-treated patients. Its precise function remains unknown, but OCN may be chemotactic for osteoclast precursors (68).

Osteocalcin is a vitamin K-dependent protein of bone and is a 5.7 kDa protein (45-50 amino acids). It contains 3 residues of the calcium binding amino acid, gamma-carboxyglutamic acid (Gla) (79).

The primary structure of osteocalcin has been determined for 11 species. Common features include the location of Gla at positions 17, 21, and 24 and the disulfide loop Cys_{23}-

Cys_{29}. Hydroxyproline occurs at position nine in eight out of 11 species, placing this protein in the ranks of collagen, elastin, complement Clq, and acetylcholinesterase as a substrate for prolylhydroxylase. The Gla-containing sequence is the locus of Ca^{2+} binding and hydroxyapatite adsorption (80).

The predicted secondary structure is two major α-helical regions. The "Gla helix" (res 16-25 or 18-25) and the "Asp Glu helix" (res 30-41) are linked by a β-turn (res 26-29) and stabilized by the Cys_{23}-Cys_{29} disulfide bond. The Gla helix is always preceded by a β-turn, with β-sheet invariably predicted for the COOH-terminal region (80).

Osteocalcin has a variety of interesting properties. These include the following: 1) a specific Gla-dependent binding of Ca^{2+} ions and adsorption of hydroxyapatite; 2) Ca^{2+}-induced transition to α-helical conformation accompanied by increased affinity for hydroxyapatite; 3) Mg^{2+} antagonism of hydroxyapatite binding; 4) interference with brushite→hydroxyapatite metamorphosis and hydroxyapatite formation; 5) biosynthesis by osteoblast-like cells; 6) appearance in bone coincident with the onset of mineralization; 7) low level circulation in blood and variations with age, sex, time of day, and bone pathology; 8) accumulation in sites of ectopic calcification; 9) chemoattractant activity toward human peripheral monocytes; 10) induction of membrane potential changes in mononuclear leukocytes; and 11) competitive inhibition of leukocyte elastase.

Table 2 summarizes studies of osteocalcin expression as regulated by calcitriol and its analogs in osteoblast-like cells.

Table 2. Regulation of Osteocalcin Expression by Calcitriol and Its Analogs

Cell Type	Factors	Osteocalcin	Ref.
ROS 17/2.8	Calcitriol	↑mRNA	63,64
	C & BT	↑mRNA	60
	AT	-mRNA	60
Human bone-derived cells	Calcitriol	↑protein	81
	BO &AS	-protein	81
hOB transfected with SV40 large T antigen	Calcitriol	↑mRNA ↑protein	82
Fetal rat calvarial cells	Calcitriol	↑mRNA	83
MG-63 cells	Calcitriol	↑mRNA	84
MG-63 with IGF-I	Calcitriol	↑mRNA	85
Mouse osteoblast	Calcitriol	↓gene	86

C: calcitriol BT: calcipotriol AT: 25-OH-16-ene-23-yne-D_3
BO: 25-OH-D_3 AS: 24,25 $(OH)_2D_3$
hOB: human osteoblast-like cells
↑: stimulate ↓: inhibit
-: no effect

Osteopontin (OPN). Osteopontin has been known by several names such as bone sialoprotein I, 44 kDa phosphoprotein, 2ar, SPP, pp69, transformation-associated secreted phosphoprotein, and Eta-1. It has been isolated and characterized from rat bone (87-89), human bone (90), porcine bone (91), chicken bone (92), rat kidney cells (93), and several transformed cells (94,95) as well as from milk (96).

The rat osteopontin, isolated by Prince et al. (87) is a 44 kDa phosphoprotein with 301 amino acid residues. It is a glycoprotein rich in aspartic acid, glutamic acid, and serine residues. It also contains 30 monosaccharides with 10 sialic acid residues presenting as one

in osteopontin provides binding surface to cells which anchor, migrate, differentiate, and grow (100).

The function of osteopontin is not yet clear. However, several studies have shown its possible role in bone formation, bone resorption, cell adhesion, resistance of bacterial infection, and tumorigenesis and metastasis.

Osteopontin has been found to be produced by osteoblasts and osteoclasts. It exists within the mineralized matrix of bone because it can only be isolated by rigorous procedures using chelating and denaturing agents. Osteopontin has high affinity for hydroxyapatite, probably contributed by its phosphate residues, sialic acid residues, sulfate residues, and the two potential calcium-binding sites. Mckee et al. (104) indicated that the level of chicken osteopontin is associated with an increasing degree of mineralization.

Reinholt and his coworkers (105) reported that osteopontin acts as a possible anchor for osteoclasts to bone. During bone resorption, osteoclasts anchor to osteopontin, which is bound to the mineral of bone matrix. Studies have shown that the vitronectin receptor on the osteoclast plasma membrane can mediate binding of osteoblasts to osteopontin and to bone.

As mentioned above, osteopontin has an Arg-Gly-Asp sequence in the cell-attachment domain of the protein which can form a bridge between cells and the mineral in the matrix.

Some studies mapped the mouse osteopontin gene to the Ric Locus that controls natural resistance to infections of Rickettsia Tsutsugamushi (RT) and indicated that mice with genetic resistance to RT have increased osteopontin expression (106-108). Mice

without resistance to RT do not express osteopontin in the first 4 days after RT infection. These results support osteopontin's role in resisting bacterial infection.

Osteopontin is secreted in elevated levels in transformed cells such as NIH 3T3 cells transformed by human bladder cancer T24-H-ras oncogene (109) and transformed epithelial and fibroblast cells of rodents and humans (110-113). Tumor promoters like TPA induce the expression of osteopontin mRNA in mouse epidermis (114,115). Chang and Prince (116) reported that TPA (12-o-tetradecanoylphorbol-13-acetate) irreversibly induces anchorage-independent growth and tumorigenicity in mouse JB6 epidermal cells. Also, cells treated with TPA synthesize and secrete a highly phosphorylated form of osteopontin.

Furthermore, Senger and his coworkers (101) found that patients with advanced metastatic cancer have high levels of osteopontin in their blood. A study by Craig et al. (109) demonstrated that cells from ras-tranfected C3H10T1/2 fibroblastic cells showed positive correlation of metastatic potential to osteopontin mRNA expression. Recent studies from Chambers and co-workers (118) have shown that circulating levels of OPN correlate with patient outcome in lung cancer. These findings indicate that osteopontin may play a role in tumorigenesis and metastasis.

Osteopontin is found in bone, kidney, inner ear, placenta and uterus, body fluids (blood, urine, and milk), and smooth muscle. Probably the most abundant sources of osteopontin are in bone, kidney, and milk.

Numerous studies indicate that osteopontin expression can be regulated by many factors such as TGFβ1, TPA, and interleukin 1α. However, still only a few reports have emphasized the regulation of osteopontin synthesis by calcitriol. Table 3 shows the effect of calcitriol and its analogs on osteopontin expression in various cells.

Table 3. Regulation of Osteopontin Expression by Calcitriol and Its Analogs

Cell Type	Factors	Osteopontin	Ref.
ROS 17/2.8	Calcitriol	↑Protein	119,120
	Calcitriol	↑mRNA	63,119,121,122
	C & BT	↑mRNA	60,64
	AT	-mRNA	60,64
RCT-3	Calcitriol	↑mRNA	123
Neonatal rat calvarial cells	Calcitriol	↑mRNA	124
Fetal rat calvarial cells	Calcitriol	↑mRNA	83
UMR-201-10B cells	Calcitriol	↑mRNA	125
Rat clonal dental pulp cells	Calcitriol	↑protein(P)(S)	126
		↑mRNA	126
JB6 Cl 41.5a	Calcitriol	↑mRNA	28,59

C: calcitriol BT: calcipotriol AT: 25-OH-16-ene-23-yne-D_3
↑: stimulate -: no effect

Alkaline Phosphatase (ALP). Serum alkaline phosphatase is the most commonly used marker of bone formation and is used as an index to monitor the activity of Paget's disease of bone. Although alkaline phosphatase is mainly found in osteoblastic cells, its precise function is still not understood.

Classical vertebrate alkaline phosphatases are a group of isozymic membrane-bound glycoproteins with molecular weights generally in the range of 100,000 to 200,000. They are usually dimers composed of two very similar, if not identical, subunits. The activity of this group of enzymes is characterized by broad substrate specificity and alkaline pH optima varying substantially with the type and concentration of the substrate.

Table 4. Regulation of Alkaline Phosphatase Expression by Calcitriol and Its Analogs

Cell Type	Factors	Alkaline Phosphatase	Ref.
HOBIT	Calcitriol	↑mRNA	82
UMR-201-10B	Calcitriol	↑mRNA	125
Caco-2	Calcitriol	↑mRNA	131
HOS-8603	C&BT	↑activity	58
Mice osteoblasts	Calcitriol	↑activity	132
Neonatal rat calvarial cells	Calcitriol	↑activity	133
	$24,25(OH)_2D_3$	-activity	133
Human osteoblast-like cells	Calcitriol & BT	↑activity	16
Human bone cells	Calcitriol	↑activity	78
	$24,25(OH)_2D_3$	-activity	78
SAOS,TE85	Calcitriol	↑activity	127

C: calcitriol
BT: calcipotriol
AT: 25-OH-16-ene-23-yne-D_3
↑: stimulate
-: no effect

HYPOTHESIS

Based on the literature review described above, we hypothesize that vitamin D analogs with high affinity for the VDR, [i.e. calcitriol and calcipotriol (BT)] will modulate the osteoblast phenotype as determined by assessment of cell proliferation and gene expression, specifically osteopontin, osteocalcin, alkaline phosphatase, and type I collagen.

SPECIFIC AIMS

The specific aims of this project were

1. To assess the effects of calcitriol, calcipotriol, AT, $25(OH)D_3$ (BO), and $24,25(OH)_2D_3$ (AS) [10^{-11} to 10^{-8}M] on cell proliferation by using β-hexosaminidase assay.
2. To determine the level of constitutive OPN, OCN, AP, and COLI expression in ROS 17/2.8, MG-63, and MC3T3-E1 cells by using $^{32}PO_4$ and ^{35}S-methionine labeling of cell culture and TCA precipitation and immunoadsorption assay for protein expression.
3. To examine the effects of calcitriol and its analogs on OPN, OCN, and COLI mRNA expression by Northern blot analyses or RT-PCR.
4. To examine the regulation at the level of transcription for those analogs that modulate OPN expression, by using reporter gene constructs (transfection assay).
5. To evaluate VDR expression to determine if effects of calcitriol and its analogs on OPN and/or other proteins are due to VDR levels by using Northern blot analysis for VDR mRNA expression.

METHODOLOGY

The methodology of this project included cell growth, cell proliferation assay, metabolic radiolabeling of cell culture, total protein by TCA precipitation, immunoadsorption, Northern and Southern analysis, RT-PCR, and transfection assay. The detailed methods and materials are described below.

Cell Growth

Materials. Alpha modification of Eagle's minimum essential medium (α-MEM), Dulbecco's modification of MEM (DMEM), Hank's balanced salt solution, and antibiotics were from Mediatech, Inc. (Herndon, VA). Fetal bovine serum was obtained from Hyclone Laboratories, Inc.(Logan, UT). L-glutamine (200 mM) and trypsin/EDTA were purchased from Irvine Scientific (Santa Anna, CA). Pronase E and EDTA were from Sigma (St. Louis, MO).

Cell Lines. The clonal rat osteosarcoma cell line (ROS 17/2.8) was provided by Dr. Gideon Rodan (Department of Bone Biology, Merk, Sharp & Dohme Research Laboratory, West Point, PA). Human osteosarcoma cells (MG-63) were purchased from American Type Culture Collection (ATCC) (Rockville, MD). Mouse osteoblast-like cells (MC3T3-E1) were generously provided by Dr. H. Kodama (Tohoku Dental College, Koriyama, Japan) and are derived from newborn C57BL/6 mouse calvaria.

Cell Culture. ROS 17/2.8 and MG-63 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2% L-glutamine, and 1% antibiotics (an antibiotic, antimycotic agent). Cells were kept in a humidified 37°C incubator in an atmosphere of 95% air and 5% CO_2. MC3T3-E1 cells were grown under similar conditions, except in α-MEM. All cells were subcultured before reaching confluence using trypsin/EDTA [MC3T3-E1 cells were subcultured using 0.001% pronase E plus 0.02% EDTA in Ca, Mg-free phosphate-buffered saline (PBS)] and were checked monthly for mycoplasma contamination by DNA fluorochrome staining (135).

Cell Proliferation Assay

To detect the effects of calcitriol and its analogs in cell proliferation, cell numbers were estimated by measuring the endogenous enzyme β-hexosaminidase activity (136).

Materials. Calcitriol and its analogs (BT, AT, BO, and AS) were obtained from Leo Pharmaceutical Products (Ballerup, Denmark). Citrate, EDTA, p-nitrophenol-N-β-D-glucosaminide, and triton X-100 were obtained from Sigma (St. Louis, MO). Glycine was purchased from Boehringer Mannheim Corporation (Indianapolis, IN).

Method. Cells were seeded at 5,000/well in flat bottom 96-well plates (Nunc, Denmark); each treatment had four duplicates. After 24 h, medium with different concentrations of calcitriol and its analogs was added to the wells. On days 2, 4, 6, and 8 (i.e., 1, 3, 5, and 7 days of drug incubation) cell proliferation was assessed by using the β-hexosaminidase assay. Sixty microliters/well substrate solution (substrate, p-nitrophenol-

N-acetyl-β-D-glucosaminide dissolved at 7.5 mM in 0.1 M citrate buffer, pH 5, and mixed with an equal volume of 0.5% triton X-100 in water) was added, and incubated at 37°C in 100% humidity. After 1 h, 90 μl/well of stop solution (50 mM glycine buffer, pH 10.4, containing 5 mM EDTA) was added. Then the absorbance was measured in a microplate reader at 405 nM. Data are expressed as mean ± SD and analyzed for statistical significance by using Sigma Stat software. Each day and dose were analyzed by 1-way ANOVA; when appropriate, multiple group comparisons were done pairwise using Student-Newman-Keuls test.

Metabolic Radiolabeling of Cell Culture

Materials. Dulbecco's phosphate buffered saline solution (PBS), Deficient DME high glucose medium, L-leucine, L-lysine, L-glutamine, and sodium pyruvate were from Irvine Scientific (Santa Anna, CA). Bovine serum albumin (BSA) was from Fisher Biotech (Fair Lawn, NJ). [^{35}S]methionine and $^{32}PO_4$ were from ICN Radichemicals (Irvine, CA). [^{35}S]-Cysteine-methionine mixture was purchased from Amersham (Arlington Heights, IL).

Method. Cells were seeded at 80,000 cells/well and grown to near confluence. Cell density was determined before and at the end of incubation with analogs for 24 h. During the last 4 h of drug incubation, cells were incubated for 1 h with methionine-free or phosphate-free medium and then [^{35}S]methionine or $^{32}PO_4$ (100 μCi/well) for the next 3 h. For detecting OCN, ALP, and COLI in MG-63 cells, cells were incubated for 1 h with cysteine-methionine-free medium, and then [^{35}S]cysteine-methionine (100 μCi/well) was

added for the next 3 h. Media were collected for determination of total secreted protein by trichloroacetic acid (TCA) precipitation or for immunoadsorption assay. Since ALP is not a cell-secreted protein, to measure the effect of calcitriol on the ALP expression, cells were lysed prior to immunoadsorption. After medium was collected, wells were washed once with PBS. Then, 0.5 ml RIPA buffer [150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, and 50 mM Tris (pH 8.0)] was added and plate was incubated on ice for 30 min with occasional rocking. After centrifugation for 10 min at 10,000g at 4°C, the lysate was removed to a fresh tube and assayed for ALP using immunoadsorption assay.

Total Secreted Protein by TCA Precipitation

The total secreted protein can be determined by TCA precipitation.

Materials. TCA was obtained from J.T. Baker Inc. (Phillipsburg, NJ). Levamisole, 6-amino-n-caproic acid, phenymethylsulfonyl fluoride (PMSF), dimethylsulphoxide (DMSO), 2,5-diphenyloxazole (PPO), and bisbenzimide were purchased from Sigma (St. Louis, MO). Absolute ethanol was from USI Chemicals Co. (Tuscola, IL). Acrylamide was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Kodak X-OMAT Film was from Eastman Kodak (Rochester, NY).

Method. Medium collected after radiolabeling was first diluted with an equal volume of protease inhibitors (10 mM levamisole, 20 mM PMSF, and 200 mM 6-amino-n-caproic acid). Then, 40% TCA was added to the medium and incubated for 30 min at 4°C. The pellet formed after centrifugation was washed 3 times with 10% TCA and once with absolute ethanol. Washed pellets were heated at 90°C for 5 minutes with reducing sample

Sepharose). The sample was centrifuged, and the pellet was washed 3 times with washing buffer (0.25% deoxycholate, 0.1% Nonidet P-40, 0.15 M NaCl, and 0.1 M Tris HCl with pH 7.4), twice with PBS, and once with double distilled water. Reducing sample buffer was added to the washed pellets and heated at 90°C for 5 min. Samples were then centrifuged, and supernatant was analyzed by 10% SDS-PAGE. The gel was stained with Coomassie Brilliant blue and destained in 10% acetic acid and 10% methanol. The destained gel was rinsed with distilled water and DMSO before being treated with 22% PPO in DMSO; then, it was dried and exposed to the X-ray film.

Western Blot Analysis

To verify the activity of human OPN antibody, we detected recombinant human OPN with three human OPN antibodies and one rat OPN antibody.

Materials. Pure nitrocellulose membrane, Western blot transfer equipment, and alkaline phosphatase conjugate substrate kit were purchased from BioRad Laboratory (Richmond, CA).

Method. Five micrograms of human or rat recombinant OPN samples were analyzed by 10% SDS-PAGE. Proteins were then electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated for 3 h at room temperature in blocking buffer: 0.3% gelatin in Tris buffered saline [TBS (20 mM Tris and 500 mM NaCl, pH 7.5)] and then incubated overnight with a 1:5000 dilution of antiserum in the same buffer. After two 15-min washes in washing buffer [TTBS (20 mM Tris, 500 mM NaCl,

and 0.05% Tween 20, pH 7.5)], the membrane was incubated for 2 h with a goat anti-rabbit secondary antibody, diluted 1:10000 in blocking buffer. Then, the membrane was washed twice with TTBS and once with TBS. Polypeptides recognized by the antipeptide antibodies were visualized by using alkaline phosphatase conjugate substrate kit.

Total RNA and mRNA Extraction

Materials. 2-Mercaptoethanol, sodium citrate, 3-[N-morpholino]propanesulfonic acid (MOPs), sodium acetate, sarkosyl, diethylpyrocarbonate (DEPC), bromophenol blue, xylene cyanole, ethidium bromide, isoamyl alcohol, isopropanol, and agarose were obtained from Sigma (St. Louis, MO). Formaldehyde was purchased from Mallinckrodt (Paris, KY). Chloroform was from Fisher Scientific (Springfield, NJ). Phenol, RNA ladder (0.24-9.5 kb), 0.5M ethylene diamine tetraacetic acid (EDTA), guanidinium thiocyanate, glycerol, and formamide were purchased from GIBCO BRL (Gaithersburg, MD). PolyATract mRNA isolation system was purchased from Promega (Madison, WI).

Method. The procedure for extraction of mRNA was described by Chomczynski and Sacchi in 1987 (137). Cells were grown for 2 days in 100-mm petri dishes (10,000 cells/cm^2). After 24 h of incubation with different concentrations of analogs, the cells were lysed with guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol). Subsequently, 2 M sodium acetate (pH 4.0), phenol, and chloroform-isoamyl alcohol mixture (49:1) were added. The mixture was vortexed, chilled for 15 min, and centrifuged at 10,000 g, 4°C for 20 min. The aqueous phase was collected and precipitated at -20°C with isopropanol. After centrifugation, the

pellet was dissolved in guanidinium thiocyanate solution and reprecipitated using isopropanol at -20°C. The RNA pellet obtained by centrifugation was washed with 75% ethanol, vacuum dried at 37°C, and dissolved in 0.1% DEPC water (containing 4 M guanidinium thiocyanate). Concentration of RNA was determined with a spectrophotometer at 260 nm, and purity was analyzed by the absorbance ratio of 260 to 280 nm. The isolated RNA was examined by electrophoresis on a 1.1% agarose gel with 2.2 M formaldehyde. Undegraded RNA samples were used for Northern blot analysis.

Total RNA 0.1-1 mg was brought to a final volume of 500 μl in RNase-free water and heated at 65°C for 10 min. Subsequently, 3 μl of biotinylated-oligo(dT) probe and 13 μl of 20X SSC were added to the total RNA. Then, the mixture was mixed gently and incubated at room temperature until completely cooled. The product, annealed oligo(dT)-mRNA hybrid, was added to streptavidin paramagnetic particles which had been washed 3 times with 0.5X SSC and resuspended in 0.1 ml of 0.5X SSC. After washing twice with 0.1X SSC and capturing by magnetic rack, mRNA was eluted with RNASE-free water.

Northern Blot and Hybridization

Materials. Nylon membrane was obtained from Oncor (Gaithersburg, MD). [α-^{32}P]-dCTP was obtained from NEN DuPont company (Wilmington, DE). Whatman 3MM paper was purchased from Whatman International Ltd. (Maidstone, England). Prime-It Random Primer Kit and NucTrap Plus Column were purchased from Stratagene (La Jolla, CA). Sodium chloride, dextran sulfate, and salmon testis DNA were purchased from Sigma (St. Louis, MO). Methylene Blue was obtained from Molecular Research Center, Inc. (Cincinnati, OH). OPN cDNA was a generous gift from Dr. D. Denhardt (Rutgers

University). Human osteocalcin, VDR, osteonectin, AP, COLI, and β-actin cDNA probes were purchased from ATCC. Rat osteocalcin cDNA probe was provided by Drs. Jane Lian and Gary Stein (Department of Cell Biology, University of Massachusetts, Medical Center).

Method. After running the gel, RNA was transferred to nylon membrane using 10x SSC as a transfer buffer. Then, the membrane was briefly washed with 2x SSC, stained with methylene blue, and destained with DEPC water. The membrane was photographed and dried at 80°C; then, the bands of RNA ladder and positions of 28S and 18S of rRNA were marked. The membrane was prehybridized in 10 ml of 50% formamide, 1% SDS, 10% dextran sulfate, 1 M sodium chloride, and denatured salmon testis DNA for 2 h at 42°C. OPN, OCN, ALP, COLI, and VDR cDNA probes were radiolabeled using a Prime-It Random primer kit and [α-^{32}P]-dCTP. β-actin cDNA was used as a control probe to evaluate the RNA loading of each sample. The radiolabeled cDNA probe was separated from unincorporated [α-^{32}P]-dCTP using a NucTrap push column. Denatured ^{32}P-labeled cDNA was added to the membrane with hybridization solution at 42°C for incubation over 16 h. After hybridization, the membrane was washed twice with 2x SSC, twice with 2x SSC and 1% SDS (65°C), and twice with 0.1x SSC. The membrane was air dried and exposed to film. The film was developed after 3 h.

Before rehybridizing, probes were removed from the membrane by boiling the membrane in DEPC water with 0.1x SSC, 0.1% SDS, and 10 mM Tris, pH 7.0 for 10 min.

RT-PCR

Materials. RNA PCR kit was purchased from Perkin Elmer (Foster City, CA). Primers were from GIBCO (Gaithersburgh, MD). PCR was performed using Perkin Elmer, Gene Amp, PCR system 2400.

Method. Total RNA was extracted from MG-63 and U-251MG (human glioma cells) cells using the method described above. The first-strand cDNA was generated with reverse transcriptase and random hexamers. The OPN cDNA was amplified by polymerase chain reaction (PCR) using a set of primers, 5'-ACCATGAGAATTGCAGTGATTTGC-3'-(OP.A)-and-5'-ATTGACCTCAGAAGATGCACTATC-3'-(OP.B)-or-5'-ATCAGTGACCAGTTCATCAGATTC (OP.C). These PCR primers were designed from the human OPN sequence (132). We used β-actin for internal control. The primers for β-actin-are-5'-ATGGATGATATCGCCGCGCTC-3'-and-5'-CTAGAAGCATTTGCGGTGGACGAT-3'. The PCR was performed for 35 cycles with the following condition: denaturation at 94°C 30 sec, annealing temperature at 60° C 45 sec, and elongation at 72°C 1 min. After PCR, 15 ul of the sample were electrophoresed on a 2.5 % agarose gel and stained with ethidium bromide.

Isolation of Genomic DNA

Materials. DNA isolation kits were purchased from Gentra Systems, Inc. (Minneapolis, MN).

Method. Cells were seeded at 10,000/cm^2 in 100-mm petri dishes and grown to confluence. A Puregene DNA isolation kit was used to extract DNA. Concentration of DNA was determined with a spectrophotometer at 260 nm, and purity was analyzed by the absorbance ratio of 260 to 280 nm.

Southern Blot Analysis

Materials. Restriction enzyme EcoRI was obtained from GIBCO (Gaithersburgh, MD).

Method. Ten micrograms of genomic DNA from MG-63 and U-251MG cells were digested with the restriction enzyme EcoRI, electrophoresed through 0.7% agarose gels, denatured in 1.5 M NaCl, and 0.5 M NaOH 2 times for 15 min, neutralized in 0.5 M Tris-HCL-3 M NaCl (pH 7.0) 2 times for 30 min, and then blotted onto nylon membranes. The blots were prehybridized and hybridized the same as Northern blots described above.

Transfection Assay

Materials. The pRSV-lacZ plasmid was a gift from Dr. Mien-Chie Hung (University of Texas, M.D. Anderson Cancer Center, Houston, TX). The original λ 9090 2ar clone was from Dr. David Denhardt (Rutgers University). The pGL2-Basic-luciferase vector, pGL2-control-luciferase, and Reporter lysis solution were purchased from Promega (Madison, WI). The lipofectin reagent and OPTI-MEM were obtained from Gibco-BRL (Gaithersburg, MD).

RESULTS

Effects of Calcitriol and Its Analogs on Cell Proliferation

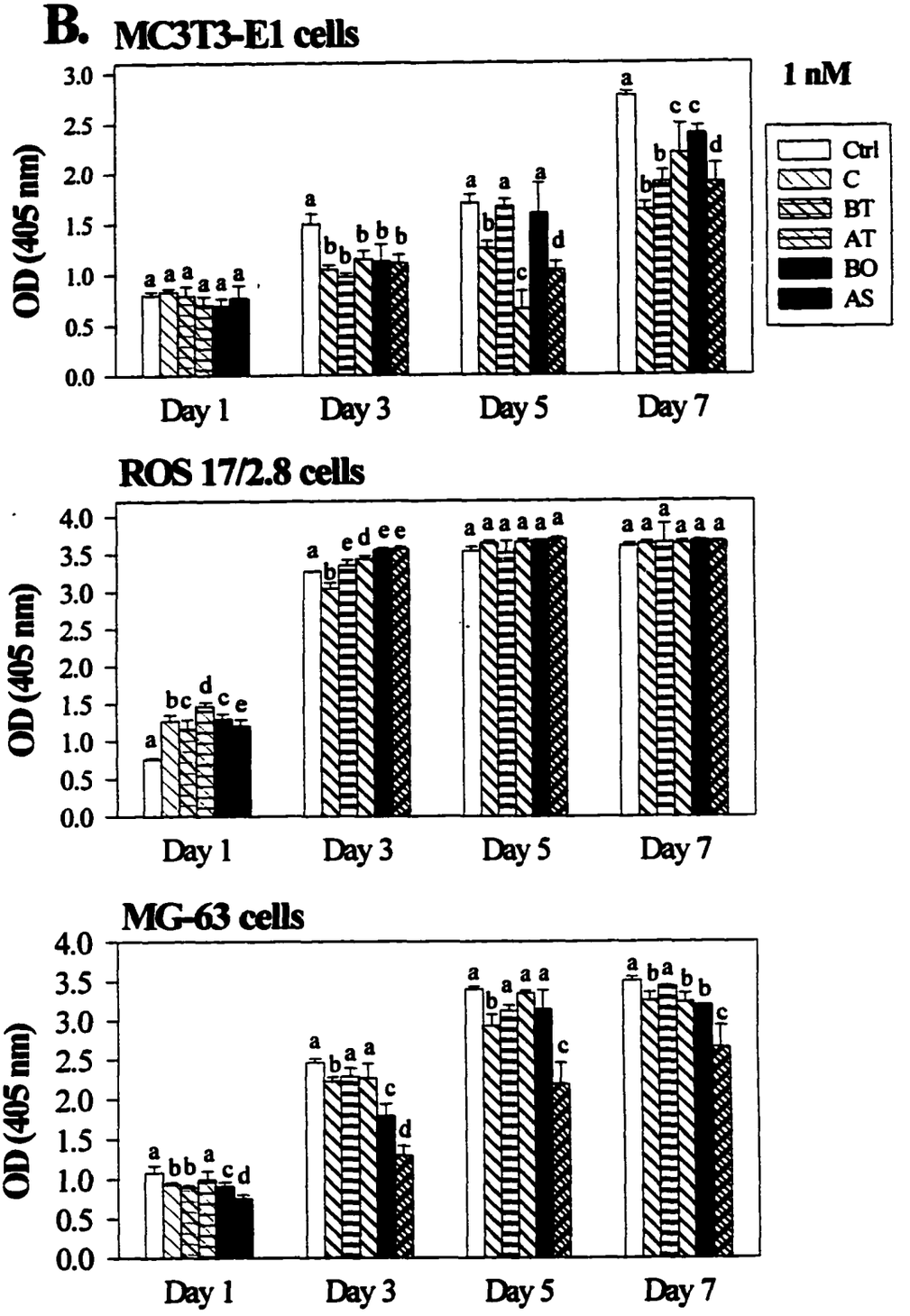
To measure the effect of calcitriol analogs on cell proliferation, cells were treated with vehicle (0.001% isopropanol) or calcitriol analogs at 10^{-8}, 10^{-9}, 10^{-10}, or 10^{-11} M. Medium containing drugs was replaced every 2 days, and cell number was estimated by assaying endogenous β-hexosaminidase activity after 1, 3, 5, and 7 days of drug treatment. Prior studies demonstrated that regression analysis for cell numbers versus β-hexosaminidase activity showed correlation coefficients of 0.99, 0.99, and 0.96 for ROS 17/2.8, MG-63, and MC3T3-E1 cells, respectively. As shown by the summary in Table 5 and the data in Figure 1, MC3T3-E1 cell proliferation was generally inhibited after 3 or more days of treatment with any concentration of calcitriol or its analogs. Calcitriol and BT tended to be the most potent, followed by AS and AT and then by BO. ROS 17/2.8 cell proliferation, on the other hand, was generally stimulated by calcitriol and all analogs, especially at the higher concentrations and at the shorter incubation times (i.e., 1 and 3 days). By day 7 and to some extent by day 5, there were few substantial differences among any of the analog and dose combinations. MG-63 cell proliferation at most time points was inhibited by nearly all analogs at 1 and 10 nM; AT and BT were the major exceptions. For the two lower concentrations, 0.1 and 0.01 nM, there were few differences among any of the groups.

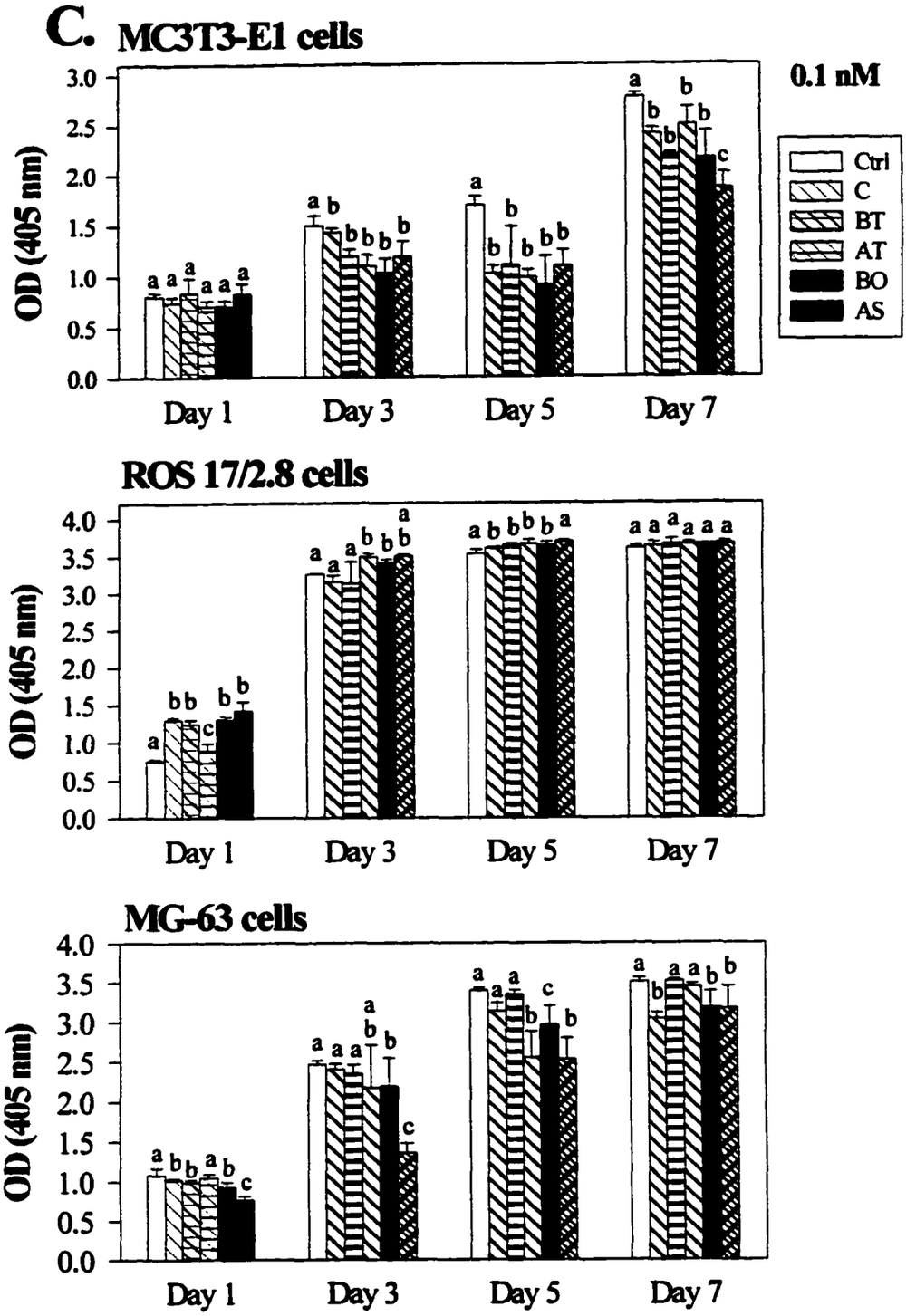
Table 5. Summary of the Effects of Calcitriol and Its Analogs on Cell Proliferation

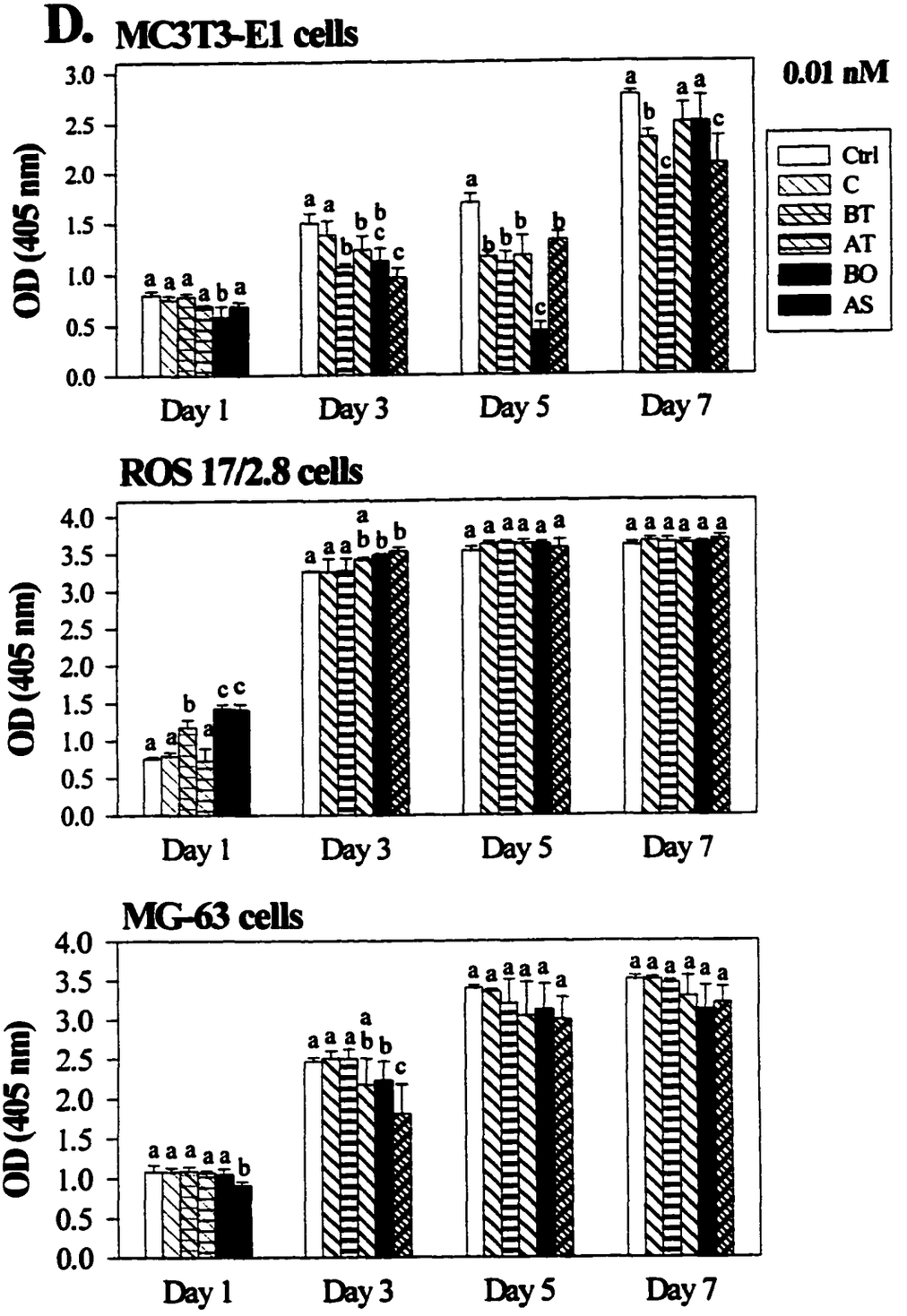
		MC3T3-E1					ROS 17/2.8					MG-63				
		C	BT	AT	BO	AS	C	BT	AT	BO	AS	C	BT	AT	BO	AS
Day 1	10 nM	—	—	—	—	—	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓
	1	—	—	—	—	—	↑	↑	↑	↑	↑	↓	↓	—	↓	↓
	0.1	—	—	—	—	—	—	—	↑	↑	↑	↓	↓	—	↓	↓
	0.01	—	—	—	—	—	↑	↑	—	↑	↑	—	—	—	—	↓
Day 3	10 nM	↓	↓	↓	↓	↓	↓	—	↑	↑	↑	↓	↓	↓	↓	↓
	1	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	—	—	↓	↓
	0.1	—	↓	↓	↓	↓	—	—	—	↑	↑	—	—	↓	↓	↓
	0.01	—	↓	↓	↓	↓	—	—	—	↑	↑	—	—	↓	↓	↓
Day 5	10 nM	↓	↓	↓	—	↓	—	—	—	—	—	↓	↓	—	—	↓
	1	↓	—	↓	—	↓	—	—	—	—	—	↓	—	—	—	↓
	0.1	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	—	—	↓	↓	↓
	0.01	↓	↓	↓	↓	↓	—	—	—	—	—	—	—	—	—	—
Day 7	10 nM	↓	↓	↓	↓	↓	—	—	—	—	—	↓	↓	↓	↓	↓
	1	↓	↓	↓	↓	↓	—	—	—	—	—	↓	—	↓	↓	↓
	0.1	↓	↓	↓	↓	↓	—	—	—	—	—	↓	—	—	↓	↓
	0.01	↓	↓	—	—	↓	—	—	—	—	—	—	—	—	—	—

— no effect relative to untreated control; ↓ decreased relative to untreated control;
↑ increased relative to untreated control

Fig. 1. Effects of calcitriol and its analogs on cell proliferation assay in MC3T3-E1, ROS 17/2.8, and MG-63 cells. Cells were seeded at 5,000/well in flat bottom 96-well plates; after 24 h, cells were changed to medium containing solvent (0.001% isopropanol) or 10 nM vitamin D analogs (A). B, 1 nM of calcitriol and its analogs. C, 0.1 nM of calcitriol and its analogs. D, 0.01 nM of calcitriol and its analogs. β-Hexosaminidase activity was measured on days 1, 3, 5, and 7 of drug treatment. Previous studies have shown that cell numbers are highly correlated with the endogenous enzyme β-hexosaminidase activity. Ctrl, control. C, calcitriol. BT, calcipotriol. AT, 16-ene-23-yne,1,25$(OH)_2D$, BO, 25$(OH)D_3$. AS, 24,25$(OH)_2D_3$. Data are expressed as mean ± SD and analyzed for statistical significance by using Sigma Stat software. Each day and dose were analyzed by one-way ANOVA, and, when appropriate, multiple group comparisons were done pairwise using Student-Newman-Keuls test. Those not sharing letters are significantly different ($p<0.05$). This figure represents one of two repeated experiments.







Metabolic Labeling and Total Protein Induction

To analyze the effects of calcitriol and its analogs on total protein secreted, cells were treated with these drugs for 24 h and radiolabeled with ^{35}S-methionine or $^{32}PO_4$, and the medium was precipitated with tricholoroacetic acid. Figure 2 shows that calcitriol and analogs had no effects on total amount of protein secreted by MC3T3-E1 and ROS 17/2.8 cells. However, we found that there were differences between control and analogs in total ^{35}S-methionine labeled-protein secreted by MG-63 cells (Fig. 2 C). There was an increase in total phosphorylated protein secreted by ROS 17/2.8 and MC3T3-E1 cells treated with calcitriol, BT, and AT (3.9-, 3.7-, and 3-fold increases, and 32-, 27-, and 15- fold increases, respectively) (Fig. 3). No phosphorylated protein secreted by MG-63 cells treated with calcitriol and its analogs was detected. There was a trend for an increase in total secreted protein by cells treated with calcitriol, BT, and AT (1.4-, 1.3-, and 1.5-fold increases, respectively), but these increases were not statistically significantly different from control or each other (Table 6).

Table 6. Total CPM Counts From Figure 2 (A, B, and C).

Cell types	Ctrl	C	BT	AT	BO	AS
MC3T3-E1	1	1	1	1	1	1
ROS 17/2.8	1	1	1	1	1	1
MG-63	1	1±0.36	1± 0.37	1± 0.05	1± 0.25	1± 0.2

$p>0.05$, between control and treatment. There were no significant differences between treatments. The ratio of analogs to control was calculated. Ctrl, control. C, calcitriol. BT, calcipotriol. AT, 16-ene-23-yne,1,25(OH)$_2$D$_3$. BO, 25(OH)D$_3$. AS, 24,25(OH)$_2$D$_3$.

Fig. 2. Total [35S]methionine-labeled protein secreted by MC3T3-E1 (A), ROS 17/2.8 (B), and MG-63 (C) cells. These are fluorographs of total protein secreted by cells which were treated with control (0.001% isopropanol) or 10 nM vitamin D analogs for 24 h; medium collected was TCA precipitated as described in "Methodology." Each lane was loaded with a volume of sample normalized to equal cell numbers. Control (lane 1), calcitriol (lane 2), BT (lane3), AT (lane 4), BO (lane 5), and AS (lane 6). K, thousands. This figure represents one of three repeated experiments.

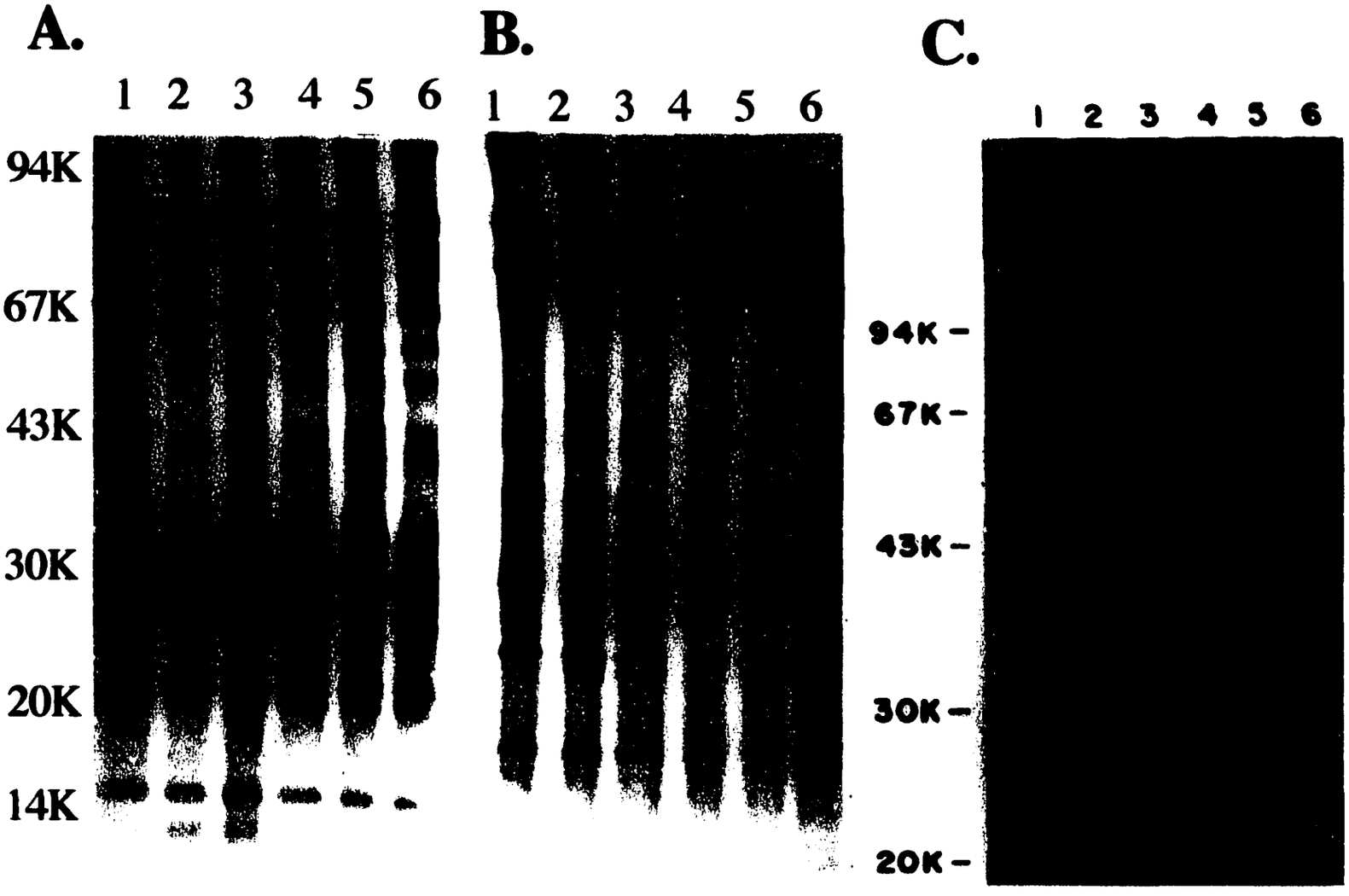
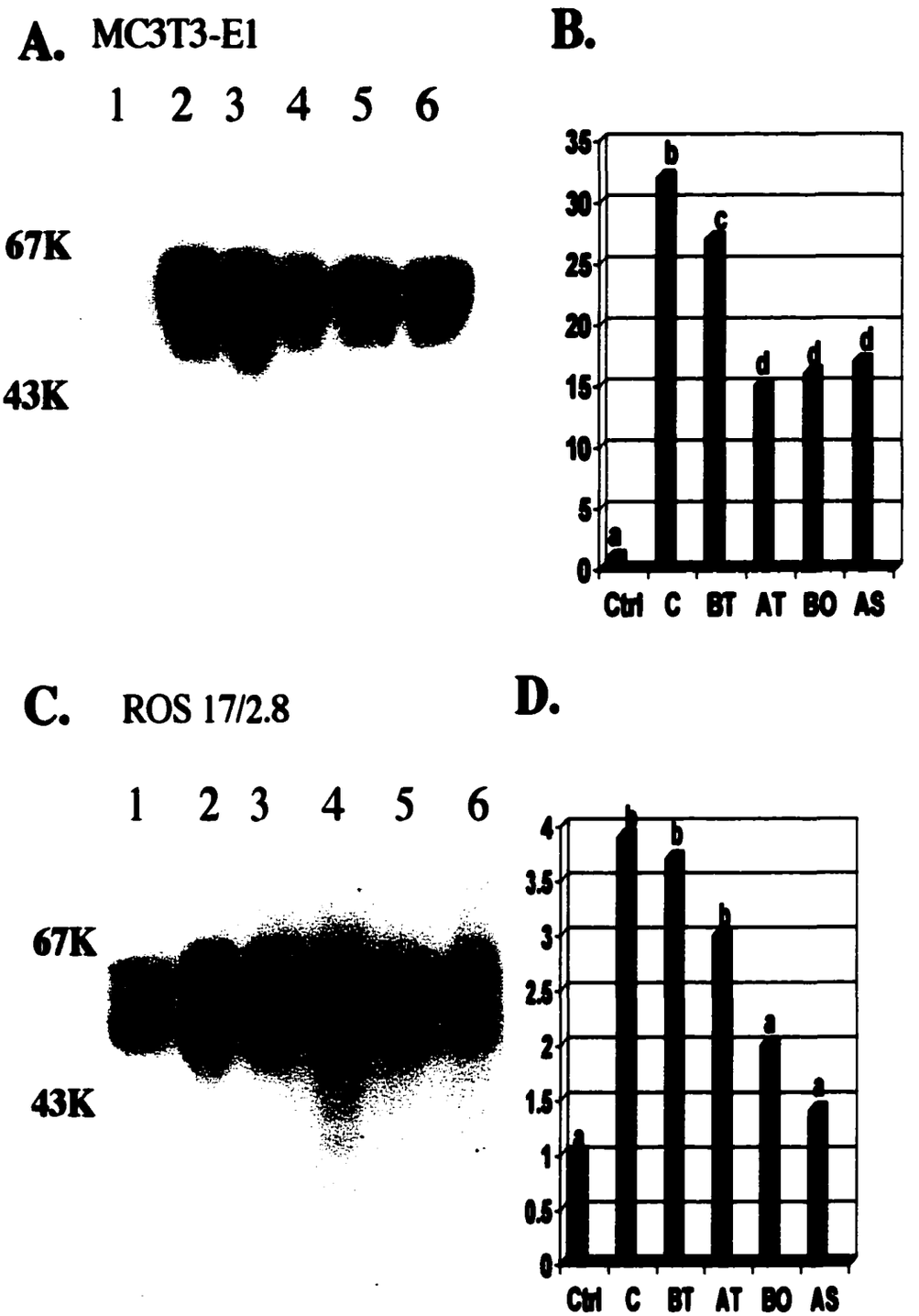


Fig. 3. Total $^{32}PO_4$-labeled protein secreted by MC3T3-E1 (A, B) and ROS 17/2.8 (C, D) cells. A and C are autoradiographies of phosphorylated protein secreted by cells which were treated with control (0.001% isopropanol) or 10 nM vitamin D analogs for 24 h. Each lane was loaded with a volume of sample normalized to equal cell numbers. Control (lane 1), calcitriol (lane 2), BT (lane 3), AT (lane 4), BO (lane 5), and AS (lane 6). B and D, graphs of ratio of analogs to control calculated from densitometric scanning of A and C, respectively. K, thousands. Those not sharing letters are significantly different ($p<0.05$). This figure represents one of three repeated experiments.



OPN Synthesis

For OPN expression, radiolabeled medium was immunoadsorbed with antiserum to OPN. Figure 4 shows that calcitriol and BT induced about a 3-fold increase of OPN expression (both ^{35}S-methionine and $^{32}PO_4$ labeled), while AT had no effect in MC3T3-E1 cells. In ROS 17/2.8 cells (Fig. 5), C, BT, AT, and BO induced OPN secretion by 3.7, 3.2, 2.3, and 1.5 fold, respectively. There was no detectable OPN expression (both ^{35}S-methionine and $^{32}PO_4$ labeled) in MG-63 cells treated with calcitriol or its analogs. We verified the activity of human OPN antibody by using recombinant human OPN as a positive control in a Western blot analysis. In both MC3T3-E1 and ROS 17/2.8 cells treated with calcitriol and its analogs, multiple bands of OPN were immunoadsorbed, suggesting that OPN is differentially glycosylated or phosphorylated by these cells.

OPN mRNA Expression in ROS 17/2.8 and MC3T3-E1 Cells

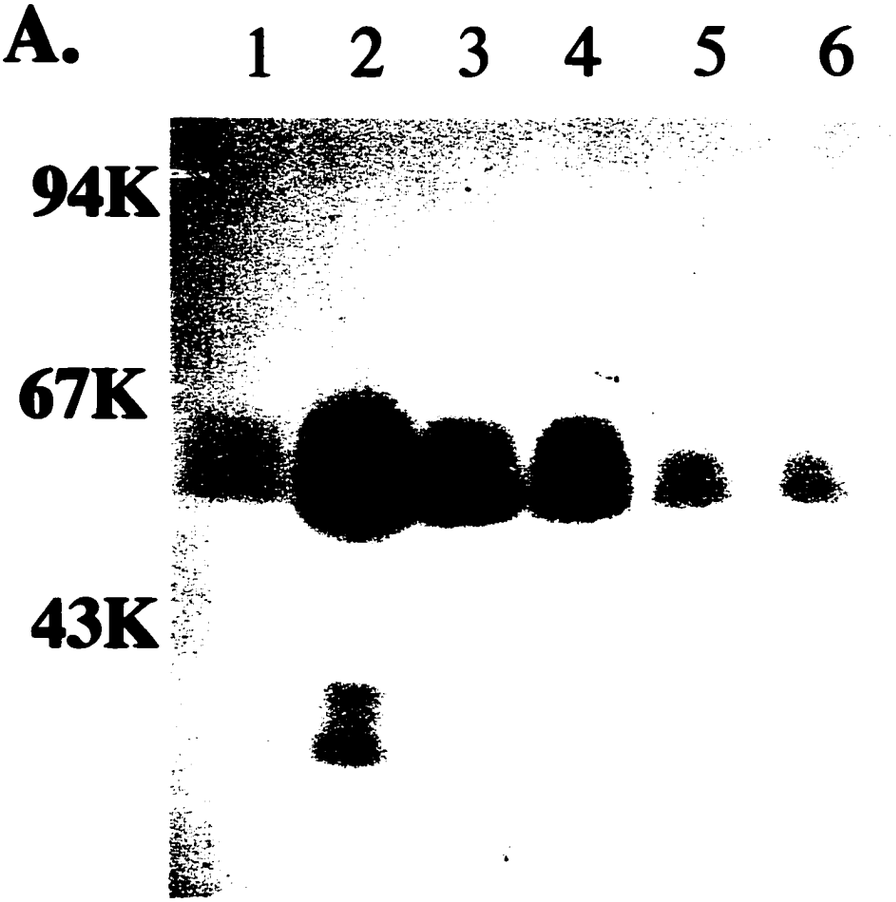
To determine if the calcitriol analogs enhanced steady-state OPN mRNA expression, cells were treated with these drugs for 24 h, and analyzed for OPN mRNA by Northern blot. Figure 6 shows that MC3T3-E1 cells treated with calcitriol, BT, and AT had 17-,16-, and 9- fold increases, respectively, in OPN mRNA expression. In ROS 17/2.8 cells, calcitriol and BT stimulated OPN mRNA by about 2 fold, but these increases were not statistically different from control or other analogs (Fig. 7).

OPN mRNA Expression in MG-63 Cells

However, there was no detectable OPN mRNA in MG-63 cells, even after examining different cell densities (Fig. 8) and different time courses of calcitriol and

Fig. 4. Effects of calcitriol and its analogs on OPN expression by MC3T3-E1 cells. Secreted protein in the medium was collected and immunoadsorbed with antiserum to OPN. A, autoradiography of $^{32}PO_4$-labeled OPN; B, fluorography of [^{35}S]methionine-labeled OPN. Each lane was loaded with a volume of sample normalized to equal cell numbers. Control (lane 1), calcitriol (lane 2), BT (lane 3), AT (lane 4). C and D are graphs of ratio of analogs to control calculated from densitometric scanning of A and B, respectively. K, thousands. A and B represent one of three repeated experiments, while C and D were compared using data from all three experiments. Those not sharing letters are significantly different ($p<0.05$).

Fig. 5. [^{35}S]methionine-labeled OPN secreted by ROS 17/2.8 cells. Cells were treated with control (0.001% isopropanol) or 10 nM vitamin D analogs for 24 h. Secreted proteins in the medium were collected and immunoadsorbed with antiserum to OPN. A, Fluorography of 10% SDS-PAGE with control (lane 1), calcitriol (lane 2), BT (lane 3), AT (lane 4), BO (lane 5), and AS (lane 6). B, graph of ratio of analogs to control calculated from densitometric scanning of A. K, thousands. A represents one of three repeated experiments while B represents the average of the three experiments. Those not sharing letters are significantly different ($p<0.05$).



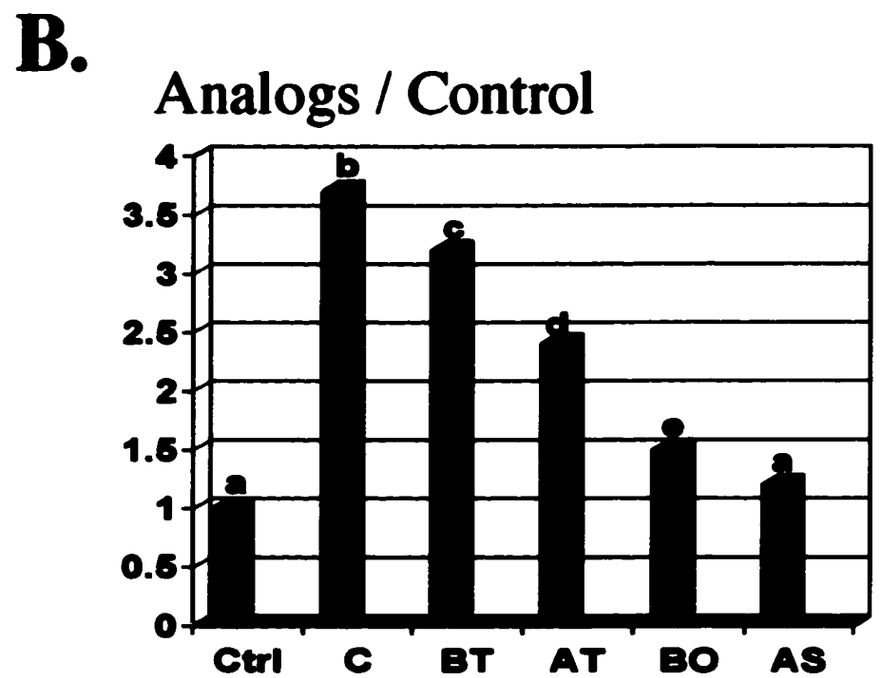
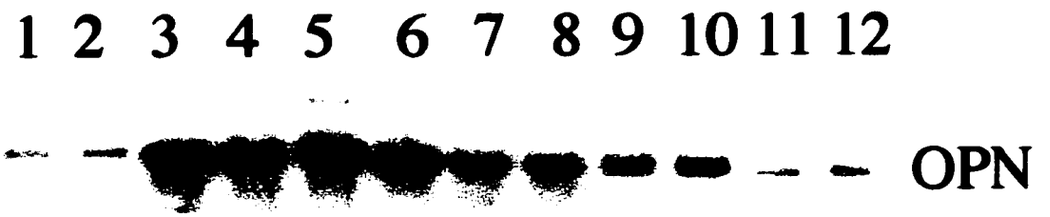


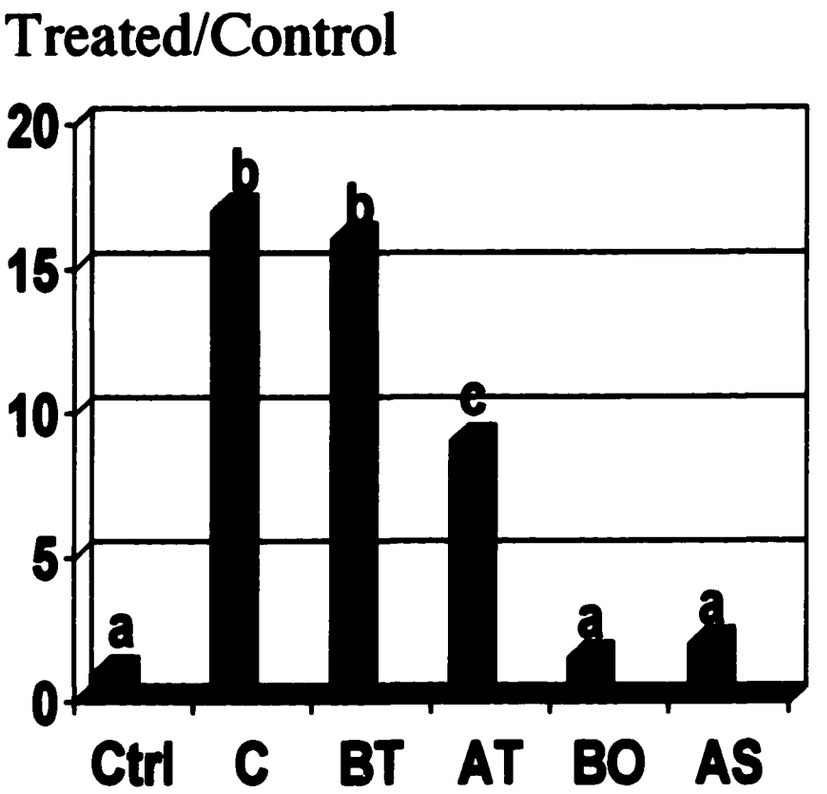
Fig. 6. Northern blot analysis of OPN mRNA in MC3T3-E1 cells. A, Northern blot of OPN mRNA from cells incubated with control, 0.001% isopropanol (lanes 1, 2), 10 nM calcitriol (lanes 3, 4), BT (lanes 5, 6), AT (lanes 7, 8), BO (lanes 9, 10) or AS (lanes 11, 12) for 24h. Each lane was loaded with 10 μg total RNA. B, Northern blot of β-actin mRNA from the same membrane as A. C, graph of data from densitometric analysis of OPN mRNA normalized against β-actin mRNA. A and B are data from one of three repeated experiments, while C presents the averages from the three experiments. Those not sharing letters are significantly different ($p<0.05$).

A.

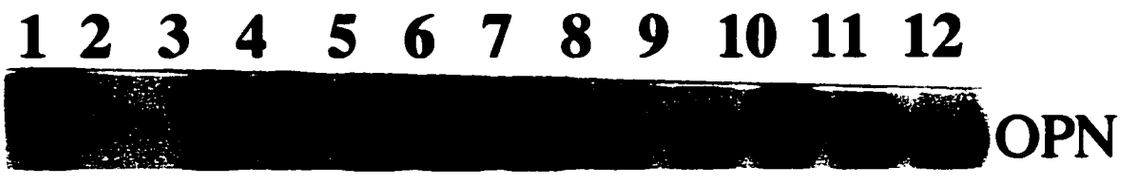


B.

C.



A.



B.

C.

Treated / Control

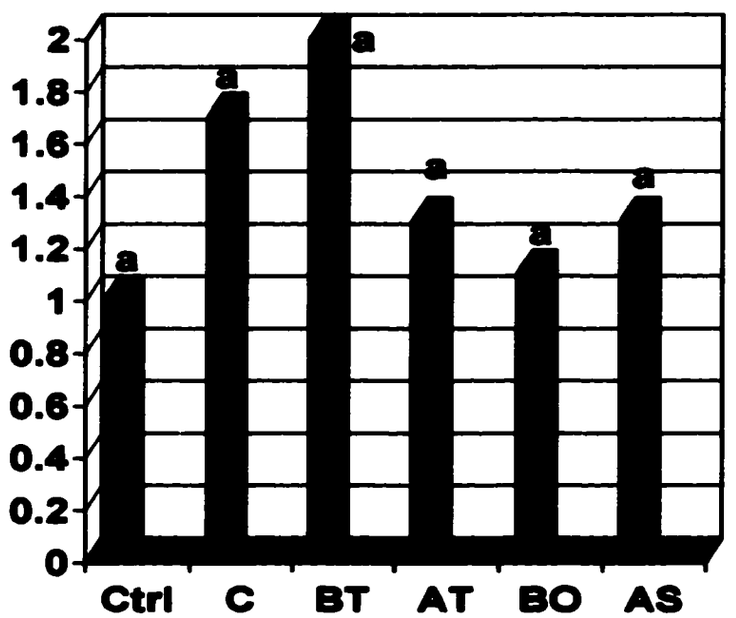


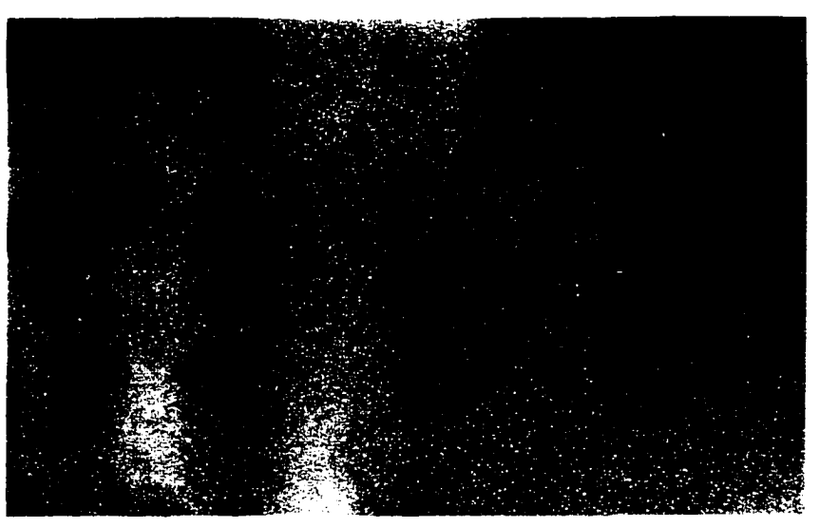
Fig.8. Northern blot analysis of OPN mRNA in MG-63 cells treated with different analogs (lanes 1 to 6) and U-251 MG cells (lane 7). Control (lane 1), calcitriol (lane 2), BT (lane 3), AT (lane 4), BO (lane 5) and AS (lane 6). A, high cell density. B, low cell density. This figure represents one of three repeated experiments.

A.

1 2 3 4 5 6 7

OPN

B.



OPN

analogs treatment (24, 48, and 72 h). Furthermore, we extracted polyA-mRNA rather than total RNA for Northern blot analysis but still detected no expression of OPN mRNA in MG-63 cells.

OPN Gene

To determine if the lack of OPN message in MG-63 cells could be due to lack of OPN gene, we did Southern blot analysis for OPN gene in this cell line, using human glioma cells as a positive control. Figure 9 shows that MG-63 cells have an OPN gene (7.1 kb) the same size as in glioma cells, a positive control.

RT-PCR for OPN mRNA

Since there was no OPN mRNA expression from Northern blot analysis in MG-63 cells, RT-PCR was used to evaluate OPN mRNA expression, again using human glioma cells as a positive control. After 35 cycles of RT-PCR, we found one OPN band in MG-63 cells which appeared to correlate to the smallest of three OPN bands in human glioma cells (Fig. 10). Therefore, we concluded that if expressed at all, the level of OPN mRNA is too weak to detect from Northern blot analysis in MG-63 cells. However, results from radiolabeling and Western blot analyses suggest that very little, if any, OPN protein is secreted by MG-63 cells.

OCN, ALP, and COLI Expression

To determine the effect of calcitriol on OCN, ALP, and COLI expression in MG-63 cells since they did not express OPN, we did immunoadsorption assay for detecting OCN,

Fig.9. Southern blot analysis of OPN gene in MG-63 and U251 MG cells. MG-63 without calcitriol (lane 1), MG-63 treated with calcitriol (lane 2), and U-251 MG cells (lane 3). Ten micrograms of genomic DNA from MG-63 and U-251MG were digested with the restriction enzyme EcoRI, electrophoresed through 0.7% agarose gels; denatured in 1.5 M NaCl and 0.5 M NaOH 2 times for 15 min, neutralized in 0.5 M Tris-HCL-3 M NaCl (pH 7.0) two times for 30 min, and then blotted onto nylon membranes. Then the membranes were prehybridized and hybridized with radiolabeled cDNA and exposed to film. This figure represents one of two repeated experiments.

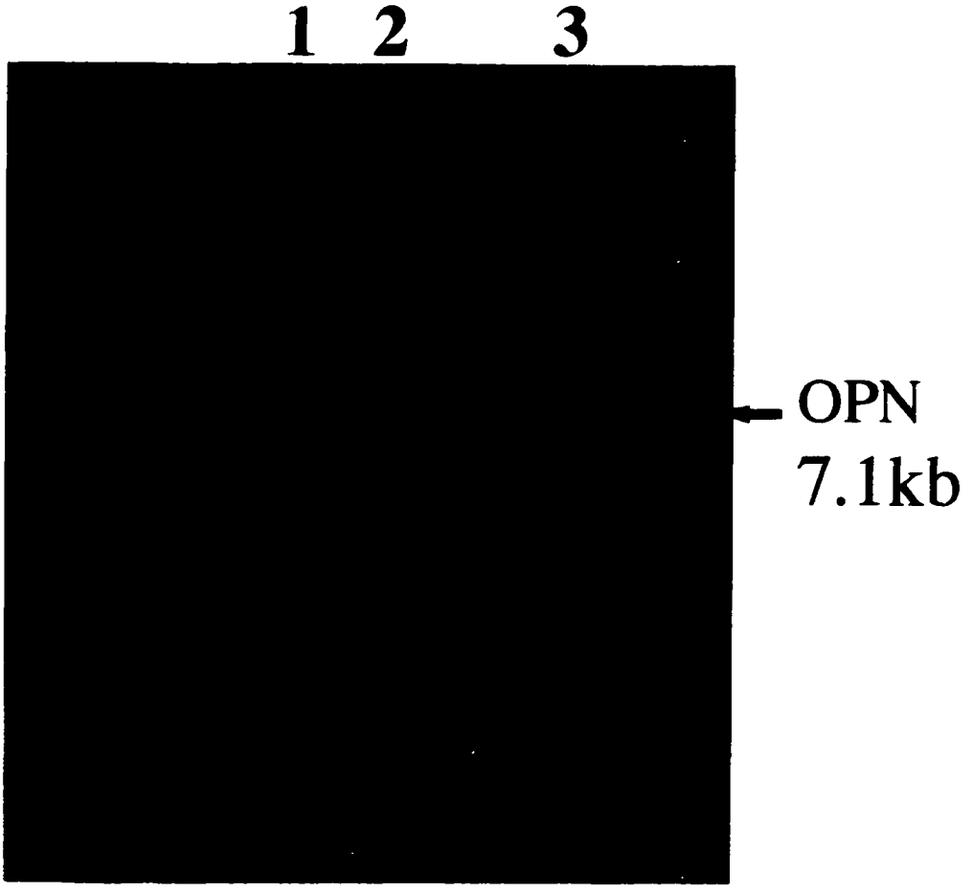
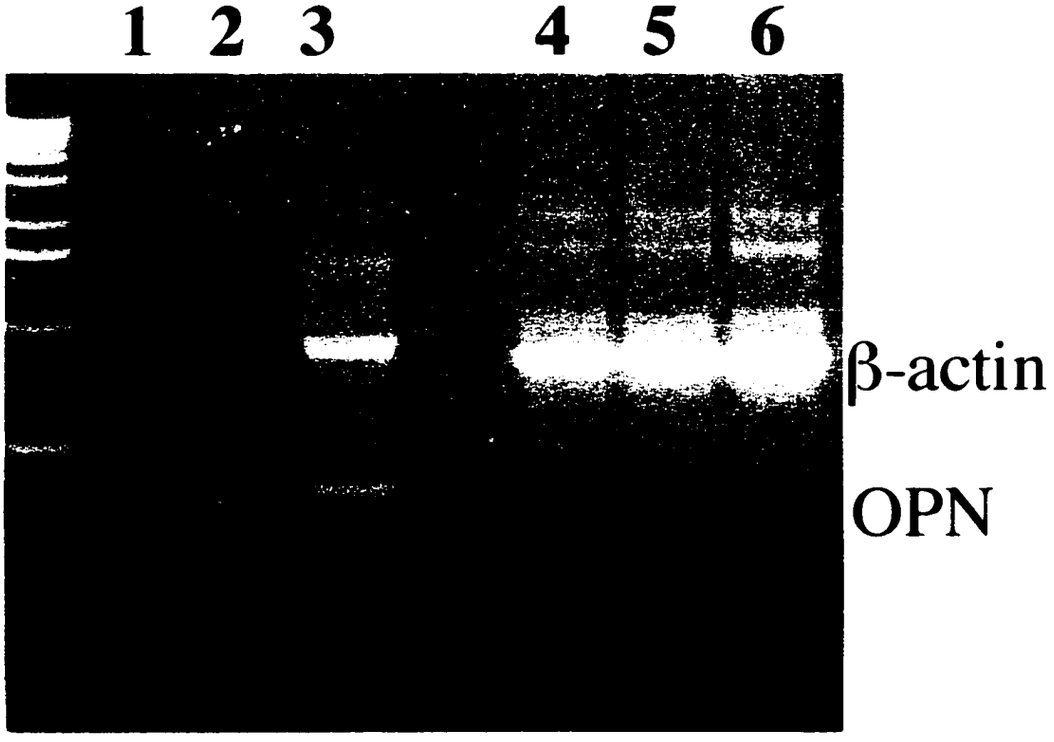


Fig.10. RT-PCR of OPN mRNA expression in MG-63 cells (lane 1: control, lane 2: calcitriol treated) and U-251MG cells (lane 3). Lanes 4-6 were β-actin mRNA expression. The OPN cDNA was amplified by polymerase chain reaction (PCR) using a set of primers,-5'-ACCATGAGAATTGCAGTGATTTGC-3'(OP.A)and5'-ATTGACCTCAGAAGATGCACTATC-3'-(OP.B)-or5'-ATCAGTGACCAGTTCATCAGATTC (OP.C). The primers for β-actin are 5'-ATGGATGATATCGCCGCGCTC-3'and5'-CTAGAAGCATTTGCGGTGGACGAT-3'. This figure represents one of three repeated experiments.



ALP, and COLI in MG-63 cells. Figure 11 shows that calcitriol stimulates the expression of OCN, ALP, and COLI in MG-63 cells.

OCN mRNA Expression

To measure the effects of calcitriol and analogs on OCN mRNA expression, we also used Northern blot analysis. Figure 12 shows that calcitriol, BT, and AT stimulated the expression of OCN mRNA by 4.5, 3.5, and 3 fold, respectively, in ROS 17/2.8 cells. We could only obtain cDNA for rat OCN, and it did not cross-react with human or mouse.

VDR and COLI mRNA Expression

Since basal expression of OPN was low in MG-63 cells and could not be induced by calcitriol, we did Northern blot analyses for VDR and COLI mRNA expression in this cell line to determine if calcitriol had effects in these cells. We found that calcitriol and BT had 50% inhibition of VDR mRNA expression (Fig.13) but stimulated COLI mRNA expression about 4.5 fold (Fig.14).

Transfection Assay

Since calcitriol and BT induced steady state OPN mRNA and OPN protein in ROS 17/2.8 and MC3T3-E1 cells, a transfection assay was used to determine if the induction of OPN expression by calcitriol and BT is due to transcriptional regulation. By using the OPN promoter containing the VDRE linked to the luciferase reporter gene, we found calcitriol and BT increased the luciferase activity by 4-fold in MC3T3-E1 cells (Fig.15A) and 2-fold in ROS 17/2.8 cells (Fig.15B). These results suggest that calcitriol and BT

increase the rate of transcription of the OPN gene, thereby producing increased levels of OPN mRNA in both ROS 17/2.8 and MC3T3-E1 cells. Table 7 shows the summary of results of effect of calcitriol and its analogs in ROS 17/2.8 and MC3T3-E1 cells.

Fig. 11. OCN, ALP, and COLI expression in MG-63 cells. [^{35}S]methionine-cysteine-labeled OCN (A), ALP (B), and COLI (C) expression in MG-63 cells. Cells were treated with control (0.001% isopropanol, lanes 1, 2) or 10 nM calcitriol (lanes 3, 4) for 24 h. Secreted protein in the medium was collected and immunoadsorbed with antiserum to OCN and COLI. Cell lysate was collected and immunoadsorbed with antiserum to ALP.

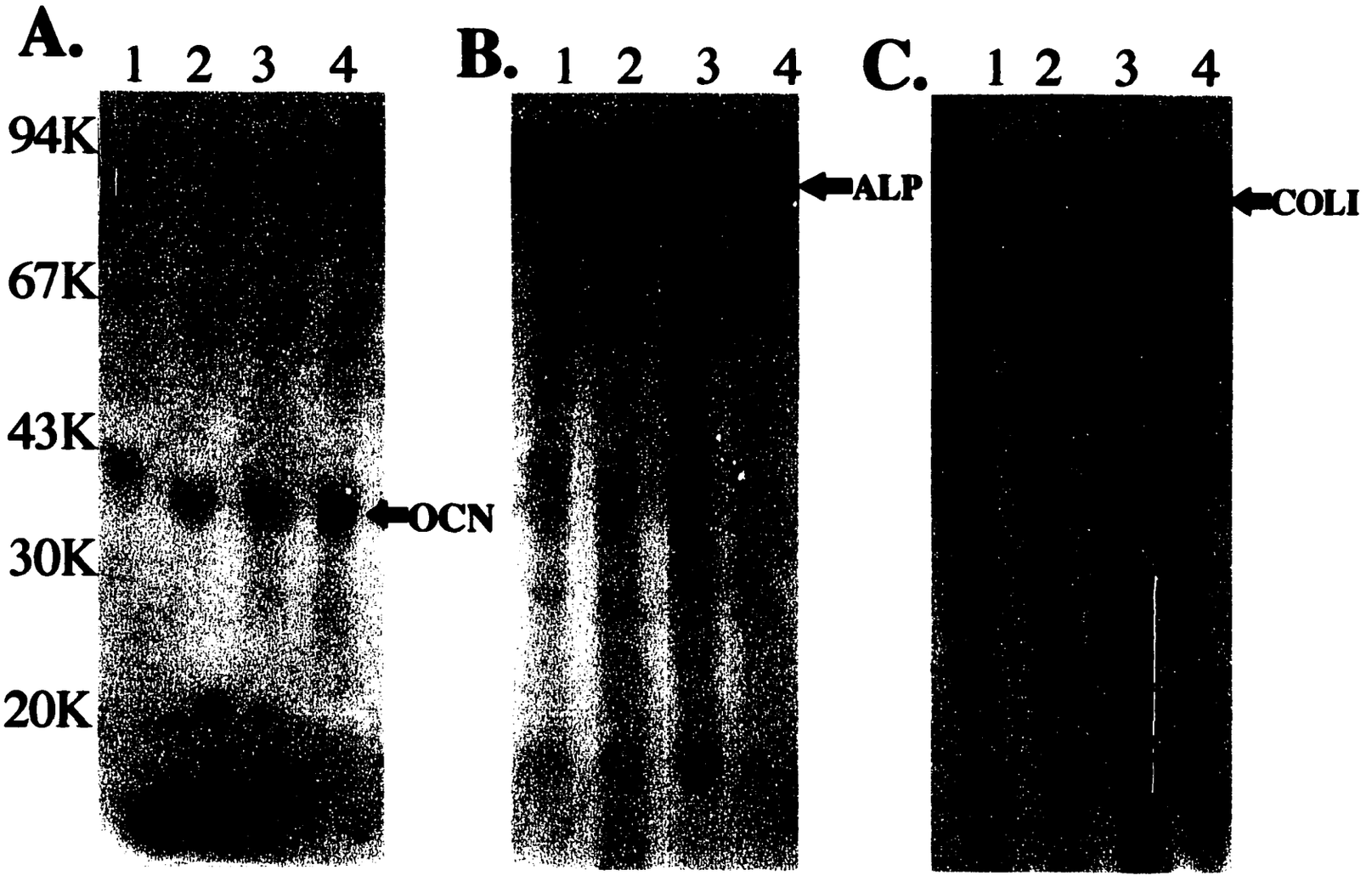
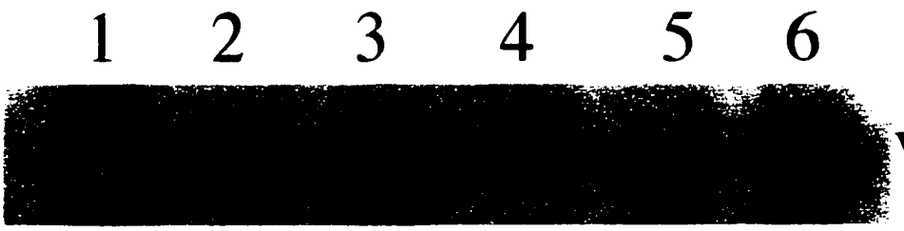


Fig. 12. Northern blot analysis of OCN mRNA in ROS 17/2.8 cells. A, Membrane probed with radiolabeled OCN cDNA. Each lane was loaded with 10 μg total RNA. Control, 0.001% isopropanol (lanes 1, 2), 10 nM calcitriol (lanes 3, 4), BT (lanes 5, 6), AT (lanes 7, 8), BO (lanes 9, 10) and AS (lanes 11, 12). B, Northern blot of β-actin mRNA from the same membrane as A. C, graph of data from densitometric analysis of OCN mRNA normalized against β-actin mRNA. OCN, osteocalcin. This figure represents one of two repeated experiments. Those not sharing letters are significantly different ($p<0.05$).

Fig. 13. Northern blot analysis of VDR mRNA expression in MG-63 treated with calcitriol and analogs. A, Northern blot of VDR mRNA from cells incubated with control, 0.001% isopropanol (lane 1), 10 nM calcitriol (lane 2), BT (lane 3), AT (lane 4), BO (lane 5), or AS (lane 6) for 24 h. Each lane was loaded with 10 μg total RNA. B, Northern blot of β-actin mRNA from the same membrane as A. C, graph of data from densitometric analysis of OPN mRNA normalized against β-actin mRNA. This figure represents one of two repeated experiments. Those not sharing letters are significantly different ($p<0.05$).

A.



B.

C.

Treated/ Control

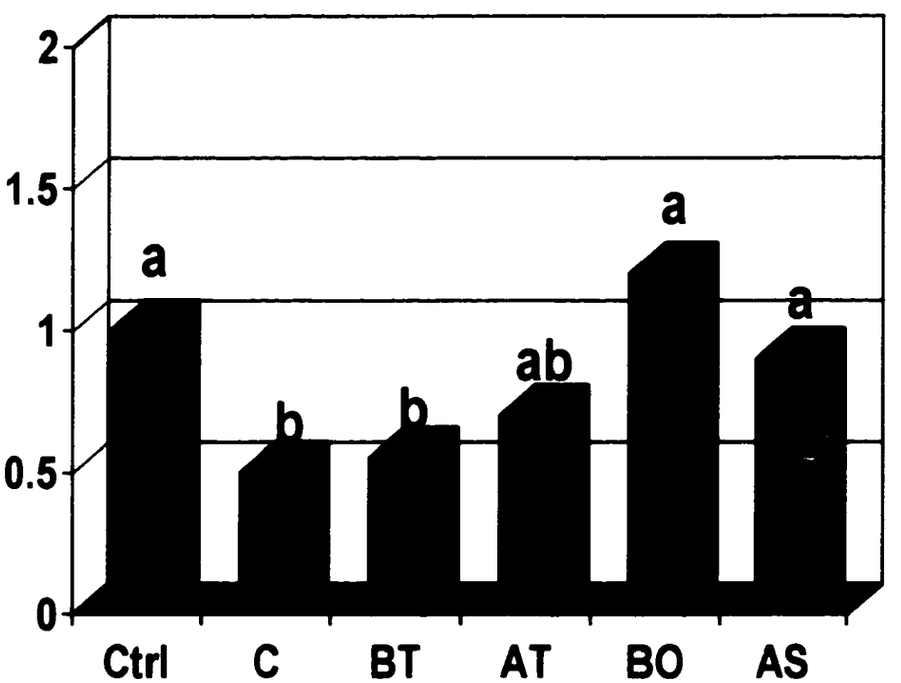
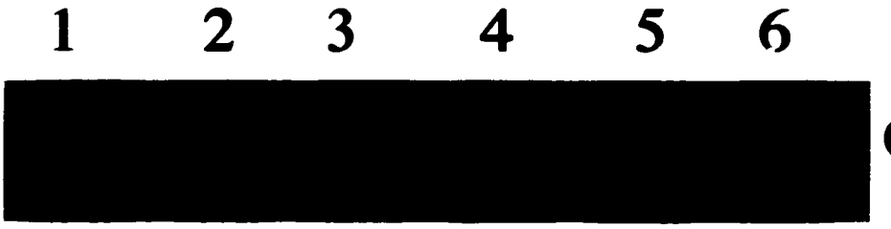


Fig. 14. Northern blot analysis of COLI mRNA expression in MG-63 treated with calcitriol and analogs. A, Northern blot of VDR mRNA from cells incubated with control, 0.001% isopropanol (lane 1), 10 nM calcitriol (lane 2), BT (lane 3), AT (lane 4), BO (lane 5), or AS (lane 6) for 24 h. Each lane was loaded with 10 μg total RNA. B, Northern blot of β-actin mRNA from the same membrane as A. C, graph of data from densitometric analysis of COLI mRNA normalized against β-actin mRNA. This figure represents one of two repeated experiments. Those not sharing letters are significantly different ($p<0.05$).

A.



B.

C.

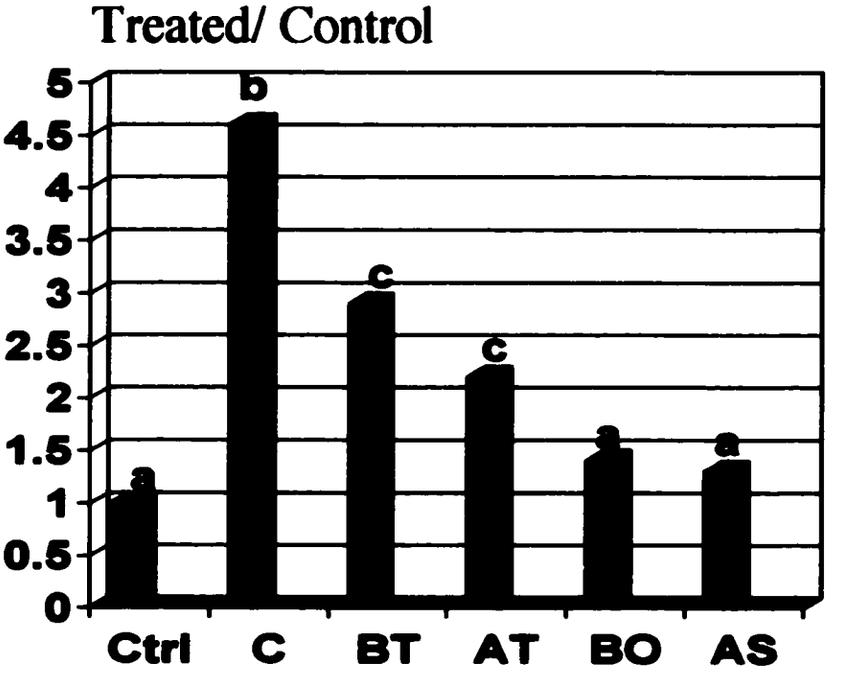
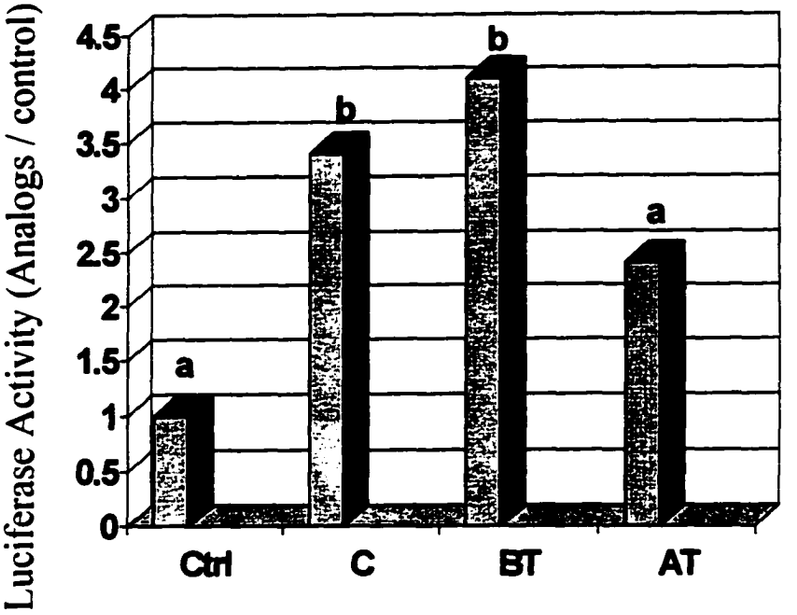


Fig. 15. Transfection assay of MC3T3-E1 and ROS 17/2.8 cells. Luciferase activity in transfected MC3T3-E1 and ROS 17/2.8 cells treated with control and calcitriol analogs. A, MC3T3-E1 cells. B, ROS 17/2.8 cells. The data are plotted as luciferase activity normalized against β-galactosidase activity vs. calcitriol analogs. The data are the average of three experiments. Those not sharing letters are significantly different ($p<0.05$).

A. MC3T3-E1Cells



B. ROS 17/2.8 cells

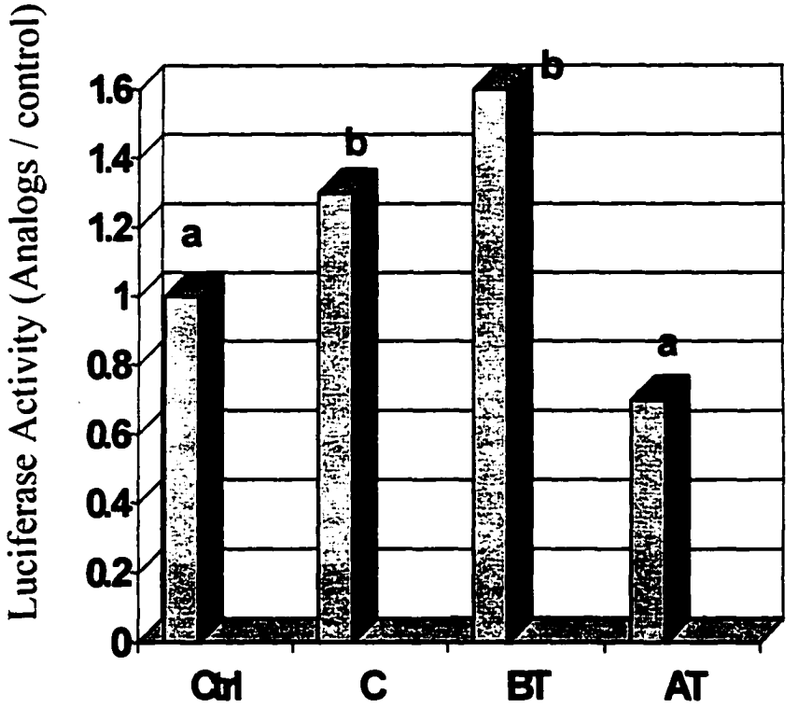


Table 7. Summary of Results of Effect of Calcitriol and Its Analogs in ROS17/2.8 and MC3T3-E1 Cells (Results are relative to control)

Cell line	ROS 17/2.8					MC3T3-E1				
Calcitriol and analogs	C	BT	AT	BO	AS	C	BT	AT	BO	AS
Total ^{35}S-Met protein	-	-	-	-	-	-	-	-	-	-
Total $^{32}PO_4$ protein	3.9↑	3.7↑	3↑	-	-	32↑	27↑	15↑	16↑	17↑
npOPN	3.7↑	3.2↑	2.3↑	1.5↑	-	3.2↑	2.1↑	-	ND	ND
pOPN	ND	ND	ND	ND	ND	2.8↑	2.7↑	-	ND	ND
OPN mRNA	1.7-	2-	-	-	-	17↑	16↑	9↑	-	-
OCN mRNA	4.5↑	3.5↑	3↑	-	-	ND	ND	ND	ND	ND
Luciferase activity	1.3↑	1.6↑	-	ND	ND	3.4↑	4.1↑	2.4↑	ND	ND

C: calcitriol
BT: calcipotriol
AT: 25-OH-16-ene-23-yne-D_3
- : no effect
ND: not determined
↑: increased
↓: decreased
BO: 25(OH)D_3
AS: 24, 25 $(OH)_2D_3$

DISCUSSION

Vitamin D: Metabolism

Vitamin D is a fat soluble vitamin. There is a family of compounds that exhibit the vitamin D activity. Provitamin D is a derivative of cholesterol, 7-dehydrocholesterol, and is converted to vitamin D_3 by the action of ultraviolet light through the skin. Vitamin D_3 is transported to the liver, where vitamin D is converted to $25(OH)D_3$ by a 25-hydroxylase enzyme. $25(OH)D_3$ is transported to the kidney, where the active form of vitamin D, $1,25(OH)_2D_3$ (also called calcitriol), is produced (1). Vitamin D can also be converted to 24,25-dihydroxyvitamin D_3 and other metabolites, but those metabolites are generally considered inactive forms of vitamin D (138).

Calcitriol acts as an osteoid hormone with many functions carried out through both receptor-mediated and local effects on intestine, bone, and kidney. It can regulate bone mineral homeostasis, cell proliferation, and cell differentiation and may also play an important role in tumor promotion and metastasis. Our study focused on the action of calcitriol in bone cells.

Bone Cells

All bone is formed by bone cells. Two main types of bone cells found in fully formed adult bone, osteoclasts and osteoblasts, have diametrically opposed actions which are influenced by PTH and calcitriol, as well as by other substances such as calcitonin and

malignant cells. Table 8 shows a summary of the effects of calcitriol on cell proliferation in several in vitro studies.

Table 8. Calcitriol and Cell Proliferation in Different Studies

Cell types	Effect	Concentration	Ref.
Human malignant melanoma Hs695T	-	13 nM	13
Human myeloid leukemia HL-60	-	10^{-10} - 10^{-8} M	10
Human osteosarcoma MG-63	-	10 nM	20
Human osteoblast-like cells	-	10^{-10} - 10^{-6} M	16
Human renal carcinoma cell KU-2	-	0.1- 100 nM	21
ROS 17/2.8	-	10^{-8} - 10^{-7} M	14,15
Rat and mouse osteoblast-like cells	-	0.13 - 130 nM	140
Chinese hamster ovary CHO cells	-	100 pM	17
Human colon carcinoma Caco-2 cells	-	10^{-8} - 10^{-7} M	145
Human epidermal keratinocytes	- ; +	$>10^{-8}$ M; $<10^{-9}$ M	47
Human breast cancer cells T47D	- ; +	$>10^{-9}$ M; 10^{-12} - 10^{-9} M	46,144
Human bone cells	- ; +	$5x10^{-9}$-10^{-6} M;$5x10^{-12}$M	81
Human thyroid carcinoma TT	+	10^{-12} - 10^{-7} M	48
Human lymphoma cell line U937	±	0.1 - 10 nM	10

- : inhibited cell proliferation + : stimulated ±: no effect on cell proliferation

Clinical research has shown that calcitriol can be useful in the treatment of psoriasis (16,17,24). Psoriasis is a hyperproliferative disease of the epidermis whose cause is unknown. The antiproliferative effect of calcitriol on keratinocytes may suggest that calcitriol is likely to be an autocrine or paracrine factor, since calcitriol is produced by the keratinocyte itself. Some calcitriol analogs such as calcipotriol (BT) also have this

Studies have shown that inhibition of proliferation could be linked to differentiation (144). Thus, osteoblasts at various stages of differentiation may respond differently in terms of cell proliferation when treated with calcitriol and its analogs. It may be that calcitriol and its analogs had no effects on ROS 17/2.8 cells because these are relatively well-differentiated cells. Also, a previous study in our laboratory found that calcitriol had no effects on ROS 17/2.8 cell growth.

On the other hand, however, we found that calcitriol and four analogs had significant inhibitory effects on human MG-63 cells (Fig. 1C, for example). While both MG-63 and ROS 17/2.8 cells are derived from osteosarcomas, they differ in terms of relative differentiation--MG-63 cells are capable of partial osteoblastic differentiation (20), while ROS 17/2.8 cells are already well differentiated. Thus, our data showing variability in response to calcitriol and analogs may result from differences in the level of differentiation of these two cell lines or from a species effect (i.e., human vs. rat).

MC3T3-E1 cells, like MG-63 cells, are capable of undergoing osteoblastic differentiation in culture, although they do so to a greater extent in that they are capable of making bone after about 15-20 days of culture. Thus, under our experimental conditions, MC3T3-E1 cells remain relatively less differentiated and therefore may retain susceptibility to the antiproliferative effects of calcitriol and its analogs. Additionally, of the three cell lines used, MC3T3-E1 cells are not tumor derived. It is likely that differences in response to growth promotion or inhibition differ significantlybetween "normal" and transformed cells (140). Further investigation will be required to elucidate the mechanisms of action of calcitriol on cell proliferation. It is possible that the VDR, a key trans-activating factor regarding gene expression and cell growth (145), may interact

with other factors such as nitric oxide synthase or multiple histone promoter factors in regulating cell growth (147).

The Mechanisms of Calcitriol Action

Calcitriol has been demonstrated to regulate biological responses by both genomic (involving the VDR) and nongenomic (not requiring a VDR but maybe involving a putative membrane receptor mechanisms) (41). Calcitriol and its analogs have differing affinities for VDR. Therefore, different calcitriol analogs may be used to study the mechanism of calcitriol action for specific cellular responses. The calcitriol analogs which have used in our study included BT, which like calcitriol has a high affinity for VDR; AT, BO, and AS lower affinity for VDR and mimic the nongenomic action of calcitriol by inducing rapid cellular responses such as calcium fluxes.

Calcitriol binds VDR to form a complex and then binds to specific VDRE genes. This binding can increase or decrease expression of the gene. Since OPN and OCN have been shown to contain VDREs in the promoter region, we studied the effects of calcitriol and analogs on the expression of these proteins. We also examined the effects of calcitriol on two other proteins secreted by osteoblasts, type I collagen and alkaline phosphatase, and regulated by calcitriol.

Total Protein

Before examining specific protein responses, we first examined the total protein secretion by cells treated with calcitriol and its analogs. Since most proteins contain methionine, we incubated culture medium with ^{35}S-Met for total protein expression.

Table 7 indicates that there were no differences in total cpm recovered by TCA precipitation between control and all analogs in ROS 17/2.8 and MC3T3-E1 cells. However, as noted before, we did find differences between control and analogs with respect to individual proteins from Figure 2. Since overall protein synthesis and secretion were not affected, it is likely that effects on individual proteins result from specific rather than generalized effects on protein expression. We also found that the pattern of proteins secreted by the different cell lines was different, supporting the idea that these three cell lines may represent different stages of osteoblast development.

We also examined total phosphorylated protein secreted by these cells. Our data showed that calcitriol increased total phosphorylated protein by 3.9-, and 32-fold in ROS 17/2.8 and MC3T3-E1 cells, respectively. BT also induced total phosphorylated protein to increase about 3.7- and 27- fold in ROS 17/2.8 and MC3T3-E1 cells, respectively. AT also had a stimulatory effect (3- and 15-fold increase) on total phosphorylated protein in these two cell lines. This demonstrates that calcitriol, BT, and AT all induced total phosphorylated protein in these two cell lines, although with stronger effects in MC3T3-E1 cells. Therefore, the different effects of calcitriol and analogs between these two cell lines may indicate different mechanisms of calcitriol action in primary or tumor cell lines. For example, analogs BO and AS stimulated total phosphorylated protein by 16- and 17-fold in MC3T3-E1 cells but in ROS 17/2.8 cells, BO had little effect (2-fold increase), and AS had no significant induction of total phosphorylated protein. However, we unexpected by detected no secreted phosphorylated protein in MG-63 cells treated with control or calcitriol and analogs.

OPN

Osteopontin is a secreted and highly acidic protein with potential sites for glycosylation, phosphorylation, sulfation, calcium binding, thrombin cleavage and cell-attachment. Several studies have suggested that OPN has important roles in bone formation, bone resorption, cell adhesion, resistance to bacterial infection, and tumorigenesis and metastasis (61,104-116,148). Recent studies also found that OPN may be related to heart disease (148) and kidney stone formation (159).

OPN has been isolated and characterized from many cells, but its detailed functions are not clear yet. OPN from rat bone indicated that this protein is phosphorylated and contains 12 phosphoserines and 1 phosphothreonine residues (87). Therefore, the most extensive form of modification for OPN is phosphorylation of serine and threonine. Some studies found that OPN exists in forms having apparent molecular masses (119, 144). This finding is consistent with our results from an immunoadsorption assay which showed that OPN has two bands in both ROS 17/2.8 and MC3T3-E1 cells. Furthermore, OPN represents almost all of the total phosphorylated proteins in MC3T3-E1 cells. Our results demonstrate that bone cells produce more than one form of OPN and suggest that posttranslational modifications such as phosphorylation may be major mechanisms to generate different forms of OPN.

Several studies have shown that calcitriol induces OPN expression in osteoblast-like cells (82,119-122). Our ^{35}S-methionine radiolabeling data showed that in ROS 17/2.8 cells, calcitriol, BT, and AT stimulated npOPN expression by 3.7-, 3.2-, and 2.3-fold, respectively. BO and AS had no significant effects on OPN expression in ROS 17/2.8 cells. However, in MC3T3-E1 cells, calcitriol and BT had about 3-fold increases

of OPN (^{32}P-OPN and ^{35}S-Met-OPN) expression, but AT had no effect on OPN expression. This indicated that these two cell lines treated with control or calcitriol analogs differ in the expression of osteopontin.

The analog AT, which binds poorly to VDR, activates calcium channels and regulates biological responses through a nongenomic pathway. Our results show that AT could induce both total phosphorylated protein and OPN in ROS 17/2.8 cells. Khoury et al. (60) reported that ROS 17/2.8 cells contain 1α-hydroxylase activity which would be expected to convert analog AT to V (1α, 25-dihydroxy-16-ene-23-yne-vitamin D_3), which is a conversion of a compound selective for nongenomic pathway to one capable of activating both genomic and nongenomic pathways. However, it is currently not possible to directly test whether AT is converted to V because of the lack of commercially available radiolabelled AT. However, others (Chang et al., in press) have suggested that this conversion is unlikely to contribute to the effects of AT. In the future, additional research is needed to find out why AT had effects on OPN expression in ROS 17/2.8 cells.

BO and AS are natural vitamin D metabolites. Some studies have found that BO and AS have lower affinity to VDR than calcitriol (63,64). Our results demonstrated that BO and AS had almost no induction on OPN protein expression in ROS 17/2.8 cells, which is consistent with other findings. Our data did not show that effect of BO and AS on OPN expression in MC3T3-E1 cells.

However, in MG-63 cells, we cannot find any OPN (either $^{32}PO_4$ or ^{35}S-Met labeled) expression with control or calcitriol analogs treatment. We repeated this experiment at least three times, all with negative results. For immunoadsorption assay

using OPN antibody, we considered this negative result may due to the problem of OPN antibody. We examined the human OPN antibody used for this experiment by Western blot analysis, using human recombinant OPN as a positive control. Data indicated that human OPN antibody can react with recombinant human OPN but not cross-react with recombinant rat OPN. Therefore, the lack of OPN expression in MG-63 cells was not due to the OPN antibody.

OPN mRNA

After detecting the effects of calcitriol and its analogs on OPN expression, we wanted to know the effects on OPN mRNA expression. Our data from Northern blot analyses indicated that calcitriol and BT also stimulated the steady-state level of OPN mRNA expression by 17-, and 16- fold, respectively, in MC3T3-E1 cells. In ROS 17/2.8 cells, calcitriol and BT had about 2-fold induction of OPN mRNA expression. AT had a 9-fold increase in OPN mRNA expression in MC3T3-E1 cells but no significant effect on the expression of OPN mRNA in ROS 17/2.8 cells. This suggests that AT has differential effects on OPN and OPN mRNA expression in these two cell lines. Furthermore, BO and AS had no effects on OPN mRNA expression in both MC3T3-E1 and ROS 17/2.8 cells.

This induction of OPN synthesis by calcitriol and BT correlated with increasing expression of OPN mRNA in both MC3T3-E1 and ROS 17/2.8 cells, suggesting that regulation of OPN expression could be at the level of transcription. We further verified that by doing transfection assay.

In MG-63 cells, there was no detectable expression of OPN mRNA in cells treated with control or analogs from repeated Northern blot analyses. We examined the effect on different time courses (not only 24-h treatment of calcitriol and its analogs but also 48- and 72-h treatment. The results indicated that MG-63 cells treated with control or calcitriol analogs for 24, 48, and 72 h all did not express OPN mRNA.

Also, we examined OPN mRNA expression with different cell density in MG-63 cells treated with control or calcitriol analogs. We still did not find any OPN mRNA expression in MG-63 cells. Since Saitoh et al. (153) reported that human gliomas express OPN, we used human glioma U251MG cells as our positive control. Northern blot analyses for OPN mRNA expression in both MG-63 and glioma cells demonstrated that there was no OPN mRNA expression in MG-63 cells.

Since we got negative results in Northern blot analyses for OPN mRNA expression from total RNA, we needed to further examine on poly-A mRNA. We extracted the poly-A mRNA from MG-63 cells treated with control and calcitriol and then did the Northern blot analysis. There was still no OPN mRNA expression in MG-63 cells ± calcitriol, even loading with 4 μg of poly-A mRNA.

Therefore, we used RT-PCR for OPN mRNA expression in MG-63 and glioma cells (positive control). Data from RT-PCR suggested that MG-63 ± calcitriol expressed OPN mRNA, as well as glioma cells. This suggests that the message of OPN expression was too weak to detect by Northern blot analysis.

OPN Gene

Osteopontin is encoded by a single copy gene. In the human genome, the OPN gene maps to chromosome 4q21-25, with additional evidence presented for the presence of a Bgl II restriction fragment length polymorphism (RFLP) (151). Studies have shown that Southern blot analysis of genomic DNA from mouse and human produced banding patterns consistent with the existence of one gene encoding for OPN (151, 152). Saitoh et al. (153) also studied the expression of osteopontin in human glioma. They found that OPN expression may be correlated with the malignancy grade of gliomas. Their data of Southern blot analysis indicated that OPN DNA most likely exists as a single copy gene in human glioma cells.

Prior to performing the RT-PCR mentioned above, our data indicated that MG-63 cells treated with control or calcitriol analogs did not express OPN or OPN mRNA. Therefore, we wanted to examine the OPN gene in this cell line, also using glioma cells as a positive control. Our data from Southern blot analysis demonstrated that MG-63 cells contain the OPN gene at same 7.1 kb as glioma cells. MG-63 cell line is a human osteosarcoma cell line which is derived from an osteosarcoma of a 14-year-old male. Studies have shown that MG-63 cells express matrix proteins such as collagen types I and III, osteocalcin, and fibronectin, as well as alkaline phosphatase activity. However, no studies found that MG-63 cells express OPN. Although MG-63.3A has been established and it may be possible to differentiate further toward the osteoblastic phenotype, our results suggest that MG-63 cells may not be a good model of osteoblast development in one critical aspect--the production of osteopontin.

Harter et al. (154) also found that another human osteosarcoma, OHS-4 cells, did not express the OPN. However, several studies have been shown that osteopontin is secreted in elevated levels in many transformed cells (109-113). For human osteosarcoma cells that likely do not secrete the OPN, it may be useful to study the regulation of OPN expression by these human osteosarcoma cells at the level of the promoter region of the OPN gene.

VDR mRNA Expression in MG-63 Cells

To determine if the effects of calcitriol and its analogs on OPN expression may be related to VDR level, we did Northern blot analysis for VDR mRNA expression in MG-63 cells. The result showed that calcitriol, BT, and AT had a significant inhibition on VDR mRNA expression but that BO and AS had no significant effects on VDR mRNA expression. This suggests that MG-63 cells, which express very low basal levels of OPN and secrete little if any OPN mRNA, could not response to calcitriol and analogs by increased OPN expression mediated by VDR.

Although OPN is synthesized by a variety of cell types in culture, it has been clearly shown that bone cells are a major site of synthesis and secretion of this phosphorylated glycoprotein (97,98). Developmental studies have reported that OPN was synthesized and secreted by bone cells in early stages of osteogenesis and that its expression preceded mineralization and the synthesis of osteocalcin (104). Since MG-63 cells are not fully differentiated with respect to the osteoblast phenotype, they should be expected to express OPN. Thus, it is important to study the reasons for their lack of OPN expression.

A recent study (155) reported that a helix-loop-helix-type transcription factor, hairy and enhancer of split homolog-1 (HES-1), is expressed in osteoblastic cells, is suppressed by calcitriol, and modulates calcitriol enhancement of OPN gene expression. HES-1 plays a crucial role in cell differentiation in certain tissues, including muscle and nerve. The overexpression of HES-1 blocked the calcitriol induction of OPN expression. This finding may suggest the weak OPN expression in MG-63 cells is due to the overexpression of HES-1. We did not have cDNA for HES-1, but in the future we can detect the HES-1 mRNA expression in MG-63 cells to determine this possible reason.

OCN

OCN is also an osteoblast gene with a functional VDRE. OCN is a major noncollagenous bone protein. Several studies have found that calcitriol and BT stimulate OCN and OCN mRNA expression (60,63,81-85,125,156). Our results for calcitriol and its analogs on OCN mRNA expression in ROS 17/2.8 cells support the findings mentioned in several studies. Calcitriol, BT, and AT stimulated the expression of OCN mRNA by 4.5-, 3.5-, and 3-fold, respectively, in ROS 17/2.8 cells. This effect was greater than OPN expression in this same cell line and confirms that AT had significant activity in this cell line. We did not have the OCN cDNA for mouse and human; however, we used rOCN cDNA for MC3T3-E1 and MG-63 cells and found that rOCN cDNA cannot cross-react with mouse or human.

Also, we looked at the effect of calcitriol on OCN protein expression in MG-63 cells; our data from immunoadsorption assay showed that calcitriol stimulated OCN

expression in MG-63 cells. Since the only antibody available was for human OCN, the finding of OCN expression was limited.

ALP and COLI Expression

We also measured the effect of calcitriol on ALP expression in MG-63 cells. ALP is a marker of bone formation. Many studies have shown that calcitriol can increase the activity of ALP and ALP mRNA expression (Table 4). Our finding also supported the role of calcitriol on bone formation, since calcitriol stimulated the expression of ALP in MG-63 cells.

Type I collagen is the basic building block of the bone matrix fiber network. Many reports have demonstrated that calcitriol inhibits type I collagen synthesis in different cell types (71-77). However, a study using a human osteosarcoma cell line (OHS-4) found that this cell line secreted COLI mRNA and that calcitriol stimulated the expression of COLI mRNA (154). We detected the effect of calcitriol on COLI expression by using immunoadsorption assay in MG-63 cells. We found that calcitriol stimulates COLI expression in MG-63 cells, similar to the effect in OHS-4 cells. This may suggest that this human osteosarcoma cell line probably has specific characterics. Also, we examined the effect of calcitriol and its analogs on the COLI mRNA expression in MG-63 cells, and found that calcitriol stimulated the COLI mRNA expression about 4.5 fold in MG-63 cells. BT and AT also stimulated the induction of COLI mRNA expression in this cell line, but BO and AS had no significant effect on COLI mRNA expression. Therefore, calcitriol not only increased the expression of COLI but also COLI

mRNA in MG-63 cells. We need to further determine whether the induction of COLI protein and mRNA by calcitriol is due to transcriptional regulation.

Regulation Level

Prince and Butler (120) first observed that OPN levels in ROS 17/2.8 cells were upregulated by calcitriol. OPN protein secretion was increased about 2-fold by calcitriol. Furthermore, the stimulation of OPN by calcitriol was concentration dependent and blocked by transcriptional inhibitor. Several studies also using osteoblast-like cells have shown an increase in OPN mRNA or protein by calcitriol and this is the stimulation of transcription. Chang et al. (59) studied calcitriol regulation of osteopontin expression in mouse epidermal JB6 Cl 41.5a cells and also found that calcitriol increases the expression of OPN mRNA and protein by stimulating transcription.

Data from transfection assay in which an OPN fragment was fused to the luciferase reporter gene showed there was an increase in the luciferase activity; a stimulation of 4 fold was seen at calcitriol and BT treatment in MC3T3-E1 cells and of 2 fold in ROS 17/2.8 cells. Our results support that calcitriol and BT induce an increased rate of transcription of the OPN gene, thereby producing increased levels of OPN mRNA in both MC3T3-E1 and ROS 17/2.8 cells. However, the effect of calcitriol and BT in the transfection assay was not as large as that found by Northern blot analysis. It is likely that the intact OPN promoter contains regions that modulate calcitriol and BT regulation of OPN expression that are not present in the promoter fragment used in our study. Ridall et al. (48) identified a second VDRE in rat OPN gene that is upstream of the construct used in our study. Therefore, the intact OPN gene needs to use and may contribute to the

greater induction of OPN expression. In the future, we need to study VDREs from OPN genes of different species such as mouse and human.

Summary

1. Calcitriol and all analogs had different effects on MC3T3-E1, ROS 17/2.8, and MG-63 cell growth.
2. Calcitriol and BT significantly increased the expression of total phosphorylated protein and OPN in MC3T3-E1 and ROS 17/2.8 cells, but MG-63 cells secreted no phosphorylated protein and OPN.
3. Analogs with genomic effect (C and BT) consistently induced the expression of OPN and OPN mRNA in both MC3T3-E1 and ROS 17/2.8 cells and also induced OCN mRNA expression in ROS 17/2.8 cells.
4. Nongenomic analog AT stimulated total phosphorylated protein, OPN, and OCN mRNA expression in ROS 17/2.8 cells and COLI mRNA expression in MG-63 cells.
5. In MG-63 cells, Northern blot analysis of total RNA and poly-A mRNA detected no OPN mRNA, even with the inclusion of different time courses and different cell densities.
6. The OPN mRNA was detected by RT-PCR in MG-63 cells using glioma cells as a positive control, and calcitriol inhibited VDR mRNA expression in MG-63 cells, which may explain the weak message of OPN expression.
7. The lack of OPN secretion by MG-63 cells suggests that they may not be suitable for studying some aspects of osteoblast metabolism.
8. Data from transfection assay showed that the induction of OPN expression by

calcitriol and BT in both MC3T3-E1 and ROS 17/2.8 cells is due to transcriptional regulation.

9. Calcitriol also stimulated OCN, ALP, and COLI expression in MG-63 cells.

Future Studies

1. For the effects of calcitriol and its analogs on cell proliferation assay, we found that calcitriol and analogs had antiproliferative effects on MC3T3-E1 and MG-63 cells but no effects on ROS 17/2.8 cells. We need to further elucidate the mechanisms of the effects of calcitriol and its analogs on cell growth, comparing the differences between cell types or differences between normal and tumor cells. It may be useful to study the clinical effects of calcitriol and its analogs on some cancer treatment.
2. We used Northern blot analyses to detect the effect of calcitriol and its analogs on OPN mRNA expression. There is one method, S1 nuclease assay, considered more sensitive than Northern blot analysis. In the future, it may be good to use S1 assay instead of Northern blot analysis.
3. The concentration of calcitriol and its analogs used in our study was 10 nM; we need to do further study using different concentrations to examine if effects of calcitriol and its analogs are concentration dependent.
4. The treatment of calcitriol and its analogs in our study was 24 h, we can study the different time courses for protein and mRNA expression to detect if the effects of calcitriol and its analogs on protein and mRNA expression are time dependent.
5. For the effects of nongenomic analog AT, we need to measure 1α-hydroxylase activity and determine the conversion of AT to V.

6. In MG-63 cells, there was no secretion of OPN and OPN mRNA; however, they do contain OPN gene. We need to study the mechanism of OPN regulation by calcitriol in this cell line. The promoter region of osteopontin may be related to this expression. HES-1 overexpression may be a possible reason for MG-63 cells, and we need to examine the HES-1 expression in this cell line.
7. For the regulation levels of OPN by calcitriol and analogs, we need to use transcription inhibitor to block the stimulation of OPN mRNA by calcitriol. It may be better for explain the transcriptional regulation only from transfection assay. We also need to study the effects of calcitriol and its analogs on mRNA stability.
8. Further study is needed to detect the effects of calcitriol and its analogs on VDR protein and mRNA expression in ROS 17/2.8 and MC3T3-E1 cells.
9. We need to evaluate the abilities of both phosphorylated and nonphosphorylated forms of OPN to mediate bone cell adhesion and function.
10. We need to develop and use antisense OPN constructs in MC3T3-E1 cells to determine if expression of OPN is necessary for normal cell function in vitro.
11. Our finding on the effects of calcitriol and its analogs on genomic and nongenomic actions is very crucial, and we need to further study the effect on the nongenomic pathway.

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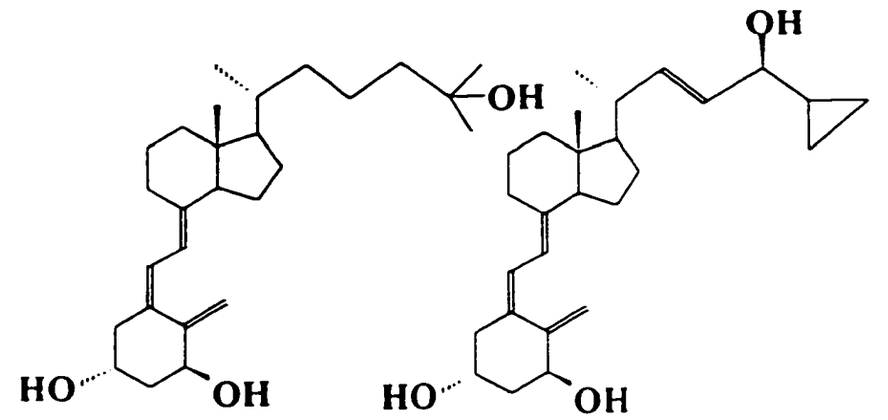
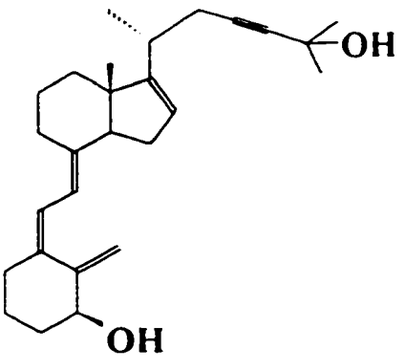
APPENDIX

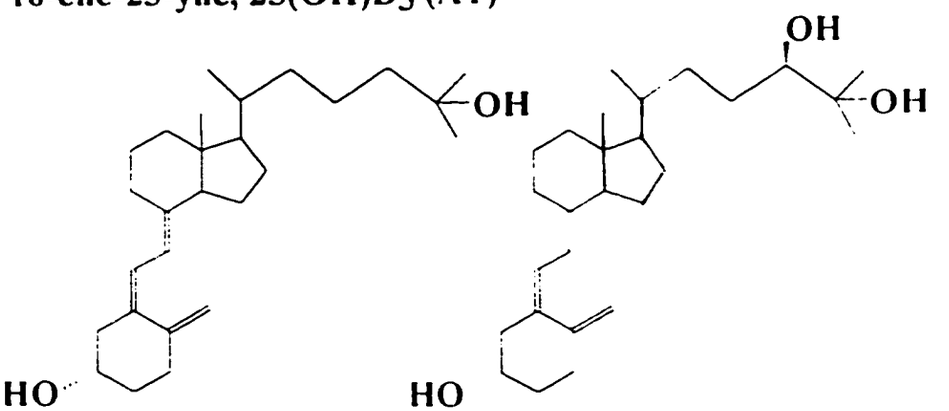
STRUCTURES OF CALCITRIOL AND ITS ANALOGS



1a, 25(OH)$_2$D$_3$ (C) calcipotriol (BT)



16-ene-23-yne, 25(OH)D$_3$ (AT)



25(OH)D$_3$ (BO) 24, 25(OH)$_2$D$_3$ (AS)

Structures of calcitriol and analogs

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Yi-Wen Chien

Major Subject Nutrition Sciences

Title of Dissertation Effects of Calcitriol and its Analogs on Protein Expression in Osteoblast-like Cell Lines: ROS 17/2.8, MG-63, and MC3T-E1

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name	Signature
Charles W. Prince, **Chair**	Charles W. Prince
Joseph E. Baggott	Joseph E. Baggott
Gary L. Johanning	Gary L. Johanning
Linda C. Lucas	Linda C. Lucas
Jerry N. Thompson	Jerry N. Thompson

Director of Graduate Program Clinton J. Grubbs

Dean, UAB Graduate School Joan F. Lorden

Date 1/7/98