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TITLE: Adjuvant Action of Hepatocyte Growth Factor in Vitamin D Therapy of Androgen-Unresponsive Prostate Cancer

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13. ABSTRACT (Maximum 200 Words)

This research is aimed at discovering new therapies for advanced prostate cancer and developing in preclinical models a treatment for androgen-independent prostate cancer that bolsters vitamin D's antitumor actions. We are examining separate and combined growth-regulatory effects of growth factors and hormones with vitamin D. While we are focused on paradoxical growth-inhibitory actions of hepatocyte growth factor (HGF), we are also seeking agents that act similarly with vitamin D. The initial year concentrated on developing information and model systems for characterizing androgen modulation of HGF's actions and interactions with vitamin D vis-à-vis growth inhibition and cell-cycle regulation in sublines transformed with stable or inducible androgen-receptor activity. From this work we hope to identify which, if any, androgenic modulating influences are mediated by signaling events related to HGF's activation of MET, the HGF receptor. In seeking agents similar to HGF, we discovered methoxyestradiol, a normal estrogen metabolite that also exerts late cell-cycle inhibitory actions. Because it is naturally occurring, readily synthesized, and reported to lack the angiogenic and other pleomorphic effects that characterize HGF activity in normal tissues, methoxyestradiol could provide a cancer-cell-specific adjuvant to vitamin D therapy of prostate cancer.

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FOREWORD

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Bernard A Roop   8-16-99

PI - Signature   Date

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DAMD17-98-1-8525
Adjuvant action of hepatocyte growth factor in vitamin D therapy of androgen-unresponsive prostate cancer

Bernard A. Roos, PI

Annual Report: "Adjuvant action of hepatocyte growth factor in vitamin D therapy of androgen-unresponsive prostate cancer," Bernard A Roos, PI (DAMD17-98-1-8525)

INTRODUCTION

No effective therapy exists for prostate cancer that has advanced after palliation with androgen deprivation. Such advanced cancer no longer is responsive to withdrawal of testosterone, the normal primary growth regulator in the prostate. Vitamin D slows the growth of normal prostate epithelium and has some growth-inhibitory action in prostate cancer that has advanced to a testosterone-unresponsive state. However, the vitamin doses required to slow cancer cause toxic side effects, notably high blood calcium. The purpose of this research is to discover new therapies applicable for advanced prostate cancer and to develop in preclinical models a treatment for androgen-independent prostate cancer that incorporates and bolsters the antitumor actions of vitamin D. The scope of our research encompasses the separate and combined growth-regulatory effects of certain growth factors and hormones with vitamin D. While the research centers on paradoxical growth-inhibitory actions of hepatocyte growth factor (HGF) in human advanced prostate cancer cell lines, we are also seeking related growth factors and hormonal agents that may also paradoxically inhibit prostate cancer cells and bolster the antitumor activity of vitamin D.

BODY

Two specific aims were originally proposed with indicated first-year tasks:

> **Aim 1. Characterize androgen modulation of HGF's actions and interactions with vitamin D in regard to growth inhibition and cell-cycle regulation in ALVA-31 clones with inducible androgen-receptor activity and identify which, if any, androgenic modulating influences are mediated by HGF/MET-dependent signaling events.**

1. *(months 1-6)* Ascertain whether early-phase cyclins, such as D and E, change in native ALVA-31 during maximal growth inhibition by HGF–vitamin D and compare the time and extent of changes to later-phase cyclins, especially cyclin B.

2. *(months 1-6)* Ascertain whether HGF–vitamin D changes maturation promoting factor (MPF) activity, based on histone kinase activity and MPF's phosphorylation; compare the time and extent of these changes with possible changes in cyclin-dependent kinases (CDKs) 2, 4, and 6 (G1/S regulators).

3. *(months 1-6)* Complete comparison of 3D6-Mab (HGF agonist) and HGF effects on cell-cycle and MET activation.

4. *(months 1-6)* Generate an androgen-receptor (AR) expression vector for retrovirus-mediated, tetracycline-regulated AR expression (Retro-On®, Clontech) to infect ALVA-31 cells. Transfect ALVA-31 and screen for antibiotic-inducible AR expression. Isolate 12 ALVA clones that show good Dox-Tc induction of AR activity.

5. *(months 5-8)* Analyze the ALVA clones for HGF/vitamin D inhibition of growth and cell-cycle regulatory factors under low AR (no antibiotic) and high AR (Dox-Tc) culture conditions.

6. *(months 9-12)* Identify the clones in which androgen responsiveness most interferes with or reverses HGF–vitamin D inhibition of growth and activity of cell-cycle regulators. Also, identify for later studies those clones that fail to show reversal despite inducible androgen responsiveness.

7. *(months 9-12)* Ascertain correlation of loss of growth inhibition with induction of androgen reporter activity and ascertain whether androgen antagonists restore inhibition.

Aim 2. Demonstrate in vivo that HGF and a novel HGF agonist (monoclonal antibody directed against MET's extracellular domain), alone or in combination with vitamin D, inhibit xenografted tumors derived from native ALVA-31 cells and ascertain whether androgenic influences on HGF and vitamin D actions seen in vitro can be demonstrated in xenografts of ALVA-31 clones with inducible androgen-receptor activity.

1. *(months 1-12)* Ascertain whether high-dose HGF (with or without dextran sulfate co-administration) affects growth of xenografted ALVA-31 cells.

2. *(months 1-12)* Ascertain whether anti-MET Mab (a documented HGF agonist with regard to hepatic growth effects in vivo as well as an in vitro growth inhibitor of ALVA-31) affects growth of xenografted ALVA-31 cells.

3. *(months 1-18)* Assess synergism of HGF and 3D6 with active vitamin D on xenografted ALVA-31 in nude mice.

We have discovered that a growth factor (hepatocyte growth factor, HGF), which influences development and maintenance of all tissues and usually stimulates cell growth, paradoxically inhibits cultured prostate cancer cells that have lost control by testosterone (Ref. 1; manuscript in appendix). In testing androgen-insensitive human prostate cancer cell lines, all but one of these lines showed paradoxical inhibition by HGF (Ref 1; appended manuscript). Moreover, in the ALVA-31 cell line, when we combined HGF with vitamin D, which by itself had only a weak anti-tumor effect, cell growth was almost totally inhibited (see Fig. 5 in Ref. 1, appended manuscript). We are engaged in a series of tissue culture and molecular biology experiments to understand why most androgen-independent prostate cancer cells paradoxically can be inhibited by HGF. The experiments to date suggest that this activity involves inhibition of later cell-cycle events (see Fig. 6 in Ref. 1, appended manuscript).

In seeking agents that might mimic the actions of HGF, we discovered that methoxy-estradiol, a normal estrogen metabolite, exerts late cell-cycle inhibitory actions similar to HGF in all human prostate cancer cell lines. Because this metabolite is naturally occurring, readily synthesized, and reported to lack the angiogenic and other pleomorphic effects that characterize HGF activity in normal tissues, methoxyestradiol could well provide a cancer-cell–specific adjuvant to bolster vitamin D therapy of prostate cancer. Accordingly, we have incorporated the studies of methoxyestradiol into our studies of HGF and its interactions with vitamin D. We have found growth inhibition (Fig. A, Appendix) and inhibitory effects on later cell-cycle events (Fig. B, Appendix). Thus, in addition to HGF agonists, our work is identifying several novel opportunities to bolster the antitumor activity of vitamin D in advanced prostate cancer.

To elucidate the mechanism of this paradoxical growth factor inhibition of androgen-insensitive prostate cancer cells, we have pursued more detailed studies in the ALVA-31 cell line. The cell-cycle control mechanisms involved differ from the usual G1/S slowing involved in growth control by hormones and growth factors. Instead of the expected G1 accumulation, marked growth inhibition of androgen-unresponsive cells is associated with a higher fraction of cells in later cell-cycle phases (Fig. 6 in Ref. 1, appended manuscript), suggesting that cell-cycle slowing by an HGF–vitamin combination involves some preferential inhibition of progression through later phases. In support of this idea, we find that combined treatment markedly reduces cyclin B, which regulates entry into cell division via activation of maturation promoting factor (MPF, or p34^{cdc2}).

HGF's switch from stimulus in normal prostate to inhibitor in androgen-unresponsive cells suggests that androgen-receptor (AR) and androgen action can influence HGF action. We have documented expression and activation of the HGF receptor (MET) in androgen-responsive and -unresponsive prostate cancer cells (Fig. 2 in Ref. 1, appended manuscript). We believe that the

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cell line differences in HGF responsiveness reflect both altered MET expression and altered regulation of intracellular targets of MET activation and/or alteration of MET's signal transduction cascade. Our work is focusing on altered regulation of intracellular MET targets.

We are now initiating production of suitable (AR-inducible) ALVA-31 sublines that will provide a model system to elucidate why HGF in combination with vitamin D markedly inhibits androgen-unresponsive cells and to begin to establish the theoretical and practical framework for enhancing vitamin D biotherapy of advanced prostate cancer with HGF and HGF agonists. However, we have experienced several problems regarding the generation of ALVA-31 clones that stably express the human AR. Because of the critical role these cell lines have for the long-term testing of the role of AR in modifying growth factor and hormone responses in our model system, we provide details of our first year's work in this area.

Since we have not previously used ALVA 31 cells for stable expression of genes, it was first necessary to establish the appropriate conditions, which included performing a "kill curve" (to determine the concentration of the antibiotic Geneticin (G418) for selection of clones) and testing function of the AR cDNA vector in transient transfection assays. Accordingly, ALVA-31 cells were plated at 2×10^5 in 24-well culture dishes (~30% confluency). The next day, the samples were treated in duplicate with G418 (at 0, 200, 300, 350, 400, 500, 600, 800, and 1,000 μg/ml) concentrations of active drug. Cells were refed every other day and stained with cresyl violet for 5 min. Cell growth was determined and the lowest drug concentration that resulted in 50-75% cell death after 7 days was chosen (G418 at 350 μg/ml).

We next assessed AR cDNA functionality by reporter gene assay. The AR cDNA was previously cloned into the expression vector pCDNA3 (Invitrogen) (Dai et al., 1996). This plasmid consists of the AR cDNA driven by the CMV promoter and contains the neo gene conferring resistance to Geneticin. To confirm that the AR encoded by pCDNA3 AR3.1 is functional in ALVA-31 cells, we cotransfected ALVA-31 cells with pCDNA3-AR and the androgen-regulated reporter plasmid MMTV-CAT (containing the mouse mammary tumor virus LTR linked to the CAT gene). Androgen (the nonmetabolizable R1881) induced CAT activity in a dose-responsive manner in these cells.

For transfection and selection of ALVA-31 stable transformants, ALVA-31 cells were plated at 4×10^6 cells/60-mm dish. Twenty-four hours later the cells were transfected with pCDNA3-AR3.1 and the parent vector pCDNA3 (5 μg/dish) using the calcium phosphate coprecipitation method. The cells were split into 96-well plates at limiting dilutions (1:10, 1:15, 1:20) and the medium was changed every other day. Clonal cell growth became apparent after 2-3 weeks and cells in these wells were passaged into 24-well dishes and then to 6-well dishes. (Note: only wells containing a single colony were expanded.)

For androgen-receptor (^{3}H-R1881 binding) assays, cells were cultured in 60-mm dishes. A whole cell binding assay was used as previously described (Dai et al., 1996). Cultures derived from 12 clonal isolates were examined, and the clone expressing the highest binding capacity was selected for further characterization. Thirty additional clonal isolates are currently being expanded and ^{3}H-R1881 binding is being assessed.

While we still continue developing tet-regulated ALVA-31(AR) sublines, our newly produced AR-containing and control stable (noninducible) sublines provide a good opportunity to identify which, if any, androgenic-modulating influences are mediated by HGF/MET-dependent signaling events. We will also use a nude mouse system based on transplantable tumors of various ALVA-31 sublines to demonstrate in vivo that HGF and a novel HGF agonist (monoclonal antibody directed against MET's extracellular domain), alone or in combination with vitamin D, inhibit xenografted tumors derived from native ALVA-31 cells and determine if androgenic influences on HGF and vitamin D actions seen in vitro can be shown in xenografts of ALVA sublines.

KEY RESEARCH ACCOMPLISHMENTS

- Discovery of HGF's paradoxical growth inhibition as a common, but not universal, feature of human androgen-insensitive prostate cancer lines.
- Discovery of synergism (in vitro) between vitamin D and HGF growth inhibition in human androgen-insensitive prostate cancer cell lines.
- Discovery that these synergistic growth inhibitory actions are directed at late cell-cycle events.
- Discovery that methoxyestradiol, a normal estrogen metabolite, exerts late cell-cycle inhibitory actions similar to HGF in human prostate cancer cell lines. Since this metabolite is naturally occurring, readily synthesized, and lacks the angiogenic and other pleomorphic effects of HGF on normal tissues, methoxyestradiol could well provide a cancer-cell specific adjuvant to bolster vitamin D therapy of prostate cancer.

REPORTABLE OUTCOMES

Manuscripts

1. Submitted to *Cancer Research* (copy appended): LR Qadan, CM Perez-Stable, RH Schwall, KL Burnstein, RC Ostenson, GA Howard, BA Roos, "Hepatocyte growth factor and vitamin D cooperatively inhibit androgen-unresponsive prostate cancer cell lines"

2. In Preparation: LR Qadan, G D'Ippolito, CM Perez-Stable, BA Roos, A naturally occurring estradiol metabolite inhibits growth of androgen-sensitive and androgen-insensitive prostate cancer cells

Cell line development

Development of androgen receptor–expressing ALVA-31 sublines

CONCLUSIONS

Our original idea that the loss of androgen receptor activity signals a new scheme of cell-cycle regulation continues to gain support. However, the creation of AR-ALVA remains critical to any definitive conclusions. These studies should be available by next year. More exciting to the larger issue of prostate cancer therapy is our discovery of a natural estrogen metabolite that selectively inhibits late cell-cycle events. We are incorporating this finding into our experimental strategy to develop adjuvant therapy that will bolster the antitumor activity of vitamin D. Thus, our original concern that clinical application of HGF's late cell-cycle inhibitory actions might prove difficult to apply clinically seems less critical. The discovery of a novel agent that can affect tumor cells but not normal cells provides us with a good additional opportunity to develop a clinical application from our basic studies of later cell-cycle events.

REFERENCES

1. Qadan LR, Perez-Stable CM, Schwall RH, Burnstein KL, Ostenson RC, Howard GA, Roos BA: Hepatocyte growth factor and vitamin D cooperatively inhibit androgen-unresponsive prostate cancer cell lines, *Under Review* (Included in Appendix)

2. Dai JL, Maiorino CA, Gkonos PJ, Burnstein KL. Androgenic upregulation of androgen receptor cDNA expression in androgen-independent prostate cancer cells. *Steroids* 61:531-539, 1996

APPENDICES

Article Submitted for Publication
LR Qadan, CM Perez-Stable, RH Schwall, KL Burnstein, RC Ostenson, GA Howard, BA Roos, "Hepatocyte growth factor and vitamin D cooperatively inhibit androgen-unresponsive prostate cancer cell lines"

Reviewing Journal's Receipt Letter

Figure Appendix
Figure A
Figure B

Hepatocyte Growth Factor and Vitamin D Cooperatively Inhibit Androgen-Unresponsive Prostate Cancer Cell Lines[1]

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Running title: **HGF inhibits hormone-refractory prostate cancer**

Key words: **cell cycle, MET, cancer biotherapy, morphogenesis, motogenesis**

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ABSTRACT

Expression of MET, the receptor for hepatocyte growth factor (HGF), has been associated with androgen-insensitive prostate cancer. Here we have evaluated MET activation by HGF and HGF action in prostate cancer cell lines. HGF causes phosphorylation (activation) of the MET receptor in 3 androgen-unresponsive cell lines (DU 145, PC-3, and ALVA-31) together with morphological change. Although HGF is known to stimulate growth of normal epithelial cells, including those from prostate, we found that HGF inhibited ALVA-31 and DU 145 (hormone-refractory) cell lines. Moreover, HGF and vitamin D additively inhibited growth in each androgen-unresponsive cell line, with the greatest growth inhibition by this combination seen in the ALVA-31 cells. Further studies in ALVA-31 revealed distinct cooperative actions of HGF and vitamin D. In contrast to the accumulation of cells in G1 seen during vitamin D inhibition of androgen-responsive cells (LNCaP), growth inhibition of the androgen-unresponsive ALVA-31 cell line with the HGF and vitamin D combination decreased, rather than increased, the fraction of cells in G1, with a corresponding increase in the later cell-cycle phases. This cell-cycle redistribution suggests that in androgen-unresponsive prostate cancer cells, HGF and vitamin D act together to slow cell-cycle progression via control at sites beyond the G1/S checkpoint, the major regulatory locus of growth control in androgen-sensitive prostate cells.

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INTRODUCTION

Prospects for prostate cancer biotherapy were brightened by the observations that prostate cancer cells express vitamin D receptors (1) and that 1,25 dihydroxycholecalciferol $(1,25(OH)_2D)$ and its active analogs (for simplicity, hereafter referred to as vitamin D) dramatically inhibit growth and promote differentiation of normal and neoplastic androgen-sensitive prostate epithelial cells (2–4). Unfortunately, only modest growth inhibition by vitamin D and its analogs has been achieved in androgen-refractory aggressive prostate cancer cell lines *in vitro* (5, 6) and *in vivo* as assessed in xenograft models (7). The mechanism for this diminished inhibition remains obscure. Neither vitamin D receptor abundance nor transcriptional activity completely explains the different responses (8–11).

In androgen-sensitive prostate epithelial cells, where growth inhibition by vitamin D is most dramatic and well characterized, several laboratories have reported an accumulation of cells in the G1 phase of the cell cycle (11, 12). This partial G1 arrest, which persists even after vitamin D withdrawal (3, 9, 11), is associated with upregulation of anti-oncogenes, such as p53, p21^{WAF1}, and p27^{KIP1}, and downregulation of cyclin-dependent kinase activities that control G1/S transit (11, 13, 14). Because expression of p53, p21$^{WAF1/CIP1}$, and other anti-oncogenes critical to G1/S checkpoint control can decrease during the progression to androgen-unresponsive carcinoma, the relative insensitivity of androgen-refractory cells to growth inhibition by vitamin D was not unexpected (9, 15, 16). Still the mechanism for vitamin D's modest inhibition of those androgen-unresponsive prostate cancer cells remains obscure, because restoration of the retinoblastoma gene did not restore vitamin D inhibition of

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cell growth (8). Some evidence suggests that vitamin D's growth-inhibitory effects in other carcinomas might involve later cell-cycle events. In the T47D estrogen-responsive breast cancer cell line, marked (> 90%) vitamin D inhibition is associated with decreased cells in G1 and accumulation of cells in later cell-cycle phases (17). Thus far, however, no evidence has emerged to support the idea that vitamin D's antiproliferative action in prostate cells involves later cell-cycle events (11, 18).

Hepatocyte growth factor (HGF, also known as scatter factor) is a mesenchymal protein with mitogenic, motogenic, and morphogenic effects on nonneoplastic as well as neoplastic epithelial cell types (19, 20). HGF's pleiotropic effects are mediated via its receptor (MET), the transmembrane tyrosine kinase encoded by the *MET* proto-oncogene (21–23). Unlike vitamin D, which usually slows growth, HGF stimulates proliferation of most normal and neoplastic cells (20, 24, 25). However, *in vitro* studies have shown that some carcinomas, such as hepatoma, melanoma, and breast carcinoma, are paradoxically inhibited by HGF (26–30). In T47D breast cancer cells, HGF mediated growth arrest and differentiation via slowing of G1/S progression and cell accumulation in G0/G1. In normal prostate, HGF is produced by stromal cells nearest to the basal epithelial cells, which have more HGF receptor and less androgen receptor compared to luminal prostate epithelium (31–36). During prostate growth the basal cells respond to a series of induced stromal growth factors, including HGF, which regenerate differentiated luminal secretory epithelium with high sensitivity to, and dependence on, androgen (31, 37–39). MET is increased early in epithelial neoplasia, including prostatic intraepithelial neoplasia (32, 33, 40), and remains highly expressed in virulent carcinomas and in derived cell lines (32, 33, 40, 41).

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The prominence of MET expression in prostate carcinoma in concert with our previous finding of HGF and vitamin D synergistic action in increasing alkaline phosphatase activity in cartilage (42) prompted our investigation of HGF's action in prostate cancer and HGF's possible interactions with vitamin D. We surveyed HGF's effects on cell morphology and growth of the androgen-sensitive LNCaP cells and on three androgen-refractory cell lines (DU 145, PC-3, and ALVA-31). Based on that survey, we pursued more detailed characterization of the separate and combined effects of HGF and vitamin D in ALVA-31, the cell line our initial survey found to be most inhibited by the growth factor–vitamin D combination. We observed that a significant inhibition was obtained by combining low doses of the agents, amounts which by themselves had no growth effect. Similarly, the HGF–vitamin D combination, but neither agent alone, decreased the fraction of cells in G1 and increased the fraction in later cell-cycle phases. These results support slowing of cell-cycle events by cooperative actions of vitamin D and HGF at loci beyond the G1/S contact point.

MATERIALS AND METHODS

Reagents. 1,25-dihydroxycholecalciferol (1,25(OH)2D) was purchased from Calbiochem (La Jolla, CA). Recombinant heterodimeric human HGF was generated and purified as previously reported (43). RNase A from bovine pancreas was purchased from Boehringer Mannheim (Indianapolis, IN) and propidium iodide from Sigma (St. Louis, MO). Biotin-conjugated antiphosphotyrosine antibody was obtained from Upstate Biotechnology (Lake Placid, NY), horseradish peroxidase (HRP)-streptavidin from ZYMED (San Francisco, CA), and tetramethylbenzidine (TMB) peroxidase solution from Kirkegard & Perry Laboratories (Gaithersburg, MD). Maxisorp 96-well plates were purchased from VWR Scientific (Atlanta, GA).

Cell Culture. Human prostate carcinoma cell lines (DU 145 [ref. 44; ATCC HTB81], LNCaP [ref. 45; ATCC CRL 1740], and PC-3 [ref. 46; ATCC CRL 1435]) were obtained from the American Type Culture Collection (Rockville, MD). Our earlier preliminary observation of HGF inhibition of PC-3 cells was performed with multiply passaged cells obtained from another laboratory (47). This inhibition was not observed with PC-3 cells from a freshly obtained ATCC preparation. Moreover, further characterization of these multiply passaged cells showed androgen receptor expression, which is uncharacteristic and does not occur in the low-passage PC-3 cells from ATCC. Thus, we are reporting only results related to the ATCC-derived lower-passage PC-3 cells. ALVA-31 cells were obtained as previously described (48). All cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 7.5% fetal bovine serum (FBS Hyclone, Logan, UT), 100 IU/ml penicillin,

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and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air.

Androgen Receptor Expression. Because it had earlier been reported that ALVA-31 cells express the androgen receptor (48), we assessed for expression of this receptor by the RNase protection technique (49). Human androgen receptor mRNA levels were measured with a ^{32}P-labeled T7 polymerase-synthesized antisense RNA probe from StuI-digested HindIII-EcoRI/Bluescript-KS. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured with a ^{32}P-labeled T7 polymerase-synthesized antisense RNA probe from StyI-digested pTRIPLEscript-GAPDH (Ambion, Austin, TX). Forty micrograms of total RNA was hybridized at 37°C for 16 h and digested with RNase mixture (Ambion) for 30 min at 37°C. RNase digestion products were separated by electrophoresis on polyacrylamide-urea gels and analyzed by autoradiography. Androgen receptor mRNA was present in LNCaP (positive control), absent in DU 145 (negative control), and absent in PC-3 and ALVA-31 cells.

Isolation of Human MET RNA by RT-PCR. Total RNA was isolated from PC-3 human prostate cancer cells by the LiCl-urea method (50) and treated with DNase (RNase-free). RT-PCR was performed for MET using the oligonucleotide primers 5'-GGTTGCTGATTTTGGTCATGC-3' (forward, residues 3905–3925 base pairs [bp]) and 5'-TTCGGGTTGTAGGAGTCTTCT-3' (reverse, residues 4146–4166 bp) (51). After the RT reaction, PCR was carried out in a DNA thermal cycler (Perkin-Elmer Cetus) under the following conditions: 1-min denature at 94°C; 1-min annealing at 55°C; 2-min extension at 72°C for 35 cycles; 7-min extension at 72°C. The expected 261-bp PCR fragment was cloned into EcoRV-digested Bluescript-KS (treated with Taq polymerase), and the correct sequence was confirmed by DNA sequencing to give plasmid hMET/BS.

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RNase Protection Analysis. RNA was isolated from the human prostate cancer cell lines LNCaP, PC-3, DU 145, and ALVA-31 by the LiCl-urea method (50). Human MET mRNA levels were measured with ^{32}P-labeled T7 polymerase-synthesized antisense RNA probe from HindIII-digested hMET/BS. Human cyclophilin mRNA was measured with a ^{32}P-labeled T3 polymerase-synthesized antisense RNA probe from pTRI-cyclophilin (Ambion). Ten μg of total RNA was simultaneously hybridized with the MET and cyclophilin probes at 37°C for 16 h and digested with RNase mixture (Ambion) for 30 min at 37°C. RNase digestion products were analyzed by electrophoresis on polyacrylamide-urea gels and autoradiography. The protected RNA fragments are 261 nt (MET) and 103 nt (cyclophilin).

Western Blot Analysis. Total protein lysates from LNCaP, PC-3, DU 145, and ALVA-31 cells were prepared as described (52) except without boiling. After separation of 10 μg of protein by SDS-PAGE, proteins were transferred by electrophoresis to Immobilon-P membrane (Millipore, Bedford, MA) and incubated in 5% nonfat dry milk, PBS, and 0.05% Tween 20 for 1 h. Rabbit polyclonal antibodies specific for human MET (h-MET C-12; 1/1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) were diluted in 5% nonfat dry milk, PBS, and 0.05% Tween 20 and incubated overnight at 4°C. Membranes were washed in PBS and 0.05% Tween 20 (3 x 10 min) and incubated with horseradish peroxidase conjugated secondary antibody (antirabbit; 1/1,000 dilution; Roche Molecular Biochemicals, Indianapolis, IN) for 1 h, washed in PBS and 0.05% Tween 20, and analyzed by exposure to X-ray film (X-OMAT; Kodak, Rochester, NY) using enhanced chemiluminescence ECL (Amersham, Arlington Heights, IL).

MET Activation (P-Tyr) Measurement by KIRA Assay. Cells were plated in 10-cm

dishes with RPMI medium supplemented with 7.5% FBS. After incubation overnight at 37°C,

semiconfluent cultures were washed three times with PBS, then treated for 10 min with HGF

at 10 ng/ml diluted in serum-free RPMI-1640 with 1 mg/ml BSA. Cells were then rinsed,

lysed in 1 ml PBS, 0.2% Triton X-100, 10 μg/ml aprotinin, 5 mM sodium fluoride, 2 mM

orthovanadate, and 0.2 mM phenylmethylsulfonyl fluoride for 30 min at room temperature.

The lysate was cleared by centrifugation, and the supernatant was collected; the protein was

quantified by the BCA protein assay (Pierce, Rockford, IL). One hundred-microliter aliquots

were transferred in duplicate to a maxisorp 96-well plate that had been coated with 5 μg/ml

protein-A-purified rabbit IgG antibody against MET-extracellular-domain (anti-MET/ECD

IgG) (53). Cell lysates were incubated for 2 h at room temperature. The bound P-Tyr was

detected after another 2-h incubation with biotin conjugated antiphosphotyrosine antibody,

followed by HRP-streptavidin and development with TMB peroxidase solution (53). The

reaction was stopped with 1 M phosphoric acid, and MET activation (i.e., phosphorylated

tyrosine) was determined based on optical density at 450–690 nm in an automatic plate

reader.

Cell and Colony Morphology. Cells were seeded in 6-cm tissue culture dishes at the

relatively low density of 5,000 cells/dish in 3 ml of tissue culture medium. After 24 h, either

vehicle or test substances (final concentration) were added: vehicle (ethanol, 0.01 or 0.1%, v/v

culture medium) or 1,25(OH)$_2$D (at final concentrations of 1–100 nM) or HGF (1–20 ng/ml

culture medium). Unless otherwise stated, cells were treated for six days, with serial

monitoring with a Nikon Diaphot inverted microscope at 50x and 250x. Additional cultures

were maintained and periodically fixed for crystal violet staining.

Assay of Cell Proliferation. Cell proliferation was assessed by cell counting. To test the separate and combined antiproliferative effects of $1,25(OH)_2D$ and HGF, cells were seeded at a density of 5,000 cells per 6-cm dish. After incubation for 24 h, medium was replaced with fresh medium containing vehicle (ethanol, final concentration of 0.01%), $1,25(OH)_2D$ (to a final concentration of 1, 10, or 100 nM), HGF (added in medium to a final concentration of 1, 10, or 20 ng/ml), or a combination of HGF and $1,25(OH)_2D$. After three days, the medium was changed and replenished, and on the sixth day cells were harvested by trypsinization and counted with a Neubauer hemacytometer. Cell numbers of each experiment were derived from the mean value of triplicate wells in an experiment. Nearly all (> 98%) cells under all treatment conditions excluded trypan blue. After six days of treatment some cell cultures were fixed with crystal violet. Photographs were obtained of stained and unstained cultures using a Nikon Diaphot inverted microscope. Although qualitatively similar results were obtained at higher plating density, the inhibitory effects were diminished at higher density in our initial experiments. Accordingly, proliferation and cell-cycle analyses were performed on low-density cultures; cells were seeded at 5,000/6-cm dish for proliferation and at 30,000/10-cm dish for analysis of cell-cycle effects (see below).

Analysis of Cell-Cycle Effects. Cell-cycle distribution and changes with HGF and $1,25(OH)_2D$ were estimated for ALVA-31 cells by analytical flow cytometry (11, 54). To accumulate sufficient cells for cell-cycle analyses, cells were seeded at 30,000 cells/10-cm dish (twice the density used for proliferation studies). They were treated 24 h later with ethanol (0.01%), HGF (10 ng/ml), $1,25(OH)_2D$ (10 nM), or a combination of (10 ng/ml)

HGF and (10 nM) 1,25(OH)$_2$D. After six days of treatment, cells were trypsinized and washed twice with ice-cold PBS containing 0.1% glucose, then fixed by drop-wise addition of 70% ethanol. After at least 12 h of ethanol fixation, DNA was stained with propidium iodide (50 μg/ml) for 30 min. RNase (100 units/ml) was included in the staining solution to degrade double-stranded RNA. Analyses were performed with a FACS Scan unit (Becton Dickinson, San Jose, CA). Excitation was at 488 nm with emission measured at 630 nm. Distribution of cells with respect to their DNA content was analyzed for 5,000 cells for each test condition. The relative proportions of cells in various cell-cycle phases were estimated by compartment analysis of DNA fluorescence using cell-cycle analysis software from the manufacturer to set cutoffs for G1, S, and G2/M (54).

Statistical Analysis. Statistical analysis was performed by analysis of variance. For single comparisons of the difference between means, unpaired Student's t test was applied. Significance levels are indicated in the figure legends.

RESULTS

MET (mRNA and Protein) in LNCaP, PC-3, DU 145, and ALVA-31 Cells. MET mRNA was previously shown by Northern blot analysis to be present in PC-3 and DU 145 cells, but not in LNCaP cells (32). We used the RNase protection technique to determine if MET was also present in ALVA-31 cells. We found the MET mRNA in ALVA-31 cells comparable to that found in PC-3 and DU 145 cells, but there was no detectable MET in LNCaP cells (Fig. 1). Similarly, MET protein was not detectable in LNCaP by Western blot, while it was present in equivalent amounts in the other cell lines (Fig. 1).

Phosphorylation of MET Tyrosine. With the double-antibody technique we analyzed whether the addition of HGF to the prostate cancer cell lines resulted in the activation of MET. Tyrosine phosphorylation of MET was readily detectable in all androgen-unresponsive cell lines (DU 145, PC-3, and ALVA-31) but not in LNCaP cells. This finding indicates activation of the MET receptor in the three cell lines in response to HGF treatment (Fig. 2).

Initial Survey of HGF Effects on Morphology and Growth in Prostate Cancer Cell Cultures. We based our initial screening on the fact that in most targets, including prostate cells, HGF action generally dissociates aggregated cells (20, 32, 55). In each of the androgen-unresponsive cell lines, we observed similar characteristic changes with HGF treatment. Longer treatment resulted in changes in cell morphology, to more cuboidal or rounded in DU 145 and ALVA-31. These morphological changes occurred with as little as 1 ng/ml of HGF, although effects were more rapid and obvious at 10–20 ng/ml. No morphological changes were noted during HGF treatment of androgen-responsive LNCaP

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cultures. We did not observe cell fragmentation or increased trypan blue dye staining of nuclei under any conditions.

We conducted initial experiments to assess the growth effects of adding HGF (10 ng/ml) and vitamin D (10 nM) alone and in combination (Table 1). After six days of treatment with HGF alone, no growth inhibition was noted in LNCaP or PC-3, but ALVA-31 and DU 145 were inhibited 49% and 35%, respectively (Fig. 3). Vitamin D alone markedly inhibited LNCaP (71% decrease from control), with much smaller effects on PC-3 (8% decrease) and ALVA-31 (42% decrease) and no significant effect on DU 145 (9% increase). While the addition of HGF together with vitamin D did not further inhibit LNCaP, HGF addition did further inhibit each of the androgen-unresponsive cell lines: PC-3 showed a 15% decrease with the combination vs. an 8% decrease with vitamin D alone; DU 145 showed a 35% decrease with the combination vs. no inhibition with vitamin D alone. Finally, ALVA-31 manifested a 78% decrease with the combination vs. 42% with vitamin D alone. Because combining HGF with vitamin D caused as great an inhibition in the androgen-unresponsive ALVA-31 cells as the marked inhibition seen with vitamin D treatment of the androgen-sensitive LNCaP cells, we selected ALVA-31 for further study.

HGF and Vitamin D (1,25(OH)$_2$D) Distinctly Alter ALVA-31 Colony and Cell Morphology. After six days, HGF-treated ALVA-31 cell morphology was more cuboidal and rounded, while vitamin D-treated ALVA-31 cells were flattened and elongated (Fig. 4). Because growth inhibition with HGF and with vitamin D was roughly comparable under these conditions, these morphological differences are not likely to reflect cell-density differences. While HGF did not markedly alter colony shape, vitamin D generated an interlaced pattern of

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smaller, closer-packed colonies. With the HGF–vitamin D combination, cultures appeared sparse, and both rounded and tapered cells were noted. Larger ovoid cells with prominent nuclei were more numerous after combined treatment than after treatment with either agent alone. None of the treatments caused cell fragmentation or appeared cytotoxic as evidenced by microscopy after trypan blue or crystal violet staining.

Vitamin D (1,25(OH)$_2$D) and HGF Exert Separate, Cooperative, and Reversible Antiproliferative Effects on ALVA-31 Cells. Vitamin D (1,25(OH)$_2$D) at concentrations of 1, 10, and 100 nM decreased cell number by 14 (NS, not significant), 45, and 67%, respectively. HGF at 1, 10, and 20 ng/ml decreased cell number by 10 (NS), 59, and 61%, respectively (Fig. 5). Inhibition with the HGF–vitamin D combination was significantly more potent than with either agent alone at all concentrations, with the maximal effect (> 85% decrease) of the combination always significantly greater than that achieved by either agent alone. While no significant inhibition occurred with 1 nM vitamin D or with 1 ng/ml HGF, combining the two agents at these low concentrations produced a significant inhibition (35% decrease). Ten-fold higher concentrations of HGF or vitamin D were needed to achieve comparable inhibition with only one agent (Fig. 5).

In contrast to the irreversible antiproliferative effects of vitamin D in androgen-sensitive cells (3, 11, 56, 57), the antiproliferative effect of the vitamin D–HGF combination is reversible in ALVA-31. After treatment for three days with the combination of 10 ng/ml HGF and 10 nM vitamin D, cultures were divided into two groups, continuing the combined treatment in one group and withdrawing treatment in the other. Cell cultures were monitored daily by microscopy, and after three more days of treatment, cells were counted. Treatment

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withdrawal prevented the morphological changes seen with continuous treatment and doubled cell number compared to cultures treated continuously.

HGF–Vitamin D Combination Treatment Alters Cell-Cycle Distribution. Cells grown in 10 nM vitamin D or in 10 ng/ml HGF did not show significant changes in cell-cycle distribution. However, with the HGF–vitamin D combination, the percentage of cells in G1 decreased from 60 ± 3 to 50 ± 2.5, and the fraction of cells in later cell-cycle phases (S/G2/M) increased from 39 ± 3.2 to 48 ± 1 (Fig. 6). We never observed any lower-DNA–mass fluorescence peaks suggestive of DNA fragmentation and apoptosis. The generalized increase in later cell-cycle phases suggests that the growth-inhibitory effects involved cell-cycle slowing at points beyond the G1/S checkpoint.

DISCUSSION

Our results indicate that HGF can inhibit growth of some androgen-independent prostate cancer cell lines. Such inhibition by HGF has been reported for only a few cancers (26, 30, 58). Growth inhibition and antitumorigenic effects have been reported in hepatoma both *in vitro* and *in vivo* (26, 29, 59, 60). Because HGF stimulates normal hepatocytes and is a potent inhibitor of hepatoma cells (59), it is likely that the intracellular signaling in response to HGF stimulation is different in normal and malignant cells. These findings in hepatoma could well pertain to prostate, because HGF stimulates normal prostate epithelium cells and inhibits androgen-unresponsive prostate cancer.

Inhibition of prostate cancer cells with HGF seems paradoxical in view of its classical role as a paracrine growth stimulator (20), as well as previous investigations that suggested an adverse role for HGF in prostate carcinoma based on several findings (31–33). First, MET expression appears to correlate with prostate tumor invasion and spread (32, 33). Second, growth stimulation by HGF was reported in nonneoplastic rat prostate cells (31) and in one androgen-refractory (DU 145) human prostate cancer cell line (32). Third, increased invasion potential in the matrigel invasion chamber assay was stimulated by HGF (61). Although we observe growth inhibition at higher plating densities, the greater effects on growth were seen at lower plating densities. However, the difference in cell density is unlikely to explain the finding of growth stimulation (32) or lack of inhibition (61) by HGF in DU 145 cells. The most likely reason for the difference from our results was the lack of serum in their experiments (20, 32). Therefore, we compared the effect of HGF on DU 145 cells grown

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under full serum and serum-free conditions. Despite an HGF-induced growth inhibition in the presence of serum, HGF had no significant effect on the growth of DU 145 cells in serum-free medium (unpublished data). A similar discrepancy of growth effects in the presence or absence of serum has been reported for LNCaP cells with vitamin D treatment (1, 5).

The lack of mitogenic, motogenic, and morphogenic responses of LNCaP cells to HGF was not surprising in view of the absence of MET RNA transcript and protein by RNase protection and Western blot, respectively. Although all three androgen-independent cell lines (ALVA-31, DU 145, and PC-3) showed equivalent MET activation (tyrosine phosphorylation) with HGF, PC-3 lacked the antimitogenic and morphogenic responses. A similar lack of proliferative and invasive response of PC-3 to HGF was reported by Nishimura *et al.* (61). Because signals given to epithelial cells by HGF are mediated through the MET receptor tyrosine kinase, the lack of response by PC-3 despite the receptor's activation points to defect(s) in the downstream signaling pathway, which might be true for other malignant cell types.

We selected ALVA-31 cells for studies of the HGF and the vitamin D interaction because they show clear-cut growth inhibition with either agent alone. The cooperative interaction of HGF with vitamin D was revealed by the significant inhibition resulting from the combination of low doses of these agents, which by themselves exerted no effect on growth. In addition, although neither HGF nor vitamin D alone significantly altered cell-cycle distribution, their combination did, with an accumulation of cells in S/G2/M phases. These results are consistent with earlier reports that despite significant antiproliferative effects on androgen-refractory cells, vitamin D alone did not alter cell-cycle distribution (10, 11, 18).

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Our finding that the HGF–vitamin D combination treatment decreased the fraction of cells in G1 and increased the fraction in later cell-cycle phases (S, G2, and M), in concert with the marked growth inhibition, suggests that slowed cell-cycle progression with these factors involves cooperative but distinct actions at loci beyond the G1/S checkpoint. Downregulation of cyclin B and/or cyclin-dependent kinase-1, which regulate late cell-cycle events, is a plausible mechanism at which future work will be directed. Slowing of cell-cycle progression through these later phases was previously noted in T47D breast cancer cells by vitamin D, an agent considered to exert its inhibition via G1/S slowing (17). In our experiments, cell loss through apoptotic mechanisms seems unlikely, given the large inhibition observed and the lack of any evidence for apoptosis during serial microscopy or from cytometry flow analyses (54); furthermore, reversibility of the effect argues against apoptosis.

Androgen-responsive prostate epithelial cells, such as LNCaP, are markedly and irreversibly inhibited by vitamin D, with decreased G1/S transit that results in accumulation of cells in the G1 phase of the cell cycle, suggesting G1/S arrest (9, 11, 12). The development of vitamin D resistance in androgen-unresponsive prostate cancer cells could signal their diminished ability for G1/S checkpoint regulation. Although decreasing vitamin D receptor expression during tumor progression to androgen unresponsiveness could explain some of the apparent vitamin D resistance (9, 62), neither vitamin D receptor loss nor diminished transcriptional activity fully accounts for this difference, because the androgen-unresponsive ALVA-31 cells have higher vitamin D receptor number and activity than the androgen-responsive LNCaP cells (10). Each androgen-unresponsive cell line has well-documented mutations (63, 64) involving Rb, p53, p21, and/or other anti-oncogenes critical to G1/S

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checkpoint control by vitamin D and other hormones (65–69). Such mutations may provide a reason for why vitamin D is less effective in inhibiting G1/S transit in androgen-unresponsive cell lines (11, 70, 71). Another reason is the loss of androgen modulation of cell-cycle control. Restoring androgen receptor activity has been shown to reinstitute G1/S checkpoint control (72). While no one regulatory or genetic alteration is likely to disrupt G1/S control (8), cumulative changes may result in loss of cell-cycle control.

In conclusion, although most of the previous work on vitamin D and HGF-mediated growth inhibition involved effects on early cell-cycle events, our results suggest that in androgen-insensitive prostate cancer cells, HGF and vitamin D can utilize distinct mechanisms to slow cell-cycle progression at loci beyond the G1/S checkpoint. Prostate neoplasia is associated with increased MET (HGF receptor) immunostaining (33). Such heightened MET expression together with the paradoxical inhibition by HGF we have observed *in vitro* might offer an opportunity to slow progression of disease that has advanced in the face of androgen deprivation. Similarly, other pleiotropic growth factors might be used to inhibit aggressive prostate cancers (which lost control of G1/S) if they gain or retain their ability to inhibit later in the cell cycle. A combination of such agents with diverse, albeit minor, cooperative inhibitory actions at these later cell-cycle loci might ultimately serve as an important adjuvant to vitamin D and other therapeutic agents in advanced prostate cancer. Notwithstanding the novel potential benefits our *in vitro* results imply, any clinical use of HGF in advanced prostate cancer will have to address problems attached to mode, duration, and cost of HGF administration (73), as well as potential adverse effects related to angiogenesis. The prospects for HGF as adjunct to vitamin D therapy should now be addressed in preclinical xenograft studies.

ACKNOWLEDGMENTS

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Table 1 *Survey of separate and combined growth-inhibitory (percentage decrease from control/vehicle only) effects of HGF and vitamin D in four prostate cancer cell lines*

Cultures were plated at an initial density of 200–400 cells/cm2 and treated for 6 days as indicated in "Materials and Methods." Values are the mean percentage decreases from control ± SD from four experiments.

	HGF (10 ng/ml)	Vitamin D (10 nM)	Combination
LNCaP	-10 ± 12	71 ± 11	74 ± 5
PC-3	-2 ± 8	8 ± 6	13 ± 9
ALVA-31	49 ± 11	46 ± 5	78 ± 8
DU 145	35 ± 13	-2 ± 10	32 ± 13

[Figure Legends]

Fig. 1. Expression of MET RNA and protein in androgen-independent but not in androgen-responsive human prostate cell lines. *A,* RNase protection analysis of human MET expression showing high levels in PC-3 (PC, *Lane 3*), DU 145 (DU, *Lane 4*), and ALVA-31 (AL, *Lane 5*) and no expression in LNCaP (LN, *Lane 2*). Sizes of the protected fragments are 261 nt (MET) and 103 nt (cyclophilin). *Lane 1* is a "no RNA" negative control. *B,* 10 μg of total protein analyzed by Western blot using a polyclonal antibody that recognizes human MET. Expression of the 190-kDa precursor and the proteolytically processed 140-kDa ß chain and the 50-kDa α chains in PC-3 (PC, *Lane 3*), DU 145 (DU, *Lane 3*), and ALVA-31 (AL, *Lane 4*), but not in LNCaP (LN, *Lane 1*). Relatively equal amounts of protein were loaded, based on Coomassie blue staining of membranes.

Fig. 2. MET activation measurement using the KIRA assay. After HGF treatment, phosphorylated tyrosine was determined in the three androgen-independent cell lines based on optical density at 450–690 nm. Treated cells were compared to controls. The results are expressed as fold increase compared to LNCaP as a negative control (because it lacks the MET receptor).

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Fig. 3. Dose-response inhibition of DU 145 with HGF treatment. *A,* Plate-growth assay of

DU 145 cells, showing fixed cells that were initially seeded at 5,000 cells/6-cm dish, treated

with HGF at 1 ng/ml, 3 ng/ml, and 10 ng/ml for six days. *B,* Corresponding dose-response

curve of three experiments, where cells were counted at the end of six days of treatment using

a hemacytometer.

Fig. 4. Distinct morphological effects of HGF and vitamin D on ALVA-31 cell cultures.

5,000 cells were seeded in 6-cm dishes; 24 h later the following test substances were added:

ethanol control (*left upper panel*), HGF at 10 ng/ml (*left lower panel*), vitamin D at 10 mM

(*right upper panel*), or a combination of HGF and vitamin D (*right lower panel*). After six

days of treatment, the cells were stained with crystal violet, and photographs were taken using

inverted-phase microscopy. Larger panels are ×40; the small insets are ×200.

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Fig. 5. Additive growth-inhibitory effects of HGF and vitamin D on ALVA-31 cells. A dose response of ALVA-31 cell proliferation was obtained for separate and combined treatment with HGF and vitamin D ($1,25(OH)_2D$). Cultures were prepared and treated as indicated for six days, as described in "Materials and Methods." *Bars* indicate the mean and SE for four to six experiments (triplicate dishes for each condition in each experiment). * = significantly different from control at $P < 0.005$; ** = $P < 0.001$ by Student's *t* test. The mean values for cells treated with HGF and vitamin D at 10 ng and 10^{-8} M or 20 ng and 10^{-7} M are significantly different from those of cells treated with vitamin D or HGF alone, by ANOVA at $P < 0.005$. (Controls averaged $2.3 \pm 0.4 \times 10^5$ cells/dish.)

Fig. 6. Cell-cycle phase distribution changes during treatment of ALVA-31 cells with the combination of vitamin D ($1,25(OH)_2D$) and HGF. Cultures were prepared and treated with vehicle (ethanol), HGF (10 ng/ml), $1,25(OH)_2D$ (10 nM), or their combination, as described in "Materials and Methods." After six days of treatment, cells were harvested and fixed in ethanol for subsequent staining with propidium iodide and cell-cycle analyses as described in "Materials and Methods." *A*, Representative FACS profiles for control and combination-treated cells. *B*, For each condition, the cell-cycle distribution between G1 and later cell-cycle phases (S, G2, and M combined) in four separate experiments. *Bars* indicate the mean and SE; * = significantly different from control at $P < 0.05$.

A.

261 ← MET

103 ← cyclophilin

- LN PC DU AL

1   2   3   4   5

B.

LN PC DU AL

←190
←140   MET

←50

1   2   3   4

Figure 1
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Figure 2
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Figure 3

Qadan et al.

Control   Vitamin D

ALVA 31

HGF   HGF + Vitamin D

Figure 4
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Figure 5
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Figure 6a
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Figure 6b
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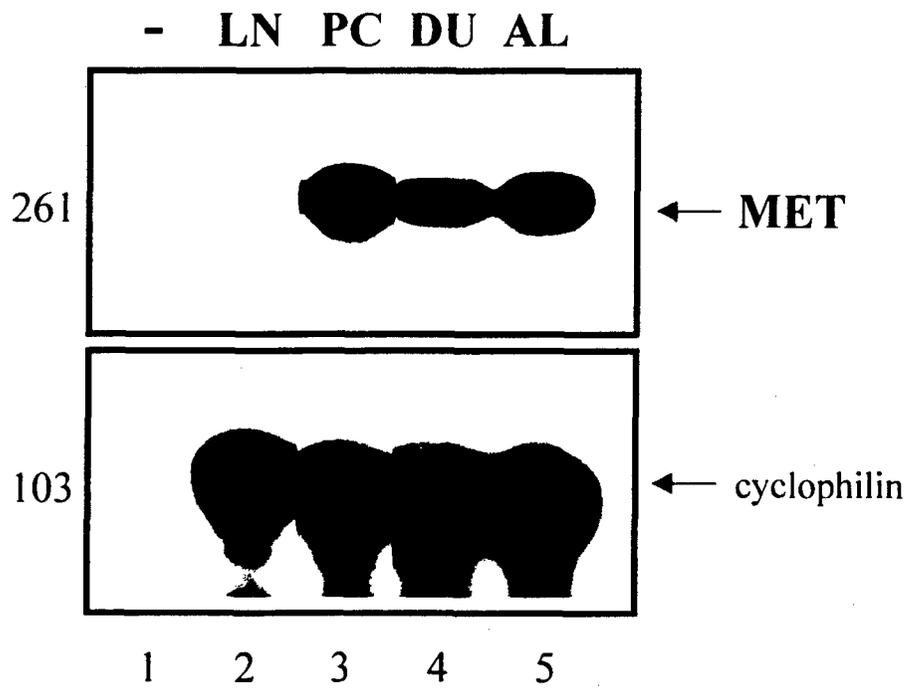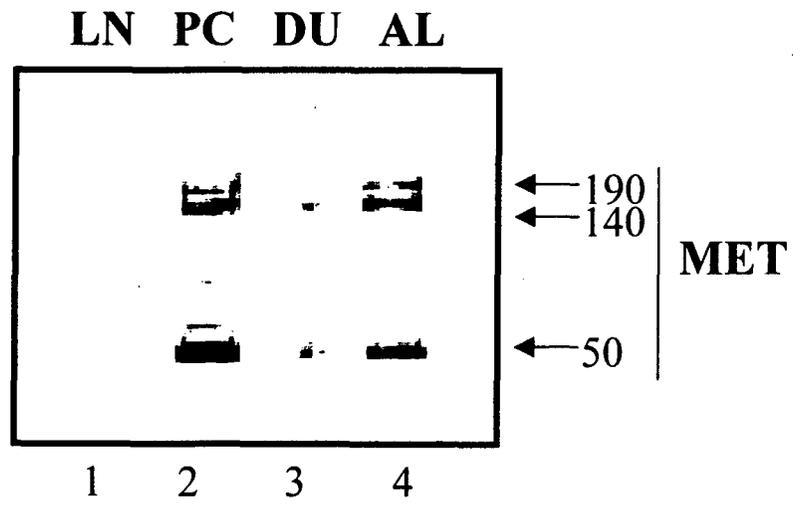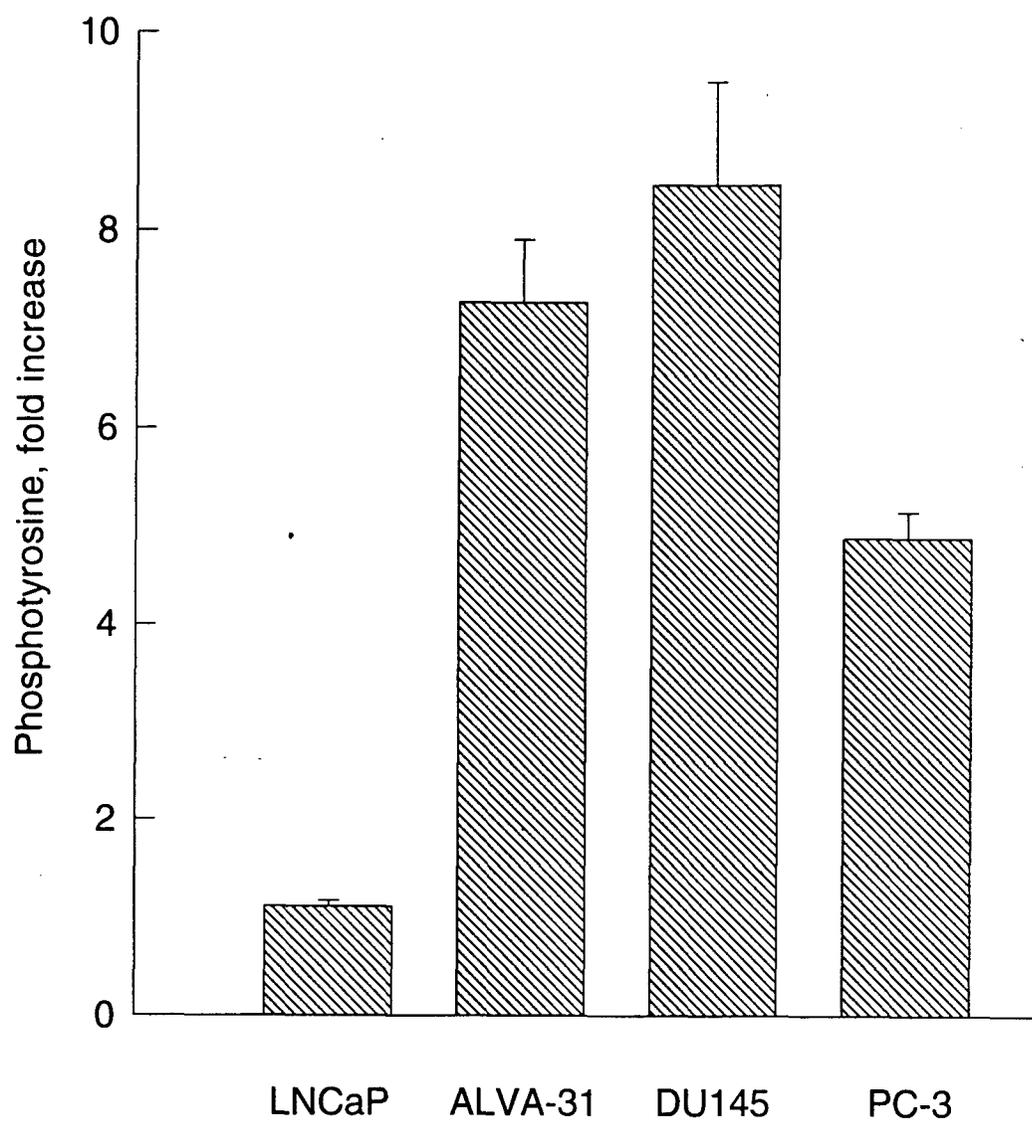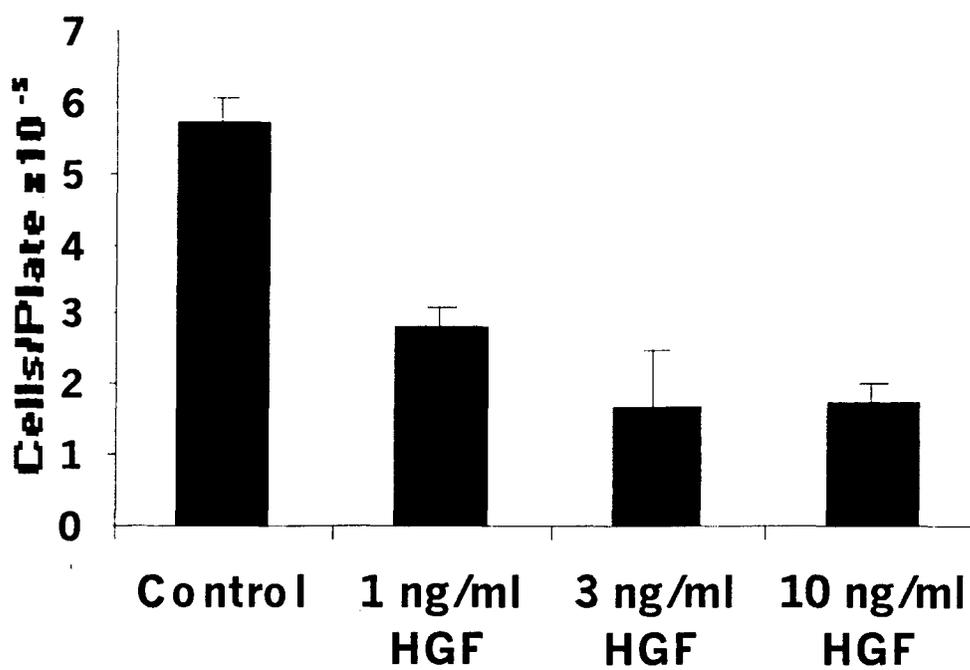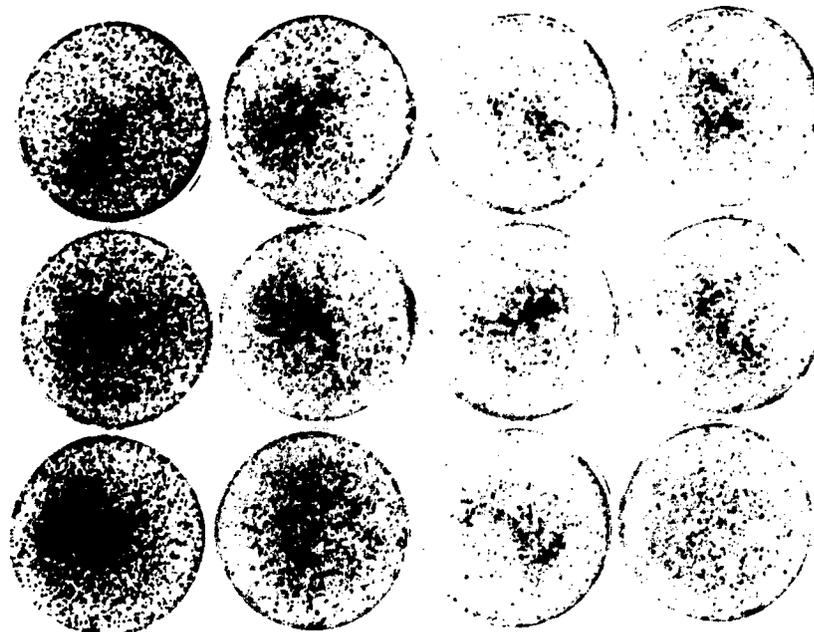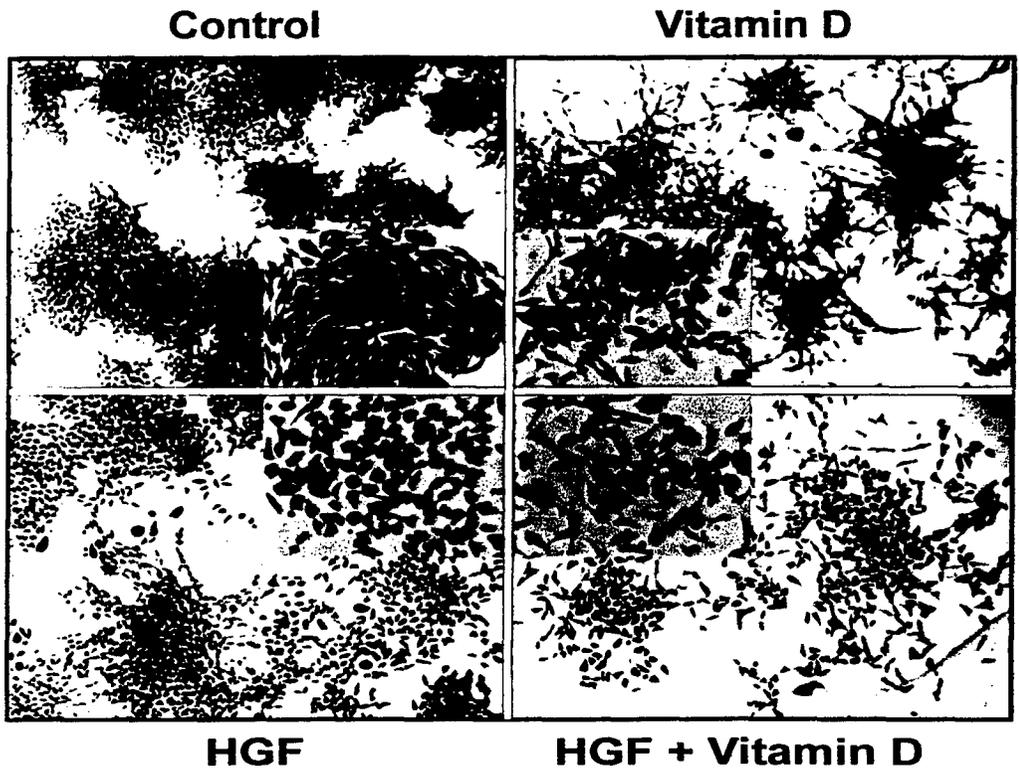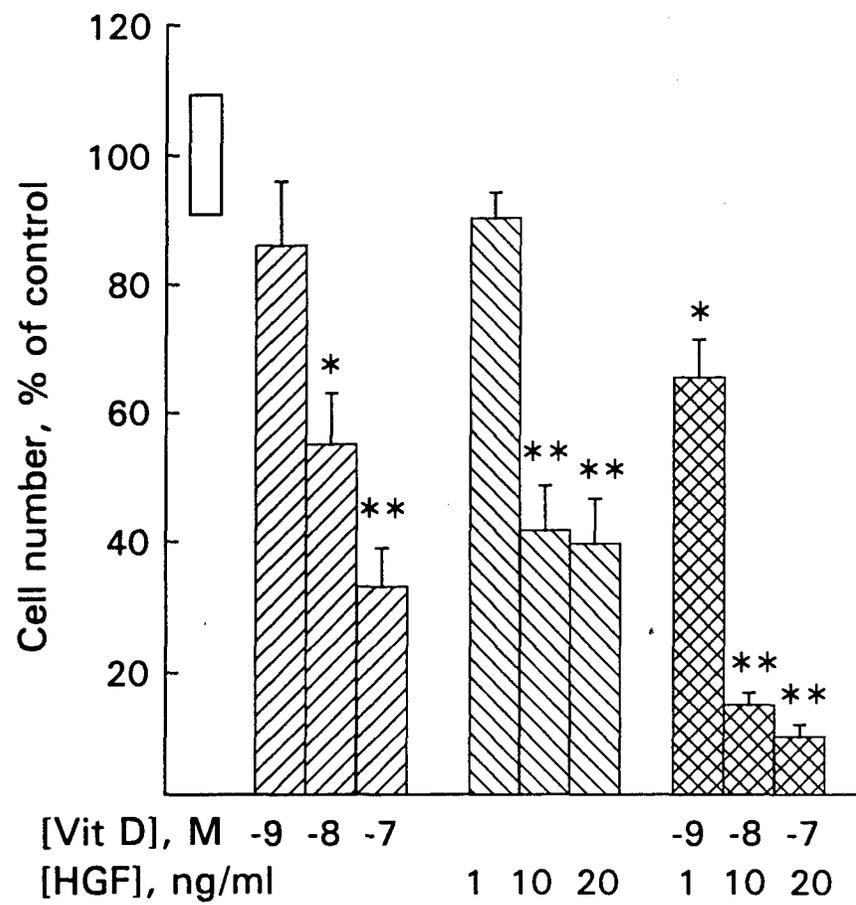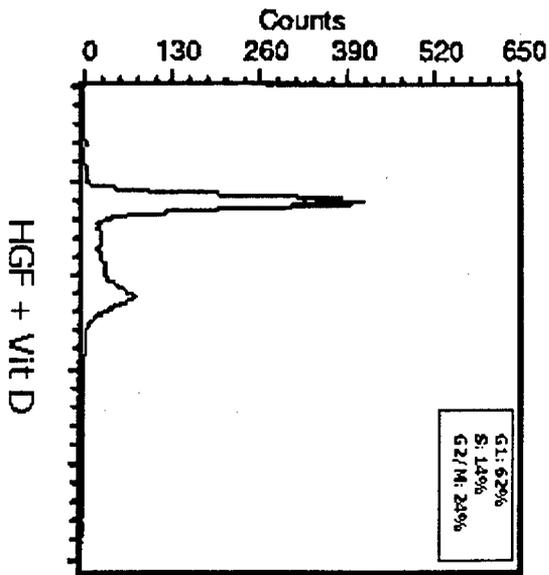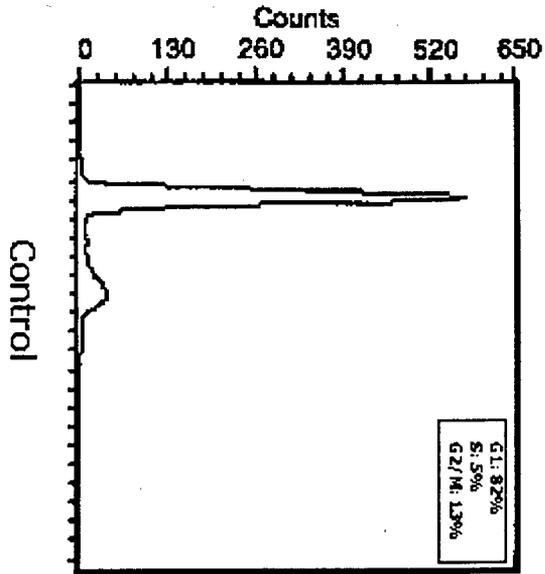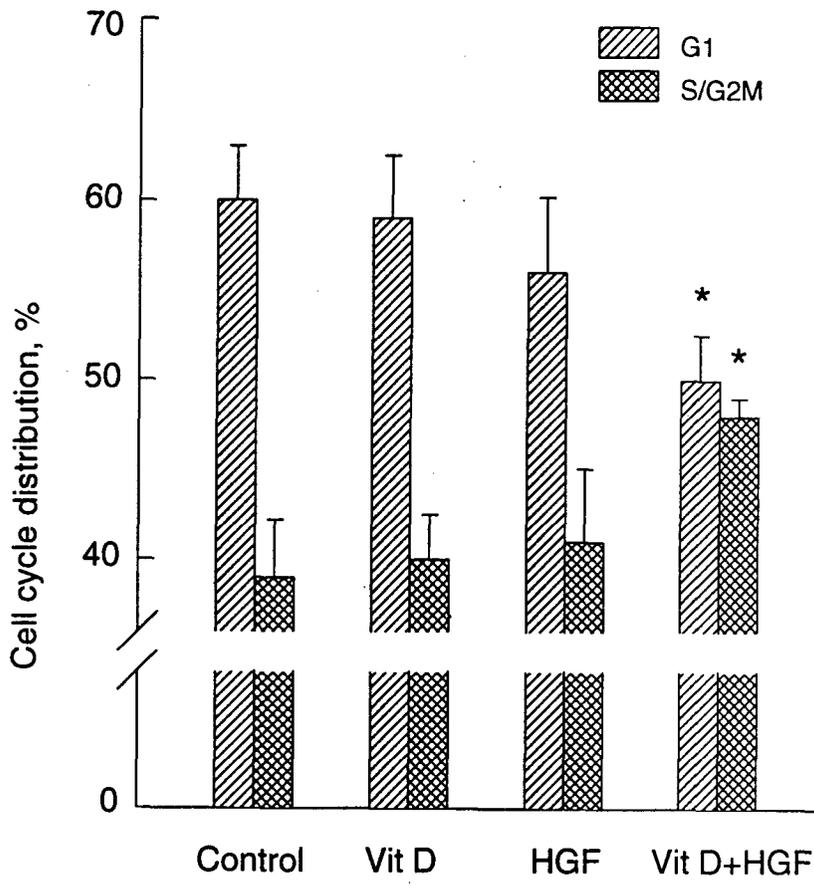
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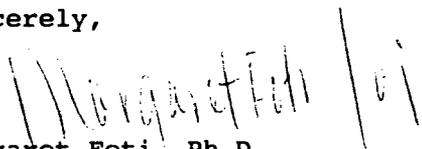
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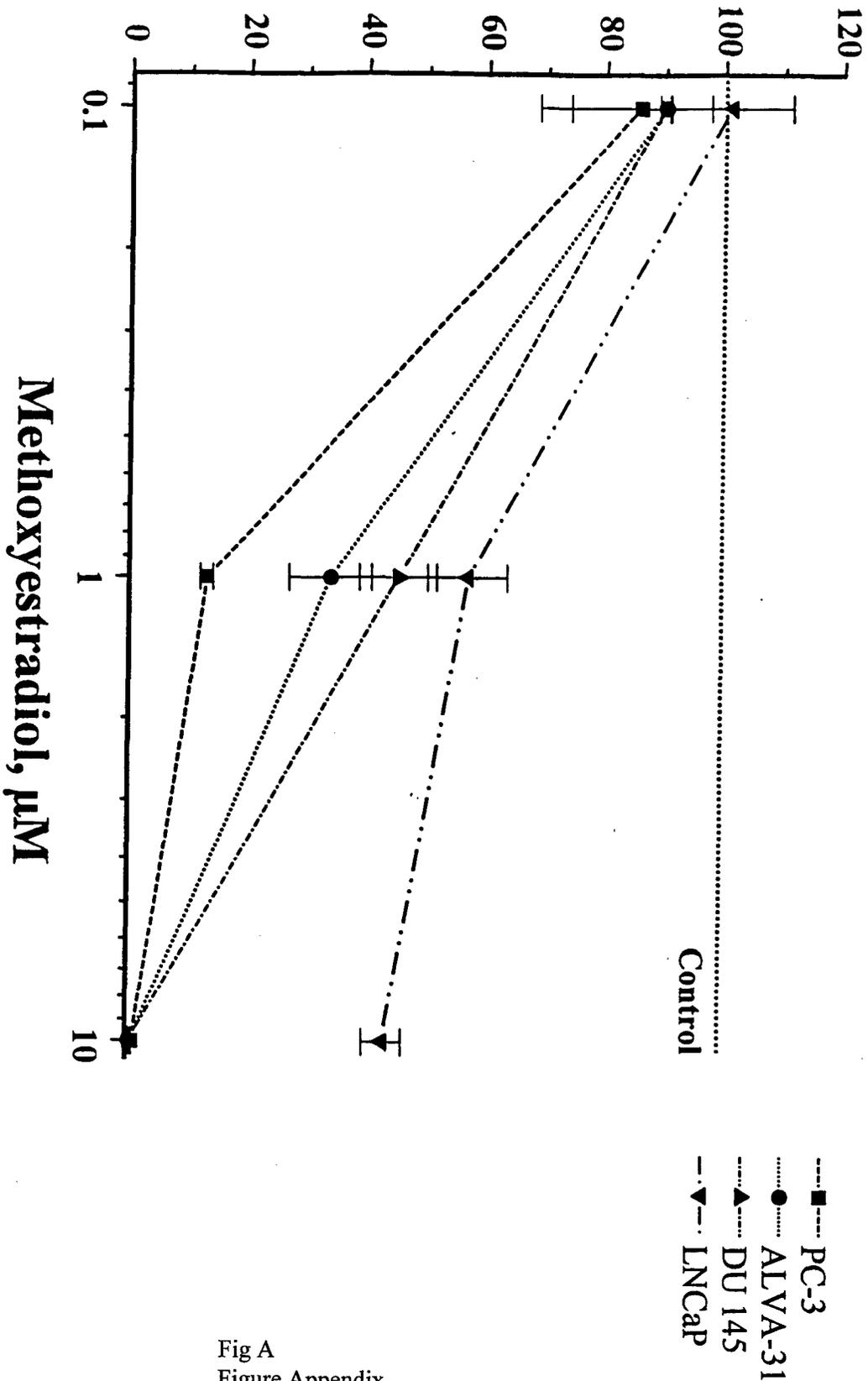
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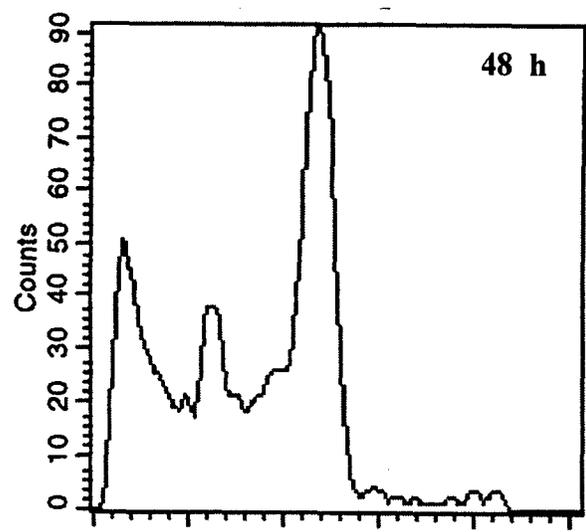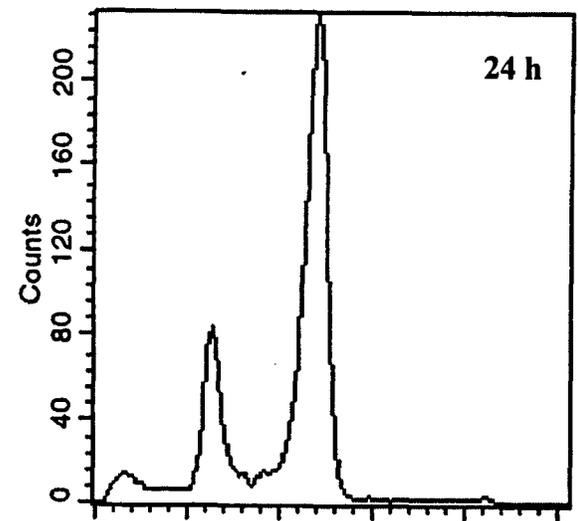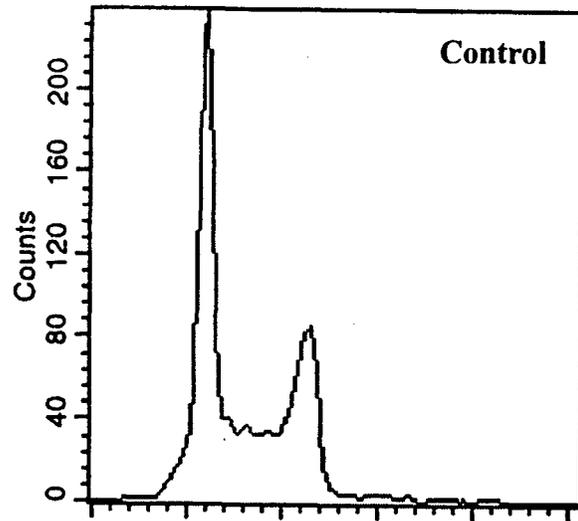
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Fig B
Figure Appendix
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MCMR-RMI-S (70-1y)   8 Jan 2003

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