

Alendronate Inhibits Invasion of PC-3 Prostate Cancer Cells by Affecting the Mevalonate Pathway¹

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ABSTRACT

Breast and prostate cancer preferentially metastasize in the skeleton, inducing locally increased bone resorption by osteoclasts. Bisphosphonates (BPs), potent inhibitors of osteoclasts and bone resorption, are able to reduce metastatic bone lesions, but the metastasis-related cellular target molecules for BPs have not yet been identified. In osteoclasts, nitrogen-containing BPs inhibit the function of the mevalonate pathway, impairing the prenylation and activation of small GTPases. In addition, direct effects of BPs on cancer cells have been suggested. In the present study, the effects of two clinically used BPs, the amino-BP alendronate and clodronate, on adhesion, invasion, and migration of human PC-3 prostate cancer cells were examined *in vitro*. We also studied the possible role of the mevalonate pathway in invasion and migration of PC-3 cells using the β -hydroxy- β -methylglutaryl-CoA reductase inhibitor mevastatin and the mevalonate pathway intermediates mevalonate (mevalonic acid lactone), geranylgeraniol, and *trans-trans*-farnesol. The results demonstrate that alendronate pretreatment very effectively inhibited *in vitro* invasion of prostate cancer cells in a dose-dependent manner, with an IC_{50} as low as ~ 1 μ M. The inhibition was similar to that of mevastatin. Clodronate also inhibited invasion, but the IC_{50} was 0.1 μ M. Importantly, geranylgeraniol and *trans-trans*-farnesol reversed the inhibitory effect of alendronate and mevastatin but not the clodronate-induced inhibition of invasion. Alendronate pretreatment also inhibited migration, which was partially reversed by geranylgeraniol and *trans-trans*-farnesol. Adhesion of PC-3 cells to various matrices was reduced, and their F-actin organization was changed. Alendronate pretreatment also inhibited invasion of human Du-145 prostate and MDA-MB-231 breast cancer cells. As a conclusion, the results demonstrate that the mevalonate pathway leading to protein prenylation is important for cancer cell invasion and migration *in vitro*. They further suggest that interference with this pathway is involved in inhibition of invasion and migration of prostate cancer cells by the amino-BP alendronate but that the mechanism of clodronate inhibition is different. It is possible that BPs have therapeutic potential in preventing the spread of prostate cancer.

INTRODUCTION

A characteristic feature of cancer is dissemination into distant, often specific tissues. Breast and prostate cancer metastasize preferentially into the skeleton, causing severe clinical problems: hypercalcemia, bone fractures, and pain (1). Metastasizing is a multistep process, which includes detachment of cancer cells from primary tumor, migration, adhesion and invasion of cancer cells into the blood or lymphatic vessels, extravasation out of the vessel with the help of MMPs,³ and, finally, interactions with the target tissue (1, 2). In bone, breast and prostate cancer cells interact mainly with bone-resorbing osteoclasts, feeding them with various stimulatory growth factors, such as parathyroid hormone-related peptide, which leads to disruption

of bone structure and release of stroma-bound growth factors, such as transforming growth factor β , that in turn stimulate growth of cancer cells (1, 3). It is also possible that tumor cells, particularly prostate cancer cells, inhibit osteoclasts or stimulate osteoblasts, which leads to formation of osteosclerotic metastatic lesions in bone (4–6).

BPs are analogues of PP_i and are well-known, potent inhibitors of bone resorption (7). Several studies show that BPs inhibit formation, adhesion, and function of osteoclasts and change their morphology (8–13). BPs bind rapidly to bone hydroxyapatite (14) and are then released and internalized by bone-resorbing osteoclasts (8). Several aspects of the molecular mechanisms of BP action have recently been clarified (15). In osteoclasts, nitrogen-containing BPs inhibit farnesyl diphosphate synthase in the mevalonate pathway, which produces isoprenylation precursors, geranylgeranyl diphosphate and farnesol diphosphate. These intermediates are needed for posttranslational prenylation (geranylgeranylation and farnesylation) of small GTPases for their activation and localization to the cell membrane in normal cellular function of cytoskeleton, signal transduction, and vesicular trafficking (16). In osteoclasts, an inhibitory action of the amino-BP alendronate is suggested to be mediated mainly via geranylgeranylation of proteins, important for normal osteoclast function (17–19). However, the primary target molecules affected by amino-BPs remain to be determined. The recent data show that amino-BP alendronate disturbs intracellular vesicular trafficking in osteoclasts, leading to impaired ruffled border formation and disturbance of the transcytotic route. Thus, rab GTPases controlling vesicular transport may be the target molecule (20). At the high concentrations, amino-BPs have been reported to induce apoptosis in osteoclasts and macrophages *in vitro* and *in vivo* (21, 22). This effect of amino-BPs also seems to be mediated via the mevalonate pathway, leading to activation of caspase-3-like proteases (23). Similarly, non-amino-BPs, such as clodronate, have toxic effects on osteoclasts and other cells due to induction of apoptosis, but the mechanism of action is different (24). It has been demonstrated that non-amino-BPs, but not amino-BPs, are metabolized to a nonhydrolyzable ATP analogue. Intracellular accumulation of this metabolite is the likely cause of the growth inhibition and cytotoxic effects (25).

BPs reduce cancer burden in bone in animal models of breast cancer bone metastasis. This has been explained by reduced release and local concentrations of matrix-derived growth factors as a result of BP inhibition of bone resorption. The vicious cycle between osteoclasts and tumor cells is thus interrupted (10, 26). Additionally, direct effects of BPs on cancer cells have been suggested. BPs have been reported to inhibit adhesion of breast and prostate cancer cells to bone matrices (27, 28) and invasion of breast and prostate cancer cells *in vitro* (29). BPs have also been shown to inhibit secretion of MMPs by prostate cancer cells (30) and the activity of MMPs *in vitro*, but the latter is evident only at high concentrations (28). Altogether, these observations suggest that BPs may have direct effects on invasion, adhesion, and migration of cancer cells.

In the present study, the effects of two BPs, clodronate and the amino-BP alendronate, on adhesion, invasion, and migration of human prostate cancer cell line PC-3 were examined *in vitro*. We also studied the possible role of the mevalonate pathway in the invasion

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³ The abbreviations used are: MMP, matrix metalloproteinase; BP, bisphosphonate; HMG CoA, β -hydroxy- β -methylglutaryl-CoA; iFBS, inactivated fetal bovine serum.

process of prostate cancer cells. The results demonstrate that alendronate inhibited the *in vitro* invasion of prostate cancer cells at very low concentrations. The results also show that the mevalonate pathway is involved in prostate cancer cell invasion and migration *in vitro*. Furthermore, the data suggest that inhibition of both protein geranylgeranylation and farnesylation is involved in amino-BP reduction of invasion and migration of prostate cancer cells.

MATERIALS AND METHODS

Cell Lines. PC-3, an androgen-independent human prostate carcinoma cell line (American Type Culture Collection), and the MG-63 osteosarcoma cell line (American Type Culture Collection) were cultured in DMEM (Life Technologies, Inc.) containing 10% iFBS (Life Technologies, Inc.), 0.03 mg/ml penicillin, and 0.05 mg/ml streptomycin (Sigma) at 37°C in a humidified atmosphere of 5% CO₂. All experiments were performed in DMEM containing 0.1–1% BSA (Sigma). For collection of conditioned medium, MG-63 cells were cultured for 10 days in DMEM-iFBS with 0.5 μg/ml ascorbic acid (E. Merck) and then cultured for 2 days in 0.1% BSA-DMEM containing 0.5 μg/ml ascorbic acid. Conditioned medium was collected from confluent cultures, centrifuged, and frozen. It was used as a bone cell-derived attractant in invasion and migration assays, causing a 2-fold increase when compared with BSA-DMEM (data not shown).

BPs and Chemical Compounds. Alendronate (4-amino-1-hydroxybutylidene-1,1-bisphonic acid) was from Merck, Sharp & Dohme (West Point, PA), and clodronate (dichloro-methylene bisphosphonic acid) was from Leiras (Turku, Finland). Mevastatin, mevalonic acid lactone, and geranylgeraniol (analogue of geranylgeranyl diphosphate) were from Sigma, and *trans-trans*-farnesol (analogue of farnesyl diphosphate) was from Sigma-Aldrich. The concentrations of BPs and other compounds that had no effects on cell growth were used in additional experiments.

Growth Rate of PC-3 Cells. PC-3 prostate cancer cells were plated in 24-well plates and cultured for 24 h in DMEM-iFBS. Then cells were treated with various concentrations of BP for 24 h, washed, and cultured for an additional 75 h without BP. The number of cells was counted with a Coulter Counter (Coulter Electronics Ltd.) before and after BP treatment.

Invasion Assay. PC-3 cancer cells were treated with the indicated concentrations of alendronate or clodronate, mevastatin (10 μM), or 1% BSA-DMEM (as a control) for 24 h. Commercial cell culture invasion inserts with 8-μm pore size (Becton Dickinson) were coated with Matrigel (30 μg/insert = 100 μg/cm²; Becton Dickinson) for 24 h to prepare an *in vitro* basement membrane. The assay was started by adding 50,000 cells in 300 μl of 1% BSA-DMEM on the upper chamber and 300 μl of 1% BSA-DMEM and 300 μl of MG-63 conditioned medium in the lower chamber as a chemoattractant to induce invasion. Mevalonate, *trans-trans*-farnesol, or geranylgeraniol were added in the upper chamber at the beginning of the incubation. The cells were incubated for 72 h at 37°C and 5% CO₂, and the insert membranes were then prepared for microscopic samples. Membranes were fixed for 10 min with 4% paraformaldehyde (J. T. Backer) and then stained with Mayer's hematoxylin (Zymed) for 24 h. After washing, membranes were cut from the inserts, the cells on the upper surface of the membrane were wiped off with cotton wool, and the membranes were mounted with glycerol-PBS (9:1; E. Merck). The number of cells on the lower surface of the membrane was counted by microscope (×10 objective) from 10 consecutive fields, representing 40% of the total area of the membrane. Experiments were repeated three times, and each treatment was done as triplicate.

Adhesion Assay. Bacteriological 96-well plates (Greiner) were coated with various extracellular matrix proteins [fibronectin (1 μg/cm²); vitronectin (300 ng/cm²); laminin (5 μg/cm²); type I collagen (5 μg/cm²); and type IV collagen (5 μg/cm²) (Becton Dickinson)] for 24 h. PC-3 prostate cancer cells were preincubated with 10 μM alendronate or 1% BSA-DMEM (control) for 24 h. Cells were then suspended in 0.5% BSA-DMEM at a concentration of 200,000 cells/ml, and adhesion to various extracellular matrix proteins was followed for up to 2 h. Adherent cells were fixed with 4% paraformaldehyde (J. T. Baker) for 10 min and stained with 0.1% crystal violet (E. Merck) for 20 min. The staining of the wells was measured with a spectrophotometric plate reader (Labsystems) after solubilization of the dye into 10% acetic acid. The proportion of adherent cells was counted as a percentage of the control, which was

assessed by allowing 200,000 cells to adhere to cell culture plates for 4 h to achieve a maximal adhesion level.

Migration Assay. Inserts were coated with laminin (5 μg/cm²; Becton Dickinson), and cells were prepared as described above ("Invasion Assay"). The migration assay was performed similarly to the invasion assay using MG-63 conditioned medium in the lower chamber as an attractant to induce migration. Mevalonate, geranylgeraniol, or *trans-trans*-farnesol was added to inserts, and the cells were incubated for 5 h. The membranes were then prepared for microscopic samples, and the number of cells that migrated on the lower surface of the membrane were counted as described above for the invasion assay.

Fluorescence Stainings. PC-3 cells were pretreated with 10 μM alendronate, 10 μM mevastatin, or 1% BSA-DMEM (control) for 24 h. After 5 h of adhesion on glass coverslips, adherent cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 5 min, and stained for 20 min with 0.2 μg/ml FITC-labeled phalloidin (Sigma), which stains F-actin, and 0.04 mg/ml Hoechst 33342 (Sigma), which stains nuclei. After washing, coverslips were mounted as microscopic samples and photographed.

Gelatin Substrate Zymography. Zymography samples were collected from the upper chambers of invasion inserts, lyophilized, and solubilized into the sample buffer. Samples were electrophoresed in nonreducing SDS-PAGE in a 12% gel copolymerized with 0.1% gelatin. After electrophoresis, the gel was washed with 50 mM Tris containing 2.5% Triton X-100 for 30 min; washed with 50 mM Tris containing 2.5% Triton X-100, 5 mM CaCl₂, and 1 μM ZnCl₂ for 30 min; and incubated for 24 h in 50 mM Tris containing 5 mM CaCl₂ and 1 μM ZnCl₂ at 37°C. Finally, the gel was fixed and stained with 0.2% Coomassie Blue solution. Enzyme-digested regions were identified as white bands against the blue background.

Statistical Analyses. Statistical analyses were done using Statistica 6.0 (1997). Normality of groups was tested with the Shapiro-Wilkis W test, and significant differences were tested with an independent *t* test.

RESULTS

The putative direct effects of two BPs, amino-BP alendronate and non-amino-BP clodronate, on cancer cells were investigated by studying their effects on invasion, adhesion, and migration of PC-3 human prostate cancer cells. Pretreatment of PC-3 cells for 24 h with alendronate at concentrations ranging from 10⁻¹² to 10⁻⁴ M inhibited PC-3 prostate carcinoma cell invasion in a dose-dependent manner in the *in vitro* invasion assay. Dramatic inhibition was obtained even with very low concentrations, and the IC₅₀ was ~1 pM (Fig. 1a). Similar results were obtained when alendronate (10 μM) was present during the invasion assay without pretreatment of PC-3 cells (data not shown). Pretreatment of PC-3 cells with clodronate also inhibited cell invasion through Matrigel (Fig. 1b). However, compared with the very potent effect of alendronate, higher concentrations of clodronate were needed, and the IC₅₀ was ~0.1 μM. We also investigated the effect of alendronate on the invasion of other cancer cell lines. Alendronate pretreatment inhibited invasion of human prostate Du-145 (IC₅₀, ~1 nM) and MDA-MB-231 breast cancer cells (data not shown).

To rule out direct cytotoxicity, the effects of various concentrations of alendronate and clodronate on the growth rates of PC-3 cells were studied by measuring the cell number in the presence or absence of alendronate or clodronate. Alendronate or clodronate had no effects on the growth rate of PC-3 cells at concentrations of <100 μM, whereas they effectively inhibited the growth of PC-3 cells at concentrations of ≥100 μM (Fig. 1, a and b). Thus, a 10 μM concentration of alendronate and clodronate was chosen for pretreatment for invasion and migration assays.

Next, we characterized the role of the mevalonate pathway for PC-3 cell invasion using mevastatin, an inhibitor of the enzyme HMG CoA reductase, and the mevalonate pathway intermediates (mevalonic acid lactone, geranylgeraniol, and *trans-trans*-farnesol). Pretreatment of PC-3 cells with 10 μM mevastatin significantly inhibited invasion

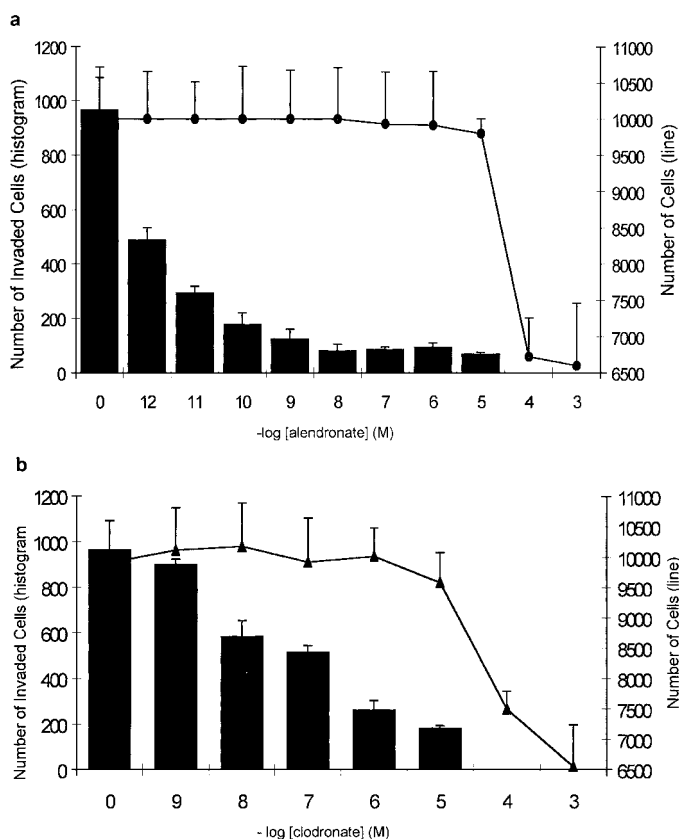


Fig. 1. Effect of BPs on PC-3 cell invasion and cell growth. PC-3 cells were pretreated with the indicated concentrations of alendronate (a) or clodronate (b) for 24 h and then incubated in invasion assay for 75 h in the absence of BPs. The number of invading cells was counted as described in "Materials and Methods" (columns). Results are the means \pm SD ($n = 4$). Separate experiments yielded similar results. For growth rate analysis, PC-3 cells were seeded at a concentration of 3000 cell/well and cultured for 24 h. Cells were then treated with the indicated concentrations of alendronate (a) or clodronate (b) for 24 h and cultured without BPs for an additional 75 h, and the number of living cells was counted. At the starting point, the number of cells was 6500 before BP treatment. Results are the means \pm SD ($n = 5$). Similar results were obtained from three separate experiments.

($P = 0.003$), suggesting that the mevalonate pathway is important for the ability of PC-3 cells to invade. Additionally, the inhibitory effect of mevastatin was effectively opposed by the addition of geranylgeraniol, *trans-trans*-farnesol, or mevalonate (mevalonic acid lactone) (Fig. 2a), demonstrating that geranylgeranylation and/or farnesylation of proteins is necessary for PC-3 cell invasion. Furthermore, the addition of intermediates to control PC-3 cells without any pretreatment had no effects on invasion (Fig. 2b).

After demonstrating the importance of the mevalonate pathway for PC-3 cell invasion, we investigated the potential involvement of this pathway in the action of BPs on cell invasion. Pretreatment of PC-3 cells with 10 μM alendronate significantly inhibited invasion ($P = 0.002$) compared with nontreated control (vehicle only). The addition of geranylgeraniol or *trans-trans*-farnesol to alendronate-pretreated cells opposed the inhibitory effect of alendronate and returned invasion to control level, whereas the addition of mevalonate had no effect on the invasion of alendronate-pretreated cells (Fig. 2c). These results suggest that protein geranylgeranylation and/or farnesylation important for PC-3 cell invasion are prevented by alendronate and that the step affected is downstream of mevalonate. Pretreatment with 10 μM clodronate also significantly inhibited PC-3 cell invasion ($P = 0.015$), but in contrast to alendronate, the addition of *trans-trans*-farnesol, geranylgeraniol, or mevalonate did not reverse the effect of clodronate on PC-3 cell invasion (Fig. 2d). Geranylgeraniol

and *trans-trans*-farnesol seemed to further inhibit the invasion of clodronate-pretreated cells when compared with the level of invasion of clodronate-pretreated cells alone (Fig. 2d). Thus, inhibition of PC-3 cell invasion by clodronate is not mediated via the mevalonate pathway. In addition to PC-3 cells, alendronate-inhibited invasion of Du-145 prostate cancer cells was opposed by geranylgeraniol and *trans-trans*-farnesol (data not shown).

Invasion requires cell adhesion, migration, and degradation of extracellular matrices. To study the effects of alendronate on these components of invasion, the effect of alendronate pretreatment on PC-3 cell adhesion properties was investigated first. An *in vitro* adhesion assay revealed that PC-3 cells adhere well to various extracellular matrix proteins and, to some extent, also to the wells coated with the medium conditioned with MG-63 osteosarcoma cells. Pretreatment with alendronate significantly inhibited PC-3 cell adhesion to all extracellular matrix proteins tested. Adhesion to fibronectin, type I and IV collagen, and laminin was inhibited most effectively ($P = 0.0005$, 0.00007, 0.0004, and 0.0004, respectively; Fig. 3). However, inhibition of adhesion to any of these matrix proteins was not as potent as inhibition in the invasion assay, suggesting that inhibition of adhesion to any single extracellular matrix protein is not the only target of alendronate and probably does not totally explain the dramatic inhibition of invasion.

Another important component of invasion is migration, which was studied next with an *in vitro* migration assay on laminin. Migration of PC-3 cells was significantly reduced by pretreatment of cells with mevastatin (Fig. 4a), alendronate (Fig. 4b), or clodronate (Fig. 4c) as compared with control treatment ($P = 0.0001$, 0.0002, and 0.004, respectively). Very similar potencies of mevastatin, alendronate, and clodronate in the migration and invasion assays prompted us to investigate the mevalonate pathway further in the migration assay. Although the mevalonate pathway seemed to be important in the migration of PC-3 cells, the addition of *trans-trans*-farnesol, geranylgeraniol, and mevalonate only partially but statistically significantly reversed mevastatin inhibition of migration of PC-3 cells (Fig. 4a). Similarly, the level of migration of alendronate-pretreated cells was partially but statistically significantly reversed by the addition of geranylgeraniol or *trans-trans*-farnesol, but not by the addition of mevalonate (Fig. 4b). One explanation for the partial effectiveness of the intermediates may be the short incubation time in the migration assay (5 h as compared with 72 h in the invasion assay), which may not be long enough to replenish the necessary/critical pools of geranylgeranylated and/or farnesylated proteins. Inhibition of migration of clodronate-pretreated cells was not reversed by any of the intermediates. On the contrary, it was potentiated further with the addition of *trans-trans*-farnesol, suggesting again that the mechanisms of action of clodronate and alendronate are different.

Thirdly, the potential effects of alendronate pretreatment on MMP-2 and MMP-9 production by PC-3 cells were studied by zymography. Zymography of samples collected from invasion inserts after invasion assay was used to analyze degradation of the extracellular matrix during invasion. Pretreatment of PC-3 cells with 10 or 0.0001 μM alendronate had no effects on the levels of MMP-2 or MMP-9 activity accumulated during the invasion assay (Fig. 5). This suggests that inhibition of invasion by alendronate was not caused by changes in gelatinase secretion or activation.

Finally, the morphology of PC-3 cells was examined. The general morphology and growth pattern of alendronate (10 μM)-treated PC-3 cells were similar to those of control PC-3 cells. However, phalloidin staining of F-actin revealed bright clusters of filopodia underneath the cell membrane (Fig. 6c), whereas control PC-3 cells lacked these aggregates (Fig. 6a). Similar F-actin clusters were also found in mevastatin-treated PC-3 cells (data not shown), suggesting that the

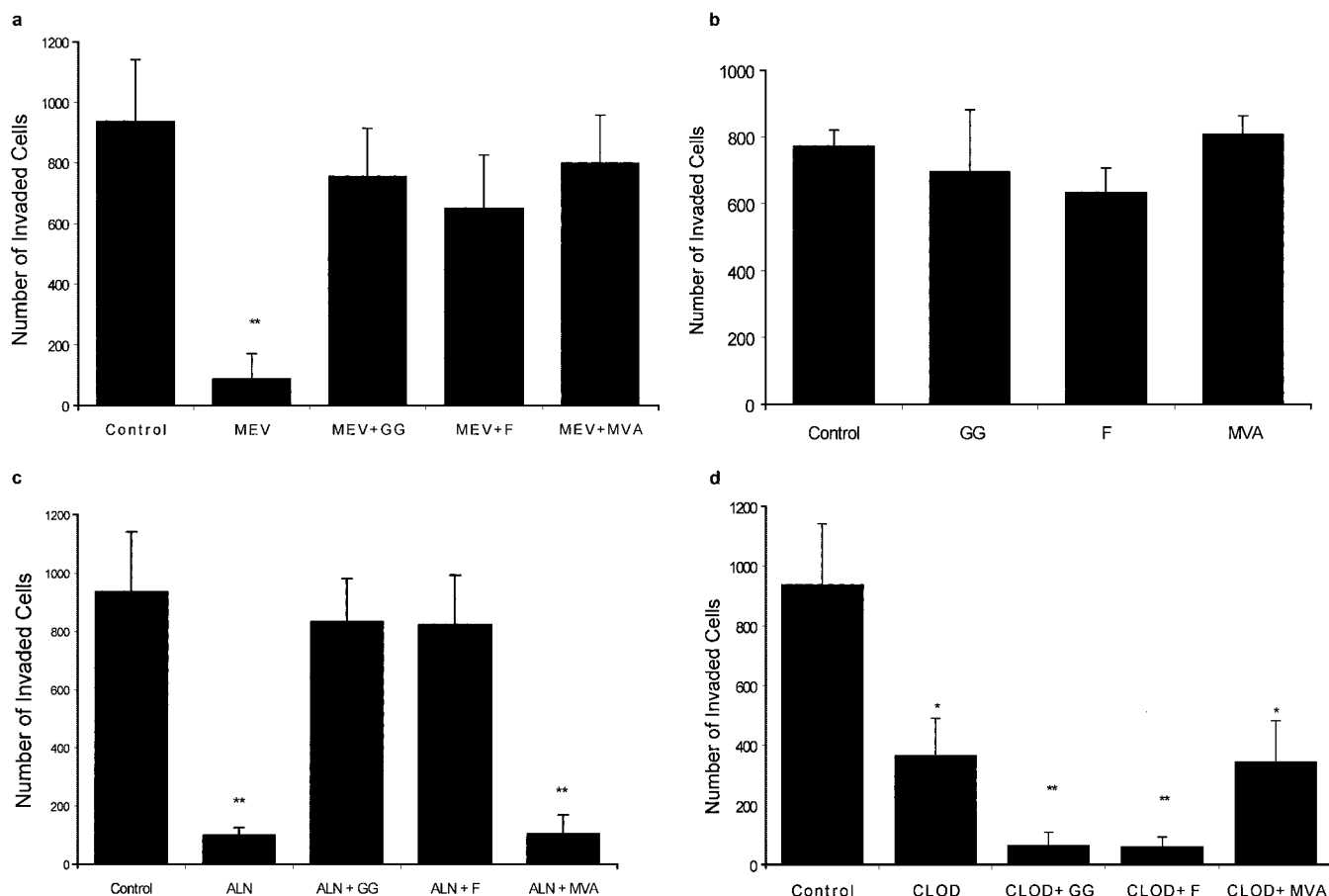


Fig. 2. Involvement of the mevalonate pathway in PC-3 cell invasion and in the effect of alendronate. PC-3 cells were pretreated with 10 μ M mevastatin (MEV; a), 1% BSA-DMEM (b), 10 μ M alendronate (ALN; c), or 10 μ M clodronate (CLOD; d). The first column on the left in a, c, and d (control) is control treatment with 1% BSA-DMEM. The mevalonate pathway intermediates 100 μ M geranylgeraniol (GG), 100 μ M *trans-trans*-farnesol (F), or 1 mM mevalonate (mevalonic acid lactone; MVA) were added to the invasion assay, and the inserts were incubated for 75 h. The number of invading cells was counted as described in "Materials and Methods." Statistical significance was calculated using an independent *t* test as compared with control treatment: *, $P < 0.05$; **, $P < 0.01$.

effect was mediated by the mevalonate pathway. Importantly, we did not find any differences in nuclear morphology by Hoechst staining after treatment of PC-3 cells with 10 μ M alendronate (Fig. 6d) compared with control treatment (Fig. 6b). This suggests, together with growth rate experiments (Fig. 1a), that under the conditions used, alendronate did not induce apoptosis.

DISCUSSION

Many studies have implied that the effects of BPs are targeted primarily to osteoclasts, with indirect actions on cancer cells (9–11). During the past few years, however, interesting new data on possible direct effects on cancer cells have emerged. In this study, we investigated the effects of BPs on early steps of metastasis: cancer cell invasion; adhesion; migration; and MMP production. Our results show that both the amino-BP alendronate and clodronate inhibit invasion, adhesion, and migration of PC-3 prostate cancer cells, but the mechanisms of action of these two BPs are distinct.

Inhibited invasion of alendronate- or clodronate-pretreated PC-3 cells clearly demonstrated the direct effect of these BPs on cancer cells *in vitro*. Importantly, only very low concentrations ($IC_{50} = \sim 1$ μ M for alendronate) were needed for complete inhibition of invasion, which suggests specific effect(s) on the function of cancer cells. Furthermore, the inhibitory effect of alendronate pretreatment sustained through the invasion assay in the absence of BPs. Corresponding inhibition of invasion was seen in alendronate-pretreated Du-145

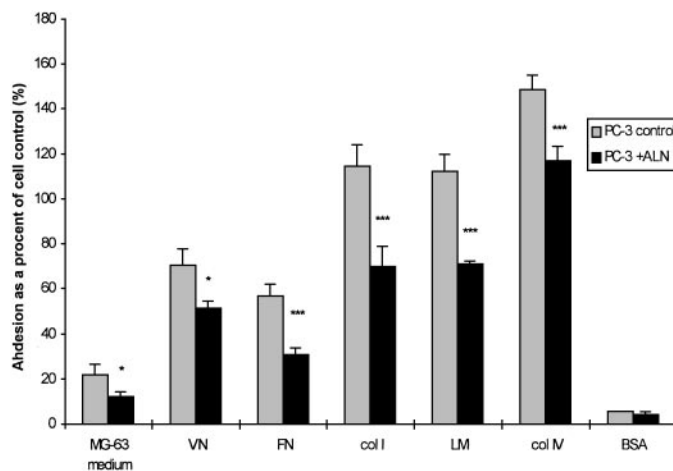


Fig. 3. Effect of alendronate on the adhesion of PC-3 cells to extracellular matrix proteins. PC-3 cells were pretreated with 1% BSA-DMEM (control) or 10 μ M alendronate (ALN), and adhesion to various extracellular matrix proteins was followed for up to 2 h. Adherent cells were stained and counted spectrophotometrically as described in "Materials and Methods." MG-63 medium is medium conditioned by MG-63 osteosarcoma cells. VN, vitronectin; FN, fibronectin; col I, type I collagen; col IV, type IV collagen; LM, laminin; BSA, BSA used as a background control. Results are the means \pm SD of five parallel wells. The experiment was repeated three times with similar results. Statistical significance was calculated using an independent *t* test to compare alendronate pretreated PC-3 with PC-3 control of the same matrix protein: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

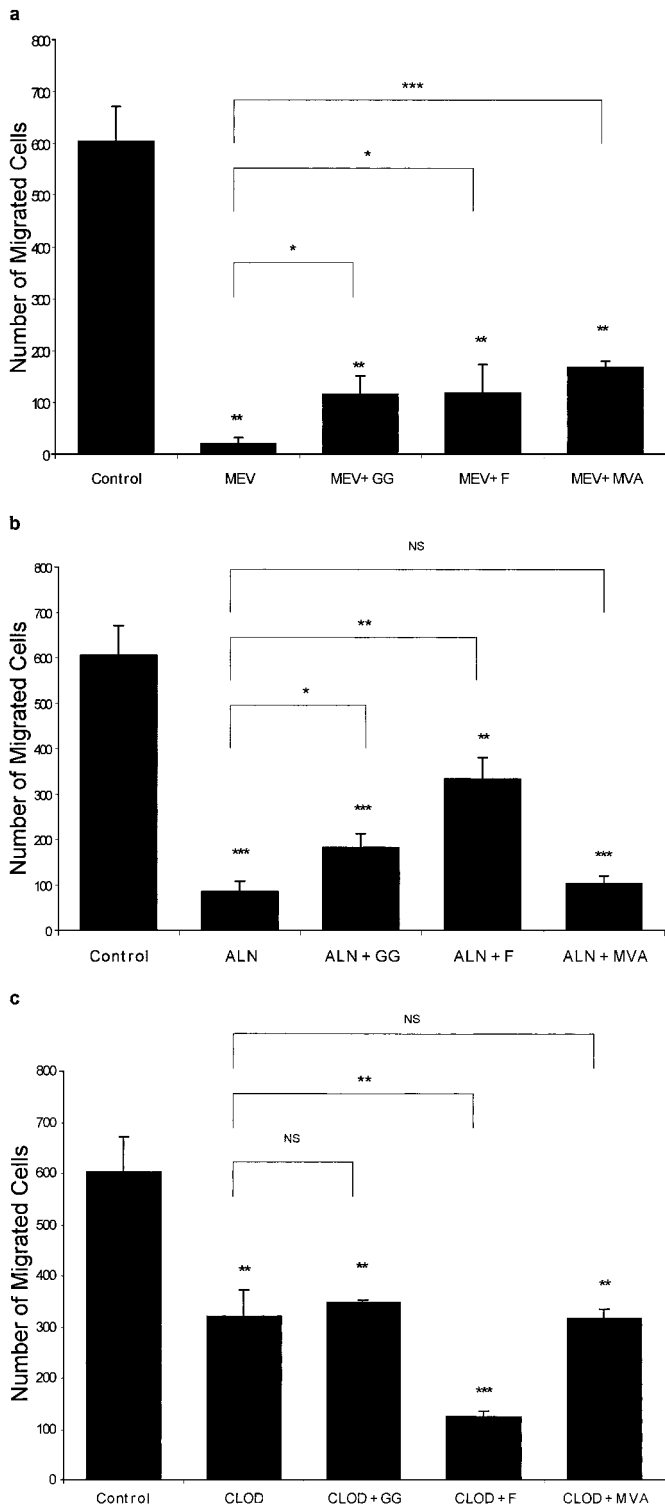


Fig. 4. Involvement of the mevalonate pathway in PC-3 cell migration. PC-3 cells were pretreated with 10 μ M mevastatin (a), 10 μ M alendronate (b), 10 μ M clodronate (c), or 1% BSA-DMEM (control, first column in a-c). The mevalonate pathway intermediates 100 μ M geranylgeraniol, 100 μ M trans-trans-farnesol, and 1 mM mevalonate were added to the migration assay. Inserts were incubated for 5 h. Results are the means \pm SD of four parallel wells. Similar results were obtained from three separate experiments. Statistical significance was calculated using an independent *t* test to compare mevastatin-, alendronate-, or clodronate-pretreated cells with control treatment, and secondly with geranylgeraniol, trans-trans-farnesol and mevalonate (lines): *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; NS, not significant.

prostate cancer cells ($IC_{50} = \sim 1$ nM) and MDA-MB-231 breast cancer cells, suggesting that the effect is not restricted to one cancer cell line. Our results are in agreement with recent studies by Boissier *et al.* (29), which showed inhibition of breast and prostate cancer cell invasion by various BPs.

Recently, several investigations have reported inhibition of cancer cell growth and induction of apoptosis *in vitro* by BPs (31–35). High concentrations (>10 μ M) of BPs have constantly been used in these studies. Hiraga *et al.* (34) reported ibandronate-induced apoptosis of MDA-MB-231 cells in bone metastasis, but not in the mammary fat pad. The differential effect was explained by a probable elevation in concentrations of BP within bone metastases as a result of locally increased bone resorption. In our study, the possibility of induction of apoptosis is not obvious because no effects on PC-3 cancer cell growth or nuclear morphology were observed at the concentration used (10 μ M).

In the present study, we show for the first time that the mevalonate pathway is important for prostate cancer cell invasion and migration *in vitro*. This was demonstrated by the ability of mevastatin, an efficient inhibitor of HMG CoA reductase, to block PC-3 cell invasion and by the ability of the mevalonate pathway intermediates downstream of the HMG CoA reductase step to overcome this inhibition. In a similar way, mevastatin prevented the migration of PC-3 cancer cells. However, the intermediates could only partially reverse the mevastatin-induced inhibition of migration.

Importantly, our results also demonstrate that the effect of the amino-BP alendronate on PC-3 cell invasion was mediated by inhibition of the mevalonate pathway. Compared with mevastatin, alen-

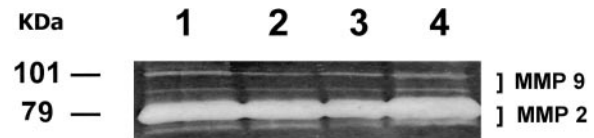


Fig. 5. MMP activities in invasion assay. Gelatin substrate zymography of conditioned medium of PC-3 cells pretreated with 10 μ M (Lane 1) or 0.0001 μ M alendronate (Lane 2) or 1% BSA-DMEM control (Lanes 3 and 4) collected from the upper chamber after the invasion assay. Positions of MMP-9 and MMP-2 are shown on the right. Molecular mass markers (in kilodaltons) are shown on the left.

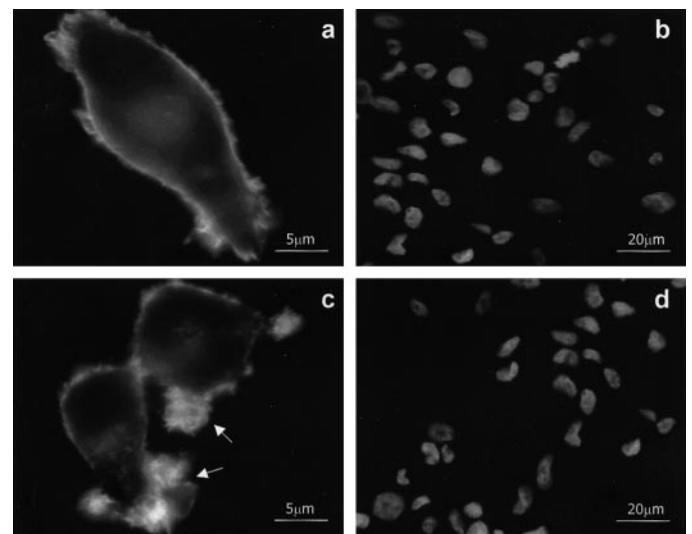


Fig. 6. Morphology of alendronate-treated PC-3 cells. Fluorescence staining for F-actin (a and c) and nuclei (b and d) of PC-3 cells treated with 1% BSA-DMEM (control, a and b) or 10 μ M alendronate (c and d) for 24 h. Note the difference in F-actin organization between control and alendronate-treated cells. Clustering of the cell membrane-associated F-actin network in alendronate-treated cells is shown with arrows. There was no difference in staining of nuclei between control and alendronate-treated cells.

dronate affected the pathway downstream of the HMG CoA reductase step because mevalonate did not reverse the effect of alendronate. This is in agreement with the findings that alendronate and other amino-BPs inhibit farnesyl diphosphate synthase *in vitro* (36–38). The IC₅₀ of alendronate inhibition of recombinant farnesyl diphosphate synthase *in vitro* is 460 nM (38). However, it is difficult to compare IC₅₀ values obtained from *in vitro* assays using pure enzyme with those obtained by measuring cellular functions. Often, cellular functions seem to be more sensitive, *e.g.*, the EC₅₀ of osteoclastic bone resorption is lower than the IC₅₀ for inhibition of farnesyl diphosphate synthase *in vitro* by many of the amino-BPs (36). Furthermore, nothing is known about the incorporation of BPs and their accumulation and intracellular concentrations in cancer cells. Our results suggest that both protein farnesylation and geranylgeranylation are impaired by alendronate in PC-3 prostate cancer cells because both *trans-trans*-farnesol and geranylgeraniol can overcome the inhibitory effect of alendronate. In osteoclasts, the effects of amino-BPs on osteoclast function and survival seem to be mediated via the mevalonate pathway required for protein geranylgeranylation (17–19, 39). This suggests that different prenylated proteins are rate limiting for osteoclast function and for cancer cell invasion.

In contrast to alendronate, the effect of clodronate was not reversed by either *trans-trans*-farnesol or geranylgeraniol, suggesting a distinct mechanism of action for these two BPs. Other studies have shown that the mevalonate pathway intermediates had no effects on cell death induced by non-amino-BPs (23). These studies, together with the present data, support the view that non-amino-BPs do not act by preventing protein prenylation. This is in agreement with several studies with macrophages, demonstrating the role of a nonhydrolyzable ATP analogue in clodronate-induced apoptosis (25). It is possible that clodronate is also metabolized into a nonhydrolyzable ATP analogue in cancer cells, which might lead to reduced invasion and migration by presently unknown mechanisms. The cause of the additional inhibition of invasion and migration of clodronate-pretreated cells by *trans-trans*-farnesol and/or geranylgeraniol remains unclear at this point and needs further investigation.

We also show for the first time that BPs inhibit cell migration in prostate cancer cells. Our results here differ from the previous observations of Boissier *et al.* (29), who did not find effects of BPs on MDA-MB-231 or PC-3 cell migration. Our results demonstrate that inhibition of migration by alendronate is at least partially mediated by protein geranylgeranylation and farnesylation. The fact that intermediates of the mevalonate pathway only partially overcome the inhibitory effect of mevastatin or alendronate on PC-3 cell migration may reflect the short treatment period during the migration assay. A longer incubation with intermediates might be needed for sufficient replenishment of depleted pools of prenylated proteins. Another possibility is that cancer cell invasion and migration are distinct functions, which are mediated through partially different mechanisms.

In addition to migration, adhesion is required for cancer cell invasion. Our present results and previous reports of inhibition of breast and prostate cancer cell adhesion to extracellular matrix proteins and bone by BPs (27, 28) suggest that this step of the metastasis process is affected by BPs. PC-3 prostate cancer cells adhered well to various extracellular matrix proteins, and alendronate reduced their adhesion to all matrices tested. On the other hand, reduced adhesion of alendronate-treated PC-3 cells may also reflect their less migratory phenotype rather than a specific impairment of cell-matrix interaction. This possibility is supported by the fact that the RGD (Arg-Gly-Asp) sequence-containing peptide further reduced the adhesion of alendronate-pretreated PC-3 and MDA-MB-231 cells to vitronectin and fibronectin (data not shown).

High concentrations of BPs ($\geq 50 \mu\text{M}$) have also been shown to

inhibit the *in vitro* activity of MMPs, which are important for invasion (29). PC-3 cells produce MMP-2 and MMP-9 at a high level, but we did not find any changes in their production by PC-3 cells treated with alendronate. This differs from the results of Stearns and Wang (30). They demonstrated decreased activity of MMP-2 and MMP-9 in femurs injected with alendronate-treated PC-3 ML cells or in femurs of alendronate-treated mice injected with PC-3 ML cells. However, the experimental system in the study of Stearns and Wang is very different from ours. In addition, there may be changes in local or cell membrane-bound MMP activities during the *in vitro* invasion assay, which cannot be detected by measuring the MMP activities accumulated in the culture medium.

The mevalonate pathway provides intermediates for prenylation of small GTPases such as ras, rho, and rab proteins. They are important for the organization of the cytoskeleton and its attachment to the cell membrane, membrane vesicle transport systems, and various signal transduction pathways (*e.g.*, leading to apoptosis; Refs. 16, 37, and 40). Impaired function of any of the above-mentioned small GTPases could affect cellular processes important for cancer cell invasion. An increasing amount of evidence indicates a role for the rho family GTPases in epithelial cell migration and cancer cell invasion (41). A rate-limiting target for protein prenylation necessary for PC-3 cell invasion and migration remains to be discovered. Based on our data regarding changes in morphology and actin distribution, a target could be one of the rho family GTPases, which regulate the organization of actin microfilaments and cellular projections. Recent data show that in osteoclasts alendronate disturbs vesicular trafficking, as manifested by impaired ruffled border formation (20). This points to the possible involvement of rab proteins in alendronate action on osteoclasts. The role of rab proteins as well as that of other small GTPases in amino-BP inhibition of cancer cell invasion needs to be clarified in additional experiments.

Taken together, our results demonstrate that the amino-BP alendronate inhibition of invasion/migration of PC-3 prostate cancer cells is mediated by the mevalonate pathway. Alendronate may thus inhibit several steps of cancer metastasis distinct from the effects on osteoclast function. Although it is difficult to estimate the BP concentrations obtained *in vivo* in primary tumors, our data suggest that amino-BPs may have therapeutic potential even at very low (picomolar) concentrations in prevention of the early steps of metastasis. In the future, identification of molecular target(s) of alendronate and the rate-limiting prenylated protein(s) necessary for tumor cell migration and invasion may provide targets for inhibition and prevention of prostate cancer invasion and metastasis.

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