0021-972X/03/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 88(12):6088–6097

doi: 10.1210/jc.2003-030712

Effects of Chemotherapeutic Agents on the Function of Primary Human Osteoblast-Like Cells Derived from Children

J. H. DAVIES, B. A. J. EVANS, M. E. M. JENNEY, AND J. W. GREGORY

Department of Child Health (J.H.D., B.A.J.E., J.W.G.), University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, United Kingdom; and Department of Paediatric Oncology (M.E.M.J.), Llandough Hospital, Cardiff CF64 2XX, United Kingdom

Studies in children treated with chemotherapy suggest that chemotherapeutic agents have deleterious effects on bone metabolism. We therefore evaluated the *in vitro* effects of clinically relevant concentrations of chemotherapeutic agents on the synthesis of type I collagen, alkaline phosphatase (AP) activity, and mineralization by primary human osteoblast-like (HOB) cells derived from children. Because serum 1,25-dihydroxyvitamin D_3 concentrations may be reduced during treatment with chemotherapy, the effect of chemotherapeutic agents on HOB cells cultured in the presence or absence of 1,25-dihydroxyvitamin D_3 was also evaluated.

Type I collagen synthesis was reduced by all agents ($P < 0.01$) other than methotrexate, whereas the relative AP activity was increased ($P < 0.01$) by all agents. The relative number of cells staining intensely for AP after culture with agents increased ($P < 0.05$), and AP mRNA expression was increased ($P < 0.01$) with vincristine. 1,25-Dihydroxyvitamin D_3 ameliorated ($P < 0.01$) the depletion of HOB cell numbers by chemotherapeutic agents. Furthermore, vincristine and daunorubicin inhibited 1,25-dihydroxyvitamin D_3-mediated AP activity ($P < 0.01$).

We conclude that chemotherapeutic agents can adversely affect HOB cell function, and we speculate that this observation may account, in part, for the osteopenia observed during and after treatment of children with chemotherapy. (*J Clin Endocrinol Metab* 88: 6088–6097, 2003)

OSTEOPENIA IS A complication that may occur during treatment with chemotherapy for childhood malignancy. Although the etiology is likely to be multifactorial, clinical studies suggest that chemotherapeutic agents have deleterious effects on bone metabolism (1–5). Furthermore, the suppression of serum markers of bone formation, such as type I collagen and bone alkaline phosphatase (AP), that occurs immediately after the administration of chemotherapeutic agents suggests a direct adverse effect on osteoblasts *in vivo* (6–8). In support of these findings, we have recently demonstrated that clinically relevant concentrations of various chemotherapeutic agents reduced human osteoblast-like (HOB) cell numbers *in vitro* and that this effect preferentially occurred in less differentiated osteoblast phenotypes (9, 10).

The process of normal bone formation involves not only adequate osteoblast numbers but also competent osteoblast function, including the synthesis of type I collagen and AP, and mineralization of an extracellular matrix. Defective bone formation may occur by impairment of these processes, even in the absence of a reduction in osteoblast numbers (11–13). Altered mineral metabolism may also predispose to osteopenia during treatment with chemotherapy (4). One contributing factor may be abnormal vitamin D homeostasis, *e.g.* some have shown that 1,25-dihydroxyvitamin D_3 concentrations may be subnormal in children receiving chemotherapy, although the mechanism is unclear (1, 4).

Children are given combinations of chemotherapeutic agents during treatment, making it impossible to determine from clinical studies the effect of each agent on osteoblasts. To date, *in vitro* studies have evaluated the effects of methotrexate and glucocorticoids on HOB cells, but there are little data available concerning the effects of the other chemotherapeutic agents on HOB cell function. We have therefore evaluated the effect of several chemotherapeutic agents on type I collagen and AP synthesis, and also mineralizing ability in primary HOB cells derived from children. Because a reduction in serum 1,25-dihydroxyvitamin D_3 concentration may occur during treatment, we have also assessed whether the presence of 1,25-dihydroxyvitamin D_3, a potent inducer of osteoblast differentiation, modulates the effects of chemotherapeutic agents on HOB cells.

Materials and Methods

Primary HOB cell culture

Ethical approval for collection of clinical samples was granted by Bro Taf Local Research Ethics Committee, Cardiff, UK. Specimens of bone were obtained from children undergoing elective orthopedic procedures. The patients underwent femoral osteotomies for Perthes' disease (4-yr-old boy and 6-yr-old girl) and fracture malunion (13-yr-old boy), whereby nondiseased (normal) bone was removed to correct the deformity of the underlying condition. These children were free from malignancy and other diseases with systemic effects likely to have adverse effects on bone physiology. HOB cells were isolated and cultured from bone explants as described previously (9, 14, 15). Briefly, bone was dissected into 0.2- to 0.5-cm pieces and washed extensively in PBS to remove bone marrow cells. The bone was placed in 24-well plates and cultured at 37 C in a humidified atmosphere of 5% CO_2-95% air in standard medium that constituted α-MEM (Invitrogen Ltd., Paisley, UK) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (250 μg/ml), 10% fetal calf serum, and 25 mM HEPES. Only

Abbreviations: AP, Alkaline phosphatase; CICP, C-terminal propeptide of type I collagen; HOB, human osteoblast-like.

cells from passage 3 or less were used for analysis. HOB cells were characterized for the expression of type I collagen, AP, osteocalcin, and Cbfa1 as described previously (9).

Chemotherapeutic agents

The chemotherapeutic agents used were representative of those used for the treatment of childhood malignancy and were chosen for their contrasting mechanisms of action. Methotrexate inhibits DNA synthesis by inhibition of dihydrofolate reductase. Etoposide induces strand breaks in DNA by inhibiting topoisomerase II, an enzyme that effects cleavage and resealing of the phosphodiester backbone of DNA. Vincristine inhibits cell division by interfering with microtubule assembly and induces a metaphase arrest. Daunorubicin inhibits DNA replication and transcription by intercalating with DNA and by the generation of free radicals. Asparaginase reduces protein synthesis in proliferating cells by reducing L-asparagine availability. The concentration of each agent used in the experiments below was determined from the available data concerning serum concentrations obtained from children receiving chemotherapy. The following concentrations were used throughout the study: 0.1 μM methotrexate, 10 μM etoposide, 0.1 μM vincristine, 0.1 μM daunorubicin, and 0.5 U/ml asparaginase (16–20). All of the chemotherapeutic agents used were freshly prepared for *in vitro* experiments by serial dilution in media.

Effect of chemotherapeutic agents on type I collagen synthesis by HOB cells

A total of 90×10^3 cells per well were seeded in six-well plates and allowed to settle for 24 h. The cells were cultured with either methotrexate, vincristine, etoposide, daunorubicin, or asparaginase for 72 h, after which the agent was removed and the cultures replenished with standard media for 24 h. Asparaginase, a known protein synthesis inhibitor, was included to make a comparison with the other agents. The supernatant was collected from each well, and C-terminal propeptide of type I collagen (CICP) was quantified using an EIA (PROLAGEN-C, Metra Biosystems, Oxon, UK) and measuring the absorbance at 405 nm by a SpectraCount Microplate Photometer (Canberra-Packard Ltd., Berks, UK). CICP is a biochemical indicator of type I collagen synthesis, and the assay measures both cleaved and uncleaved CICP. The amount of cell protein was determined from each corresponding well using a Bio-Rad protein assay (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Six replicate wells were used for each agent, and each experiment was performed on two separate occasions. The results are expressed as CICP synthesis in the presence of a chemotherapeutic agent (nanograms of CICP per milligram of cell protein) as a percentage of control.

Effect of chemotherapeutic agents on AP activity in HOB cells

Cells were seeded into 96-well plates at a density of 3×10^3 cells per well and allowed to settle for 24 h. For each experiment, a duplicate plate was set up to assay for cell numbers. The HOB cells were then cultured with a chemotherapeutic agent for 72 h, after which the AP activity and cell numbers were evaluated. AP activity was evaluated using reagents that together comprise the 104-LL Sigma Diagnostic Kit (Sigma-Aldrich Co. Ltd., Dorset) as described previously (9). Briefly, cells were washed with PBS, and 50 μl of a 1:1 mixture of 4 mg/ml para-nitrophenyl phosphate and the alkaline buffer 2-amino-2-methyl-1-propanol (1.5 M, pH 10.3) was added. After incubation at room temperature, the absorbance of the developing yellow color was read at 20, 30, 40, and 50 min on a SpectraCount Microplate Photometer (Canberra-Packard Ltd.) at 405 nm. The change of absorbance per hour was calculated for each well. These rates were adjusted for differences in cell numbers between treatments.

Cell numbers were measured using a CellTiter96 Non-Radioactive Cell Proliferation assay (Promega, Southampton, UK) (9, 21, 22). This method compares favorably with [^{3}H]-thymidine incorporation and direct cell counting for quantification of cell numbers (23, 24). After washing in PBS, cells were incubated for 2 h at 37 C with 120 μl of a 2-mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate in phenol-red-free DMEM (Invitrogen Ltd.) according to the manufacturer's instructions. The resulting OD was measured at 490 nm on a SpectraCount Microplate Photometer (Canberra-Packard Ltd.). Twenty-four replicate wells were used for each agent, and each experiment was performed on two separate occasions. Results are expressed as AP activity in the presence of a chemotherapeutic agent (AP activity per cell number) as a percentage of control.

Effect of chemotherapeutic agents on numbers of AP-positive stained cells in a HOB cell population

Because there is variable expression of AP by HOB cells within a cell population (25), we examined the effect of culture with a chemotherapeutic agent on the numbers of AP-positive cells in a HOB cell population. Cells were aliquoted onto sterile coverslips, cultured with either vincristine or etoposide for 72 h, and then fixed using formal saline. The cells were stained using nitroblue tetrazolium/5 bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich Co. Ltd., Poole, UK), for 16 h at room temperature, after which any excess stain was removed (26). Cells were counted in random microscopic fields using image analyzer software (Image proplus 3, MediaCybernetics, DataCell, Finchampstead, UK) interfaced with a color camera (JVC TK1070E, JVC Professional, London UK) mounted on a microscope (Olympus BH2, Olympus Optical Co. Ltd., London, UK). Cells were counted and graded according to their intensity of staining: grade I, uniformly lightly stained cells; grade II, patchy stained cells; grade III, darkly stained cells with nucleus distinct; grade IV, uniformly darkly stained cells with nucleus not distinct. The number of AP stained cells was expressed as a percentage of the total cell number.

Effect of chemotherapeutic agents on AP mRNA expression in HOB cells

Cells were seeded into six-well plates at a density of 90×10^3 cells per well, and three wells were assigned to each treatment. A chemotherapeutic agent was added after 24 h, and total RNA was extracted after 72 h using the Purescript RNA isolation kit (Flowgen, Leicestershire, UK). RT-PCR was then performed using standard techniques and specific β-actin and AP primers (β-actin, 5′-AGCCATGTACGTTGCTA-3′, 5′-AGTCCGCCTAGAAGCA-3′; AP, 5′-ACGTGGCTAAGAATGTCATC-3′, 5′-CTGGTAGGCGATGTCCTTA-3′) (27). cDNA was prepared using 1 μg RNA in a 20-μl reaction and a Reverse Transcription Kit System (Promega). Reaction times were 1 h at 42 C.

Semiquantitative RT-PCR

Aliquots (1 μl) of the total cDNA were amplified in each PCR in a 20-μl reaction mixture that contained PCR buffer (1 mM Tris pH 9.0, 0.01% Triton X-1000, 5 mM KCl), 10 pmol of each AP primer and 0.7–0.9 pmol of each β-actin primer, deoxycytidine triphosphate, deoxy-GTP, deoxy-ATP, and thymidine 5′-triphosphate (each at 0.2 mM), 1.5 mM $MgCl_2$, and 0.5 U of Taq polymerase (Promega). Amplifications were performed in a 480 DNA Thermal Cycler (Applied Biosystems, Cheshire, UK) for 20–30 cycles, after an initial denaturation at 94 C for 2 min. The number of cycles used was determined by preliminary studies to investigate the number of cycles needed for both the β-actin and AP products to be on the linear part of the reaction. The reaction profile was 94 C for 30 sec, 55 C for 2 min, and 72 C for 2 min.

Reaction products were analyzed by electrophoresis of the 20-μl sample in TBE (90 mM Tris pH 8.3, 90 mM boric acid, 2.5 mM EDTA) buffer and 1.5% agarose gels. The amplified DNA fragments were visualized by ethidium bromide staining and quantified using a densitometer (Model GS69, Bio-Rad Laboratories Ltd.). The quantitative differences in AP amplification between cDNA samples were calculated after normalizing for the amplification in the corresponding β-actin PCR product. Experiments were performed in triplicate, and the results are expressed as a ratio of the density of the AP band to that of the β-actin band in the presence of a chemotherapeutic agent and compared with control.

Effect on in vitro mineralization after exposure of HOB cells to individual chemotherapeutic agents

To promote mineralization *in vitro*, cells were cultured in standard medium (as above) supplemented with 0.1 μM dexamethasone (Sigma-

Aldrich Co. Ltd.), 10 mM β-glycerophosphate (Sigma-Aldrich Co. Ltd.), and 0.2 μM L-ascorbic acid-2-phosphate (Sigma-Aldrich Co. Ltd.). HOB cells were seeded into six-well plates at a cell density of 90×10^3 cells per well. After 24 h, individual chemotherapeutic agents were added to the cultures for 72 h and then removed. The mineralizing medium was changed twice weekly; and once there was evidence of mineralization in the control cultures, the medium was removed and cultures stained with alizarin Red-S as described previously (28). Alizarin Red-S selectively binds to and stains calcium salts. The amount of staining was quantified using image analyzer software (Image proplus 3, MediaCybernetics, DataCell). To adjust for the changes in the amount of staining occurring as a result of changes in cell numbers, a duplicate experiment was performed to assay cell protein using the Bio-Rad protein assay (Bio-Rad Laboratories Ltd.). Three replicate wells were used for each agent, and each experiment was performed on two separate occasions. Results were expressed as a percentage of control and adjusted for the changes in cell numbers.

Effect of preincubation with 1,25-dihydroxyvitamin D_3, followed by co-incubation with a chemotherapeutic agent and 1,25-dihydroxyvitamin D_3, on HOB cell numbers

Cells were seeded in 96-well plates at a density of 3×10^3 cells per well and cultured in the presence of 0.1 nM 1,25-dihydroxyvitamin D_3 (Sigma-Aldrich Co. Ltd.) for 72 h. The medium was changed, and a chemotherapeutic agent and 1,25-dihydroxyvitamin D_3 were added to the cultures for a further 72 h, after which cell numbers were evaluated. The same study protocol was used for control cultures but without the addition of 1,25-dihydroxyvitamin D_3 at any time. Cell numbers were determined, as described previously, using the CellTiter96 Non-Radioactive Cell Proliferation assay (Promega). Twenty-four replicate wells were used for each chemotherapeutic agent, and each experiment was performed on two separate occasions.

Effect of 1,25-dihydroxyvitamin D_3 on AP activity in HOB cells previously exposed to chemotherapy

Cells were seeded at 3×10^3 cells per well in 96-well plates and allowed to settle for 24 h. A chemotherapeutic agent was added to the cultures for 72 h and then removed. Chemotherapeutic agents were not added to the control cultures; 1,25-dihydroxyvitamin D_3 (0.1 nM) was added to the cultures, including control cultures, for a further 72 h. AP activity was then evaluated as above, and results were corrected for changes in cell numbers using the CellTiter96 Non-Radioactive Cell Proliferation assay (Promega) and expressed as a percentage of control values. Twenty-four replicate wells were used for each chemotherapeutic agent, and each experiment was performed on two separate occasions.

Statistics

The data were analyzed by comparing means, using the Student's unpaired *t* test. Differences were considered to be significant when $P \leq 0.05$, and data are presented as the mean ± SEM.

Results

Effect of chemotherapeutic agents on type I collagen synthesis by HOB cells

CICP concentrations were reduced in the supernatant of those wells in which HOB cells were cultured with either vincristine, etoposide, daunorubicin, or asparaginase ($P < 0.01$) (Fig. 1A). These reductions were accompanied by a reduction in cell number as reflected by the diminished cell protein concentrations in the corresponding wells (Fig. 1B). After adjustment for the changes in cell numbers, all agents other than methotrexate reduced CICP synthesis ($P < 0.01$) (Fig. 1C). Vincristine demonstrated the maximal ability to reduce CICP synthesis to 51.2 ± 3.7% of control values (Fig. 1C).

Effect of chemotherapeutic agents on AP activity in HOB cells

All agents other than methotrexate reduced the AP activity per well ($P < 0.01$) (Fig. 2A). This effect was greatest with daunorubicin, which reduced AP activity to 57.6 ± 0.1% of control values. As in the previous experiment, the agents also caused a reduction of cell numbers in the corresponding wells ($P < 0.01$) (Fig. 2B). The AP activity was thus expressed adjusted for the changes in HOB cell numbers and was shown to be increased after exposure to the different agents ($P < 0.01$) (Fig. 2C). This effect was greatest with vincristine, which increased AP values to 185.9 ± 5.3% of control values, whereas methotrexate increased AP values to only 133.1 ± 3.6% of control values ($P < 0.01$) (Fig. 2C).

Effect of chemotherapeutic agents on numbers of AP-positive stained cells in a HOB cell population

The variable staining of AP within a HOB cell population is shown in Fig. 3A. Vincristine and etoposide were chosen for these studies because they demonstrated the greatest increase in AP activity/cell number (Fig. 2C) and also because of their contrasting mechanisms of action. After culture with either agent, the relative proportion of HOB cells staining intensely (*i.e.* grade III and grade IV HOB cells) for AP increased when compared with the control cultures ($P < 0.05$). At the same time, the relative proportion of HOB cells staining weakly (*i.e.* grade I and grade II cells) for AP was reduced when compared with the control cultures ($P < 0.05$) (Fig. 3B).

Effect of chemotherapeutic agents on AP mRNA expression in HOB cells

The intraassay and interassay coefficients of variation for the RT-PCR were 6.8% and 8.1%, respectively, for the controls. HOB cell AP mRNA abundance was increased, when compared with control, after exposure to vincristine ($P < 0.01$), whereas no increase was observed with methotrexate, etoposide, or daunorubicin (Fig. 4).

Effect on in vitro mineralization after exposure of HOB cells to individual chemotherapeutic agents

In vitro mineralization by HOB cells was reduced by vincristine, etoposide, and daunorubicin ($P < 0.01$) but was unaffected by culture with methotrexate (Fig. 5B). The effect was greatest when HOB cells were cultured with daunorubicin, such that mineralization was reduced to 5.2 ± 1.4% of control values (Fig. 5, A and B).

Effect of preincubation with 1,25-dihydroxyvitamin D_3, followed by co-incubation with a chemotherapeutic agent and 1,25-dihydroxyvitamin D_3, on HOB cell numbers

Protection from drug cytotoxicity was demonstrated with all chemotherapeutic agents tested after culture of HOB cells with 1,25-dihydroxyvitamin D_3 (Fig. 6). This modulation of cytotoxicity was greatest with methotrexate. Methotrexate alone reduced cell numbers to 40.1 ± 1.7% of control values; whereas when cells were cultured with 1,25-dihydroxyvita-

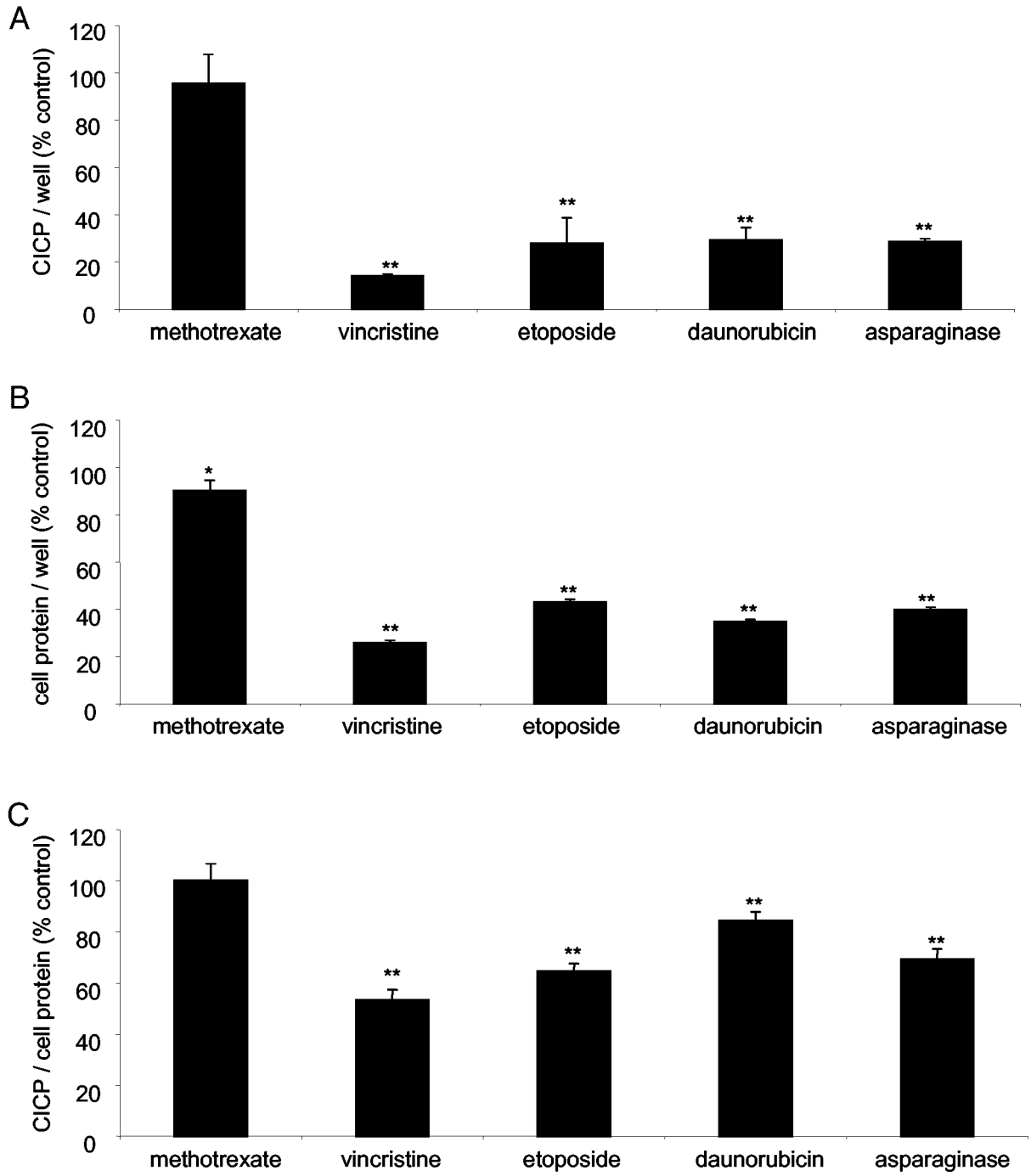

FIG. 1. A–C, Effect of chemotherapeutic agents on CICP synthesis in HOB cells. Cells were cultured with each agent for 72 h, after which the agent was removed, and CICP was quantified in the supernatant after the next 24 h. Changes in cell protein were determined at the same time point. The y-axis shows values expressed as a percentage of control; A, y-axis = CICP/well (percent control); B, y-axis = cell protein/well (percent control); C, y-axis = CICP/cell protein (percent control). Each point is the mean of six replicates, and *error bars* represent ± SEM. *, $P < 0.05$ *vs.* control; **, $P < 0.01$ *vs.* control.

min D_3, cytotoxicity was attenuated, such that cell numbers were reduced to only 61.2 ± 2.1% of control ($P < 0.01$) (Fig. 6).

Effect of 1,25-dihydroxyvitamin D_3 on AP activity in HOB cells previously exposed to chemotherapy

The AP activity/cell number with 1,25-dihydroxyvitamin D_3 was no different from control cultures in HOB cells previously cultured with either methotrexate or etoposide (Fig. 7). However, when HOB cells were cultured with 1,25-dihydroxyvitamin D_3, the AP activity/cell number was reduced when cells were previously exposed to either vincristine or daunorubicin, with AP activity/cell number only 80.1 ± 3.3% and 87.8 ± 3.1% of control values, respectively (both $P < 0.01$) (Fig. 7).

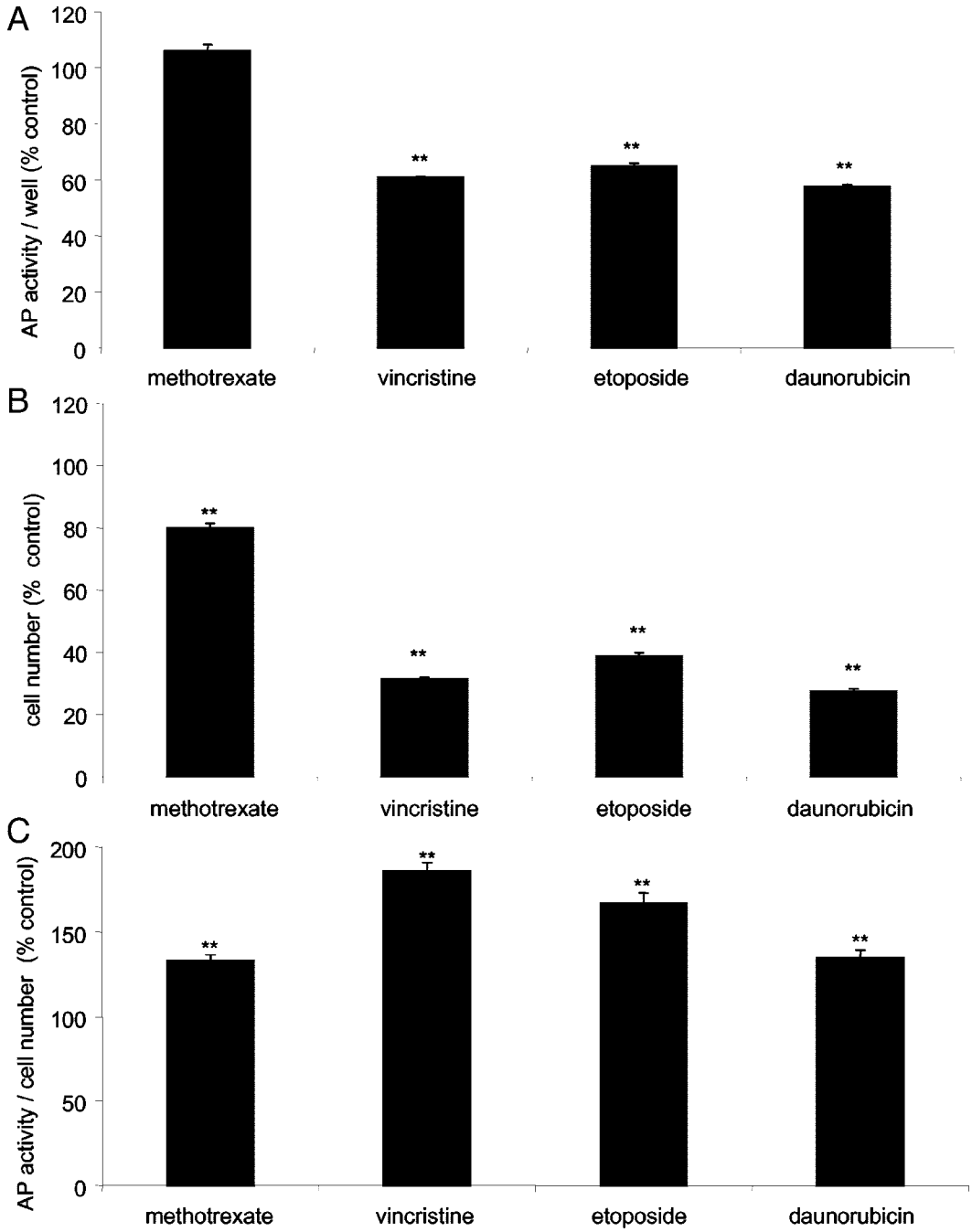

FIG. 2. A–C, Effect of chemotherapeutic agents on AP activity in HOB cells. Cells were cultured for 72 h with a chemotherapeutic agent, after which changes in AP activity and cell numbers were determined. The y-axis shows values expressed as a percentage of control; A, y-axis = AP activity/well (percent control); B, y-axis = cell number (percent control); C, y-axis = AP activity/cell number (percent control). Each point is the mean of 24 replicates, and *error bars* represent ± SEM. **, $P < 0.01$ *vs.* control.

Discussion

Because clinical studies demonstrate worsening of osteopenia during treatment of children with chemotherapy (1–5), we have hypothesized that these agents contribute to osteopenia by direct adverse effects on osteoblast function and that these agents may impair the normal accretion of skeletal mass in childhood.

Most of the agents tested caused a reduction in CICP synthesis that was partly attributable to the diminished cell numbers (Fig. 1, A and B). However, after adjusting for the changes in cell number, all the agents other than methotrexate reduced CICP synthesis (Fig. 1C). It is, therefore, likely that these chemotherapeutic agents caused impaired collagen biosynthesis in HOB cells. In fibroblasts for example, daunorubicin has been shown to inhibit prolidase, an exopeptidase that cleaves imidodipeptides containing C-terminal proline, thus reducing the amount of proline available for collagen synthesis (29). Furthermore, in the same

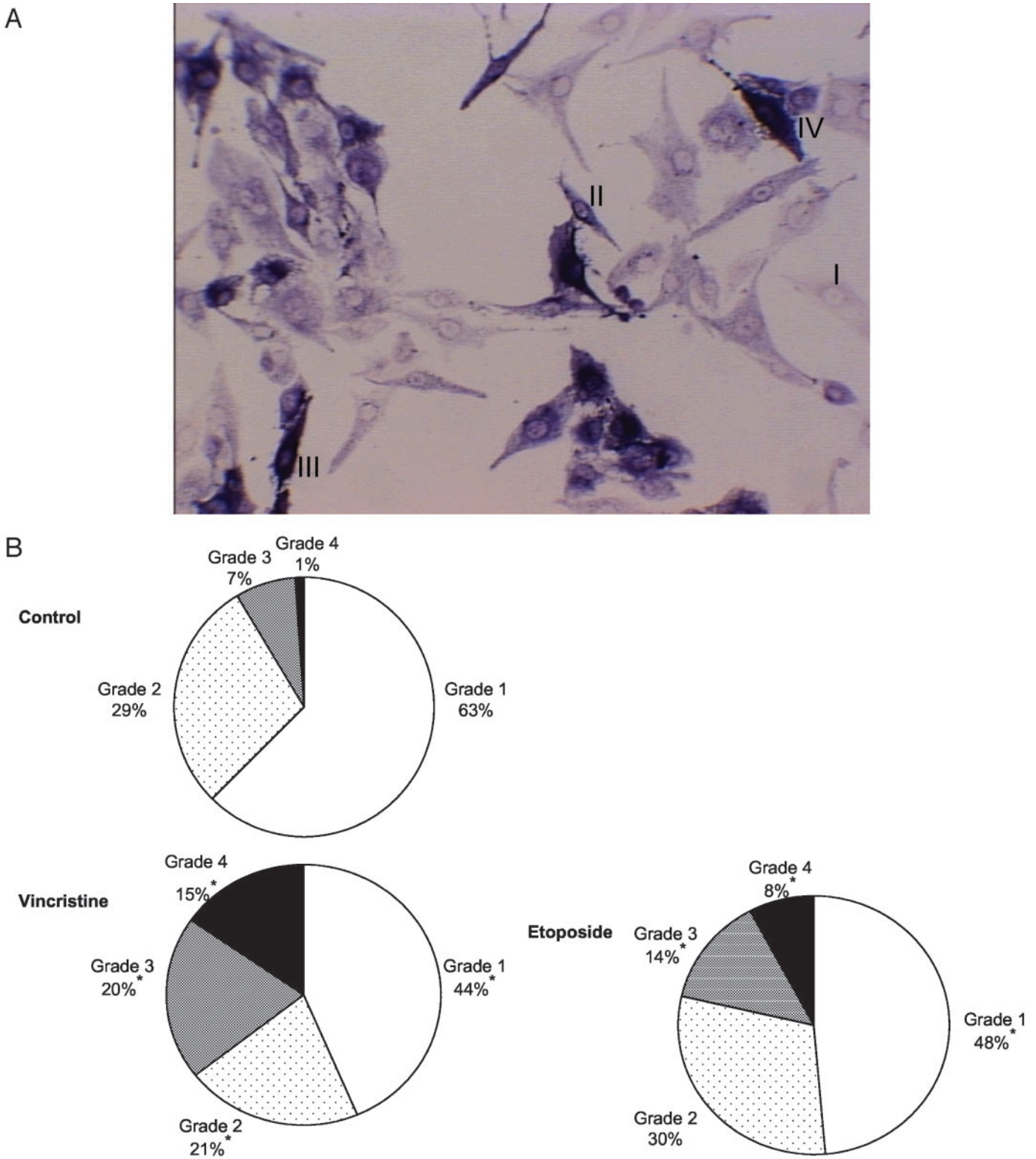

FIG. 3. A, Examination by light microscopy (×20 magnification) of a HOB cell population stained for AP using nitroblue tetrazolium/5 bromo-4-chloro-3-indolylphosphate. AP staining is indicated by *blue staining*. Grade I, Uniformly lightly stained cells; grade II, patchy stained cells; grade III, darkly stained cells with nucleus distinct; grade IV, uniformly darkly stained cells with nucleus not distinct. B, Effect of chemotherapeutic agents on AP-positive stained cells in a HOB cell population. Cells were cultured with a chemotherapeutic agent for 72 h and then stained for AP. *, $P < 0.05$ *vs.* control.

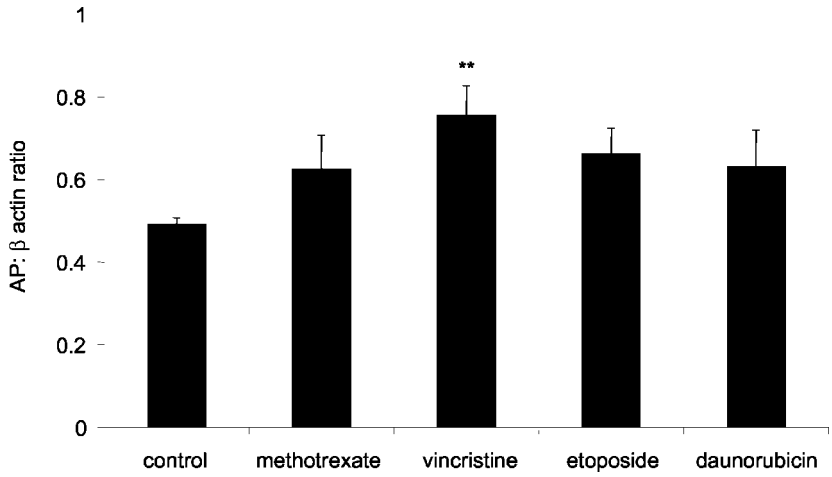

FIG. 4. Effect of chemotherapeutic agents on AP mRNA expression in HOB cells. Cells were cultured with each agent for 72 h, after which mRNA was extracted. The y-axis shows the ratio of the density of the AP band to that of the β-actin band. Each point is the mean ratio of three separate RT-PCRs, and *error bars* represent ± SEM. **, $P < 0.01$ *vs.* control.

cells, this agent also inhibits prolyl hydroxylation by inhibition of prolyl hydroxylase, which results in compromised stability of the procollagen triple helix (30). Because the synthesis of type I collagen by HOB cells during their proliferative phase is necessary for new bone matrix formation, reduced synthesis may predispose to diminished bone formation.

Studies of the effects of chemotherapeutic agents on AP activity in osteoblasts have been conflicting and limited to the effects of methotrexate. Some have shown a dose-dependent reduction of cell numbers by methotrexate with no effect on AP expression in human osteoblasts, whereas others observed suppression of AP activity in murine osteoblastic cells (31, 32). We have shown that agents other than methotrexate caused a reduction in AP activity that was largely attributable to the accompanying reduction in cell numbers (Fig. 2, A and B). However, although the expression of AP by HOB cells is a marker of a more differentiated osteoblast phenotype, within HOB cell populations there is a variable expression of AP, indicative of HOB cells at various stages of maturation (25). Figure 2C shows that, after exposure to the various agents, there was a relative increase in AP activity after adjusting for the reduction in HOB cell numbers. Consistent with this observation were the findings of a relative increase in the proportion of HOB cells that stained intensely for AP and an increase in AP mRNA after exposure to vincristine (Figs. 3 and 4). Taken together, these data suggest that chemotherapeutic agents have less adverse effects on the more differentiated, less proliferative cells that are characterized by high levels of AP activity; and agents may, therefore, selectively target the proliferating fraction of cells that express reduced AP activity.

Another possible explanation may be that the relative increase in AP activity was the consequence of chemotherapy-induced cellular differentiation, as occurs in other cell phenotypes (33, 34). However, we found that, apart from vincristine, the other agents did not increase AP mRNA synthesis despite increases in AP activity, as measured by biochemical and cytochemical analysis (Figs. 2–4). The absence of increased AP mRNA with the other agents may have resulted from the limited sensitivity of semiquantitative RT-PCR, because there was a trend to increased AP mRNA, although this did not reach significance (Fig. 4). A less likely explanation may be that chemotherapeutic agents cause a reduction in AP synthesis by interfering with transcriptional mechanisms in the surviving cells (Fig. 4). Impaired AP synthesis may predispose to osteopenia, because bone AP is necessary for normal skeletal mineralization. Furthermore, there was a reduction in mineralization after culture with some chemotherapeutic agents, which may be the result of impaired osteoblast function, such as from reduced type I collagen and AP synthesis (Fig. 5). Although methotrexate had no effect on *in vitro* mineralization, the culture conditions may not have simulated the *in vivo* situation of weekly oral methotrexate administration given, for example, during the treatment of childhood acute lymphoblastic leukemia. Moreover, a reduction in mineralization and osteocalcin synthesis has been demonstrated in murine osteoblasts given a more prolonged exposure to this agent (35).

A reduction in serum 1,25-dihydroxyvitamin D_3 concentration may occur in children receiving chemotherapy (1, 4). This may contribute to the development of osteopenia, because 1,25-dihydroxyvitamin D_3 has anabolic effects on bone and up-regulates AP activity in HOB cells. Consistent with its known effects (14), 1,25-dihydroxyvitamin D_3 increased AP activity/HOB cell number when compared with HOB cells cultured in its absence (data not shown). We have demonstrated that, in HOB cells cultured with a physiological concentration of 1,25-dihydroxyvitamin D_3, some chemotherapeutic agents caused a reduction in the AP activity/cell number (Fig. 7). This hormone, however, ameliorated the cytotoxic effects of chemotherapeutic agents on HOB cell numbers (Fig. 6). This latter effect was observed with all agents tested and was thus independent of their differing mechanisms of action. How 1,25-dihydroxyvitamin D_3 mediates protection of HOB cells from cytotoxicity is unclear and may involve antiproliferative effects or induction of cellular differentiation, because more differentiated osteoblast phenotypes are more chemoresistant than immature phenotypes (9, 36). Because chemotherapeutic agents may promote cell loss by apoptosis in human osteoblasts (10, 37), the protective effect of 1,25-dihydroxyvitamin D_3 on cell numbers may occur by an antiapoptotic mechanism, a process which has been demonstrated in human osteosarcoma

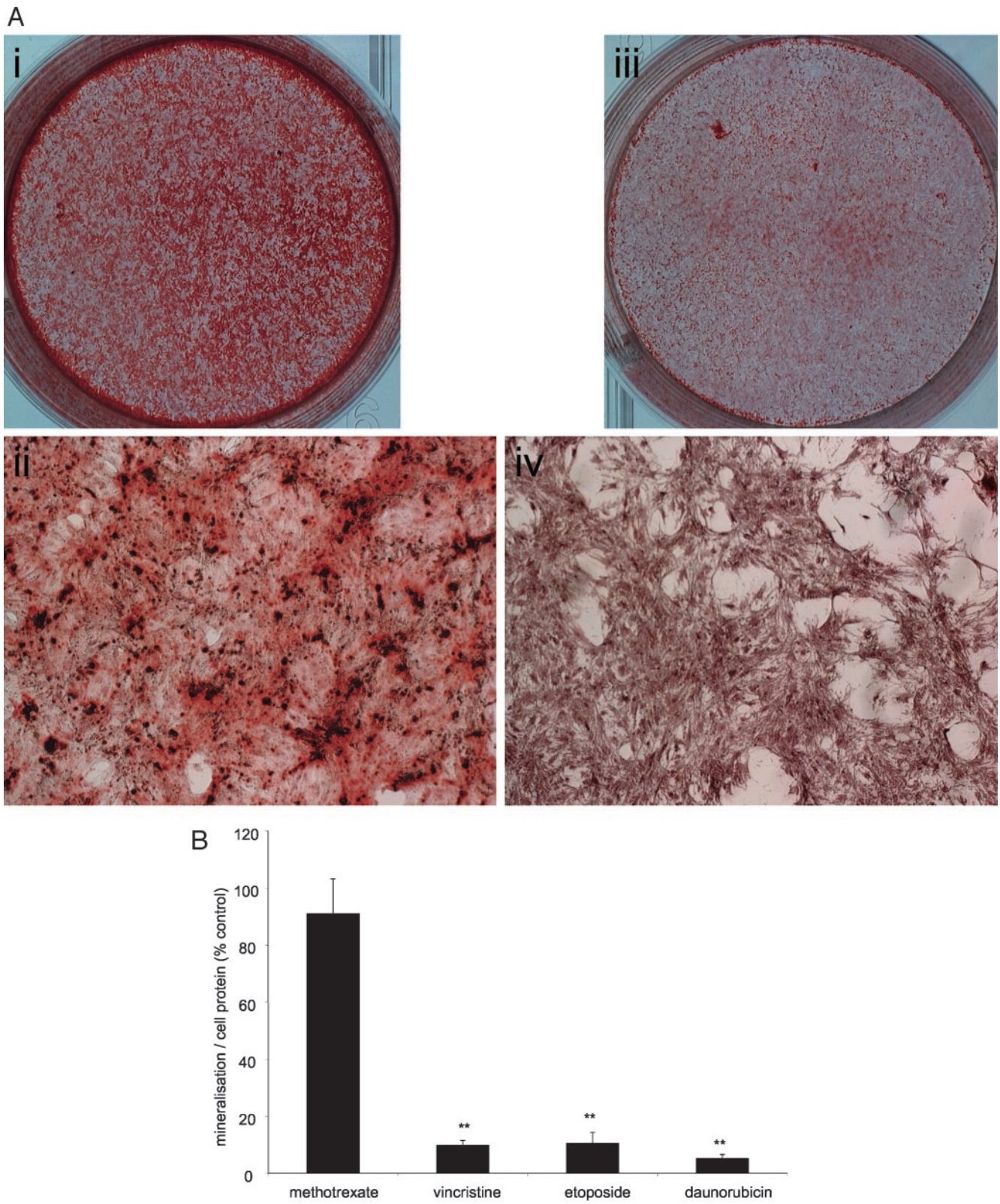

FIG. 5. A, Representative photomicrograph demonstrating alizarin Red-S staining of *in vitro* mineralization by HOB cells when cultured with daunorubicin, compared with control. Mineralization is indicated by staining *red*: i) staining of control cultures in one well of a standard six-well plate; ii) examination of control cultures by light microscopy (×10 magnification); iii) staining of cultures previously treated with daunorubicin in one well of a standard six-well plate; iv) examination of 3) by light microscopy (×10 magnification). B, *In vitro* mineralization after culture of HOB cells with individual chemotherapeutic agents. The y-axis shows mineralization/cell protein expressed as a percentage of control values. *Error bars* represent ± SEM. **, $P < 0.01$ *vs.* control.

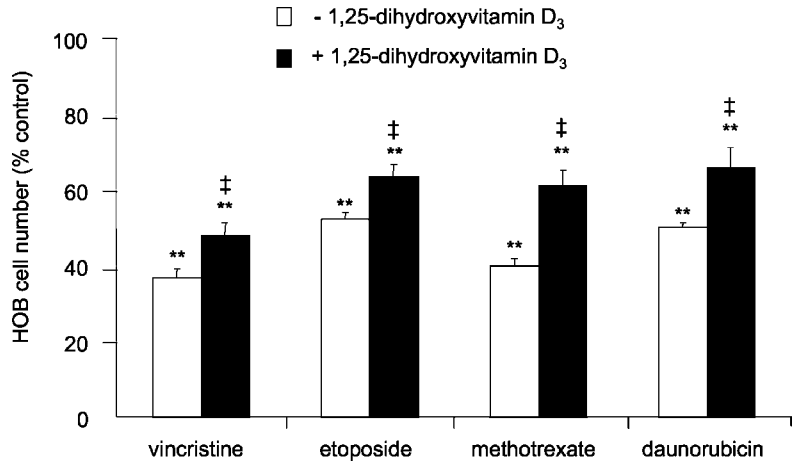

FIG. 6. Effect of preincubation with 1,25-dihydroxyvitamin D_3, followed by co-incubation with a chemotherapeutic agent and 1,25-dihydroxyvitamin D_3, on HOB cell numbers. The y-axis shows cell numbers expressed as a percentage of control. Each point is the mean of 24 replicates, and *error bars* represent ± SEM. **, $P < 0.01$ *vs.* control; ‡, $P < 0.01$ *vs.* treatment with a chemotherapeutic agent but without 1,25-dihydroxyvitamin D_3.

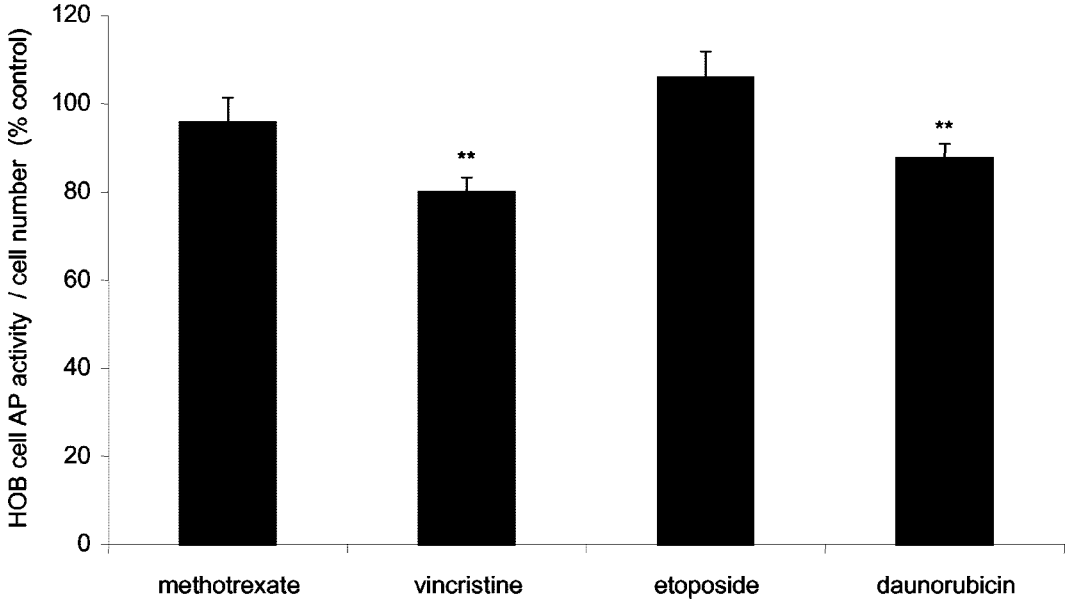

FIG. 7. Effect of 1,25-dihydroxyvitamin D_3 on AP activity/cell number in HOB cells previously exposed to chemotherapy. Cells were cultured with a chemotherapeutic agent for 72 h, the agent was removed, and 1,25-dihydroxyvitamin D_3 was added to the cultures for a further 72 h, after which AP activity was determined. The y-axis shows AP activity as a percentage of control and adjusted for the changes in cell numbers. Each point is the mean of 24 replicates, and *error bars* represent ± SEM. **, $P < 0.01$ *vs.* control.

cells (38). Furthermore, in osteoblasts, 1,25-dihydroxyvitamin D_3 promotes the transcriptional activation of the cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$, which is a mediator of growth arrest and differentiation (39). $p21^{Cip1/WAF1}$ also has an apoptosis inhibitory action in osteoblasts mediated by inhibition of stress-activated apoptosis signal-regulating kinase 1 activity; thus, $p21^{Cip1/WAF1}$ up-regulation may have accounted, in part, for the protective effects demonstrated (40, 41).

Our *in vitro* studies indicate that chemotherapeutic agents can interfere with osteoblast function, impair osteoblast responsiveness to 1,25-dihydroxyvitamin D_3, and cause adverse effects on mineralization by osteoblasts. These observations, coupled with a reduction in osteoblast numbers, may account for the suppression of serum markers of bone formation in children receiving chemotherapy and contribute to the resultant osteopenia (2, 6, 7). Because 1,25-dihydroxyvitamin D_3 provided some protection from drug cytotoxicity, optimizing patients' serum 1,25-dihydroxyvitamin D_3 concentration during administration of chemotherapy may have beneficial effects on bone. Although 1,25-dihydroxyvitamin D_3 has a growth inhibitory effect on a variety of different malignant cells *in vitro*, the effect of such therapy on malignancy *in vivo* would require further investigation (42, 43).

Acknowledgments

The authors thank Mrs. Carole Elford and Mrs. Jill Matthews for technical support and Mr. D. O'Doherty for providing the clinical samples.

Received April 22, 2003. Accepted September 3, 2003.

Address all correspondence and requests for reprints to: Dr. J. H. Davies, Lecturer in Child Health, Department of Child Health, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, United Kingdom. E-mail: daviesjh@*cf*.ac.uk.

This work was supported by LATCH (Llandough Hospital aims to treat children with cancer with hope), Llandough Hospital, Penarth, Cardiff CF64 2XX, United Kingdom and Novo Nordisk.

References

1. **Arikoski P, Komulainen J, Riikonen P, Voutilainen R, Knip M, Kroger H** 1999 Alterations in bone turnover and impaired development of bone mineral density in newly diagnosed children with cancer: a 1-year prospective study. J Clin Endocrinol Metab 84:3174–3181
2. **Arikoski P, Komulainen J, Riikonen P, Parviainen M, Jurvelin J, Voutilainen R, Kröger H** 1999 Impaired development of bone mineral density during chemotherapy: a prospective analysis of 46 children newly diagnosed with cancer. J Bone Miner Res 14:2002–2009
3. **Boot AM, van den Heuvel-Eibrink MM, Hahlen K, Krenning EP, de Muinck Keizer-Schrama SM** 1999 Bone mineral density in children with acute lymphoblastic leukaemia. Eur J Cancer 35:1693–1697
4. **Halton JM, Atkinson SA, Fraher L, Webbern C, Gill GJ, Dawson S, Barr RD** 1996 Altered mineral metabolism and bone mass in children during treatment for acute lymphoblastic leukemia. J Bone Miner Res 11:1774–1783
5. **Henderson RC, Madsen CD, Davis C, Gold SH** 1998 Longitudinal evaluation of bone mineral density in children receiving chemotherapy. J Pediatr Hematol Oncol 20:322–326
6. **Crofton PM, Ahmed SF, Wade JC, Stephen R, Elmlinger MW, Ranke MB, Kelnar CJH, Wallace WHB** 1998 Effects of intensive chemotherapy on bone and collagen turnover and the growth hormone axis in children with acute lymphoblastic leukemia. J Clin Endocrinol Metab 83:3121–3129
7. **Crofton PM, Ahmed SF, Wade JC, Elminger MW, Ranke MB, Kelnar CJH, Wallace WHB** 2000 Bone turnover and growth during and after continuing chemotherapy in children with acute lymphoblastic leukemia. Pediatr Res 48:490–496
8. **Sorva R, Kivivuori SM, Turpeinen M, Marttinen E, Risteli J, Risteli L, Sorva A, Siimes MA** 1997 Very low rate of type I collagen synthesis and degradation in newly diagnosed children with acute lymphoblastic leukaemia. Bone 20: 139–143
9. **Davies JH, Evans BAJ, Jenney MEM, Gregory JW** 2002 In vitro effects of chemotherapeutic agents on human osteoblast-like cell numbers. Calcif Tissue Int 70:408–415
10. **Davies JH, Evans BAJ, Jenney MEM, Gregory JW** 2002 *In vitro* effects of combination chemotherapy on osteoblasts: implications for osteopenia in childhood malignancy. Bone 31:319–326
11. **Manolagas SC, Jilka RL** 1995 Bone marrow, cytokines, and bone remodelling. Emerging insights into the pathophysiology of osteoporosis. N Engl J Med 332:305–311
12. **Friedlaender GE, Tross RB, Doganis AC, Kirkwood JM, Baron R** 1984 Effects of chemotherapeutic agents on bone. J Bone Joint Surg Am 66:602–607
13. **Marie PJ, De Vernejoul M, Connes D, Hott M** 1991 Decreased DNA synthesis by cultured osteoblastic cells in eugonadal osteoporotic men with defective bone formation. J Clin Invest 88:1167–1172
14. **Beresford JN, Gallagher JA, Russell RGG** 1986 1,25-Dihydroxyvitamin D3 and human bone-derived cells *in vitro*: effects on alkaline phosphatase, type I collagen and proliferation. Endocrinology 119:1776–1785
15. **Auf'mkolk B, Hauschka PV, Schwartz ER** 1985 Characterization of human bone cells in culture. Calcif Tissue Int 37:228–235
16. **Balis FM, Savitch JL, Bleyer WA** 1983 Pharmacokinetics of oral methotrexate in children. Cancer Res 43:2342–2345
17. **Crom WR, de Graaf SS, Synold T, Uges DR, Bloemhof H, Rivera G, Christensen ML, Mahmoud H, Evans WE** 1994 Pharmacokinetics of vincristine in children and adolescents with acute lymphocytic leukemia. J Pediatr 125:642–649
18. **Lowis SP, Price L, Pearso ADJ, Newell DR, Cole M** 1998 A study of the feasibility and accuracy of pharmacokinetically guided etoposide dosing in children. Br J Cancer 77:2318–2323
19. **Paul C, Liliemark J, Tidefelt U, Garrton G, Peterson C** 1989 Pharmacokinetics of daunorubicin and doxorubicin in plasma and leukemic cells from patients with acute nonlymphoblastic leukemia. Ther Drug Monit 11:140–148
20. **Boos J, Werber G, Ahlke E, Schulze-Westhoff P, Nowak-Gottl U, Wurthwein G, Verspohl EJ, Ritter J, Jurgens H** 1996 Monitoring of asparaginase activity and asparagine levels in children on different asparaginase preparations. Eur J Cancer 32:1544–1550
21. **Finlay GJ, Baguley BC, Wilson WR** 1984 A semiautomated microculture method for investigating growth inhibitory effects of cytotoxic compounds on exponentially growing carcinoma cells. Anal Biochem 139:272–277
22. **Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR** 1988 Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res 48:4827–4833
23. **Tada H, Shiho O, Kuroshima K, Koyama M, Tsukamoto K** 1986 An improved colorimetric assay for interleukin 2. J Immunol Methods 93:157–165
24. **Cory A, Owen T, Barltrop J, Cory J** 1991 Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun 3:207–212
25. **Matsuyama T, Lau W, Wergedal J** 1990 Monolayer cultures of normal human bone cells contain multiple subpopulations of alkaline phosphatase positive cells. Calcif Tissue Int 47:276–283
26. **Horwitz J, Chua J, Noel M, Donarth J, Freisler J** 1966 Substrates for cytochemical demonstration of enzyme activity. II. Some dihalo-3-indoyl phosphates and sulfates. J Med Chem 9:447
27. **Rickard DJ, Kassem M, Hefferen TE, Sarkar G, Spelsberg TC, Riggs BL** 1996 Isolation and characterization of osteoblast precursor cells from human bone marrow. J Bone Miner Res 11:312–324
28. **Bodine PV, Trailsmith M, Komm BS** 1996 Development and characterization of a conditionally transformed adult human osteoblastic cell line. J Bone Miner Res 11:806–819
29. **Muszynska A, Wolczynski S, Palka J** 2001 The mechanism for anthracycline-induced inhibition of collagen biosynthesis. Eur J Pharmacol 411:17–25
30. **Sasaki T, Holeyfield KC, Uitto J** 1987 Doxorubicin-induced inhibition of prolyl hydroxylation during collagen biosynthesis in human skin fibroblast cultures. Relevance to impaired wound healing. J Clin Invest 80:1735–1741
31. **Scheven BAA, van der Veen MJ, Damen CA, Lafeber FPJG, Van Rijn HJM, Bijlsma JWJ, Duursma SA** 1995 Effects of methotrexate on human osteoblasts *in vitro*: modulation by 1, 25-dihydroxyvitamin D_3. J Bone Miner Res 10:874–880
32. **Uehara R, Suzuki Y, Ichikawa Y** 2001 Methotrexate (MTX) inhibits osteoblastic differentiation *in vitro*: possible mechanism of MTX osteopathy. J Rheumatol 28:251–256
33. **Morceau F, Chenais B, Gillet R, Jardillier JC, Jeannesson P, Trentesaux C** 1996 Transcriptional and posttranscriptional regulation of erythroid gene expression in anthracycline-induced differentiation of human erythroleukemic cells. Cell Growth Differ 7:1023–1029
34. **Takeshita H, Kusuzaki K, Murata H, Suginoshita T, Hirata M, Hashiguchi S, Ashihara T, Gebhardt MC, Mankin HJ, Hirasawa Y** 2000 Osteoblastic differentiation and P-glycoprotein multidrug resistance in a murine osteosarcoma model. Br J Cancer 82:1327–31
35. **May KP, Mercill D, McDermot MT, West SG** 1996 The effect of methotrexate on mouse bone cells in culture. Arthritis Rheum 3:489–494
36. **Minaur NJ, Jefferiss C, Bhalla AK, Beresford JN** 2002 Methotrexate in the treatment of rheumatoid arthritis. I. *In vitro* effects on cells of the osteoblast lineage. Rheumatology 41:735–744
37. **Fellenberg J, Mau H, Nedel S, Ewerbeck V, Debatin KM** 2000 Drug-induced apoptosis in osteosarcoma cell lines is mediated by caspase activation independent of CD95-receptor/ligand interaction. J Orthop Res 18:10–17
38. **Hansen CM, Hansen D, Holm PK, Binderup L** 2001 Vitamin D compounds exert anti-apoptotic effects in human osteosarcoma cells *in vitro*. J Steroid Biochem Mol Biol 77:1–11
39. **Zenmyo M, Komiya S, Hamada T, Hiraoka K, Kato S, Fujii T, Yano H, Irie K, Nagata K** 2001 Transcriptional activation of p21 by vitamin D3 or vitamin K2 leads to differentiation of p53-deficient MG-63 osteosarcoma cells. Hum Pathol 32 :410–416
40. **Asada M, Yamada T, Ichijo H, Delia D, Miyazono K, Fukumuro K, Mizutani S** 1999 Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. EMBO J 18:1223–1234
41. **Bellido T, O'Brien CA, Roberson PK, Manolagas SC** 1998 Transcriptional activation of the p21(WAF1, CIP1, SDI1) gene by interleukin-6 type cytokines. A prerequisite for their pro-differentiating and anti-apoptotic effects on human osteoblastic cells. J Biol Chem 273:21137–21144
42. **Consolini R, Pala S, Legitimo A, Crimaldi G, Ferrari S** 2001 Effects of vitamin D on the growth of normal and malignant B-cell progenitors. Clin Exp Immunol 126:214–219
43. **Moffatt KA, Johannes WU, Hedlund TE, Miller GJ** 2001 Growth inhibitory effects of 1 alpha, 25-dihydroxyvitamin D(3) are mediated by increased levels of p21 in the prostatic carcinoma cell line ALVA-31. Cancer Res 61:7122–7129