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Vitamin D Receptor and p21/WAF1 Are Targets of Genistein and 1,25-Dihydroxyvitamin D_3 in Human Prostate Cancer Cells

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ABSTRACT

We investigated mechanisms by which genistein and 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] act synergistically to inhibit the growth of the human prostate cancer cell line LNCaP. We demonstrate that 1,25$(OH)_2D_3$ and genistein cooperate to up-regulate the vitamin D receptor protein by increasing the stability of the vitamin D receptor. Genistein and 1,25$(OH)_2D_3$ also cooperate to up-regulate the levels of p21/WAF1 (p21). Small interfering RNA-mediated knockdown of p21 expression showed that p21 is essential for significant growth inhibition of LNCaP cells in response to either compound or their combination. We conclude that one mechanism of synergism between genistein and 1,25$(OH)_2D_3$ is through genistein modulation of vitamin D signaling.

INTRODUCTION

Epidemiological, *in vitro*, and *in vivo* studies have suggested that the soy and vitamin D-rich Asian diet plays an important role in preventing the progression of latent prostate cancer into the clinically relevant disease (1–3). Genistein is an isoflavone found in high quantities in serum after ingestion of a soy-rich meal and has been demonstrated to have potent inhibitory actions on prostate and breast tumor growth in mice (4). 1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the active metabolite of vitamin D_3, is primarily obtained from endogenous synthesis and from dietary sources such as ocean fish oils (5). The growth-inhibitory actions of 1,25$(OH)_2D_3$ are mediated by the nuclear vitamin D receptor [VDR (6, 7)]. However, the exact molecular signaling pathways by which genistein or 1,25$(OH)_2D_3$ exert their biological effects on growth have not been clearly elucidated. Both compounds are known to cause inhibition of breast and prostate cancer cell growth by inducing apoptosis and by inhibition of cell cycle progression. The cyclin-dependent kinase inhibitors p21 and p27 have been implicated in cell cycle-regulatory effects of both 1,25$(OH)_2D_3$ and genistein (8–11). The presence of a functional vitamin D response element in the p21 promoter suggests that p21 is a direct target of VDR transcriptional regulation (12). Furthermore, we demonstrated previously that genistein and 1,25$(OH)_2D_3$, when combined, cause synergistic growth inhibition of prostate cancer cells through cell cycle arrest (13).

Here we investigated the possible mechanisms by which 1,25$(OH)_2D_3$ and genistein may interact to cause synergistic growth inhibition of LNCaP cells. We found that 1,25$(OH)_2D_3$ and genistein cooperate to up-regulate VDR and p21 protein levels. Experiments with cycloheximide demonstrate that genistein increases the stability of VDR. Additionally, by silencing expression of p21 using small interfering RNA (siRNA), we demonstrate that the presence of p21 is essential for the growth-inhibitory effects of 1,25$(OH)_2D_3$ and genistein, both alone and in combination.

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MATERIALS AND METHODS

Tissue Culture, Media and Supplements. LNCaP human prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum. Source and storage of 1,25$(OH)_2D_3$ and genistein (4,5,7-trihydroxyisoflavone) were as reported previously (13). Cycloheximide was from Sigma (St. Louis, MO). Stocks were prepared in 100% ethanol and stored at −20°C.

Preparation of Cell Lysates and Western Blot Analyses. LNCaP cells were plated on 10-cm tissue culture plates at 6×10^5 cells/plate. Cells were incubated for 48–60 h and then treated with experimental media containing control (0.1% ethanol and 0.1% DMSO), 10 nM 1,25$(OH)_2D_3$, 5 μM genistein, or the combination of 10 nM 1,25$(OH)_2D_3$ and 5 μM genistein. At the indicated times, cells were harvested, and cell lysates were prepared. Immunoblots with 50 μg total protein/lane were performed using previously reported protocols (14). Protein signal was detected using the enhanced chemiluminescence detection kit (Amersham-Pharmacia, Buckinghamshire, England). Signals for p21 (PharMingen, San Diego, CA), p27 (BD Transduction Laboratories, Lexington, KY), and VDR (9A7; Neomarkers, Fremont, CA), were normalized to β-actin (Sigma).

To determine the stability of VDR protein, LNCaP cells were treated as described above, with the addition of 10 μM cycloheximide to every treatment. Cell lysates were prepared at the indicated time points, and Western blot analysis for VDR was performed as described above.

p21 Silencing Using siRNA. LNCaP cells were plated at 2.5×10^5 cells/60-mm plate. Twenty-four h after plating, cells were transfected with either control siRNA (against luciferase) or p21 siRNA (Dharmacon Research, Lafayette, CO). The sequence of the luciferase siRNA used as control was 5′-CGU-ACG-CGG-AAU-ACU-UCG-A-dTdT and dTdT-GCA-UGC-GCC-UUA-UGA-AGC-U-5′. The sequence of the siRNA used to "knock-down" p21 expression was 5′-AA-UGG-CGG-GCU-GCA-UCC-AGG-A-dTdT and dTdT-UU-ACC-GCC-CGA-CGU-AGG-UCC-U-5′.

Transfection of siRNA was carried out according to manufacturer's instructions (Gibco, Grand Island, NY) after optimization of the amount of siRNA to maximize p21 suppression. Briefly, either siRNA (1.92 μM) was mixed with OPTI-MEM medium (Gibco). In a separate tube, 1.68 nM Oligofectamine (Gibco) was mixed with OPTI-MEM and incubated at room temperature for 10 min. The siRNA- and Oligofectamine-containing tubes were mixed and incubated for 25 min at room temperature. Plates were then rinsed with OPTI-MEM once, and 1 ml of OPTI-MEM was added to plates. One ml of this Oligofectamine-siRNA mix was added drop-wise to cells. After 4 h, the Oligofectamine-siRNA mix was replaced with RPMI 1640/10% fetal bovine serum. Thirty-six h later, cells were treated with vehicle control (0.1% ethanol and 0.1% DMSO), 10 μM genistein, 100 nM 1,25$(OH)_2D_3$, or the combination of the two agents. Cells were incubated in experimental media for 72 h with a media change after 48 h. At the end of incubation, the number of viable cells was determined by trypan blue exclusion. Each treatment was carried out in triplicate. Differences between experimental treatment groups were detected by ANOVA with *post-hoc* analysis by Fisher's test. *P*s of <0.05 were considered significant.

RESULTS

Up-Regulation of VDR in LNCaP Cells by 1,25$(OH)_2D_3$ and Genistein. Using flow cytometry, we have shown previously that 1,25$(OH)_2D_3$ and genistein synergistically inhibit prostatic cell growth via cell cycle arrest (13). Further exploration of these cell cycle effects suggested that genistein increased the block at G_0-G_1 induced by 1,25$(OH)_2D_3$ (data not shown). Because the action of

$1,25(OH)_2D_3$ is mediated by the VDR, we carried out immunoblot analyses to examine the levels of VDR in LNCaP cells treated with 10 nM $1,25(OH)_2D_3$ and 5 μM genistein, alone or in combination. Fig. 1*A* is a representative immunoblot for VDR expression, and Fig. 1*B* is the quantitative data from two independent experiments. As seen in Fig. 1, *A* and *B*, LNCaP cells treated with 10 nM $1,25(OH)_2D_3$ alone exhibited a modest (25–50%) up-regulation of VDR levels within 6 h that persisted for at least 48 h. At 48 h, there was a 1.5-fold increase in VDR expression in $1,25(OH)_2D_3$-treated cells compared with control. Treatment with 5 μM genistein had no effect. However, when cells were treated with the combination of $1,25(OH)_2D_3$ and genistein, there was a more robust up-regulation of VDR within 4 h that was sustained throughout the entire time course, suggesting a potentiation by genistein on $1,25(OH)_2D_3$ regulation of VDR levels. At 4 h, there

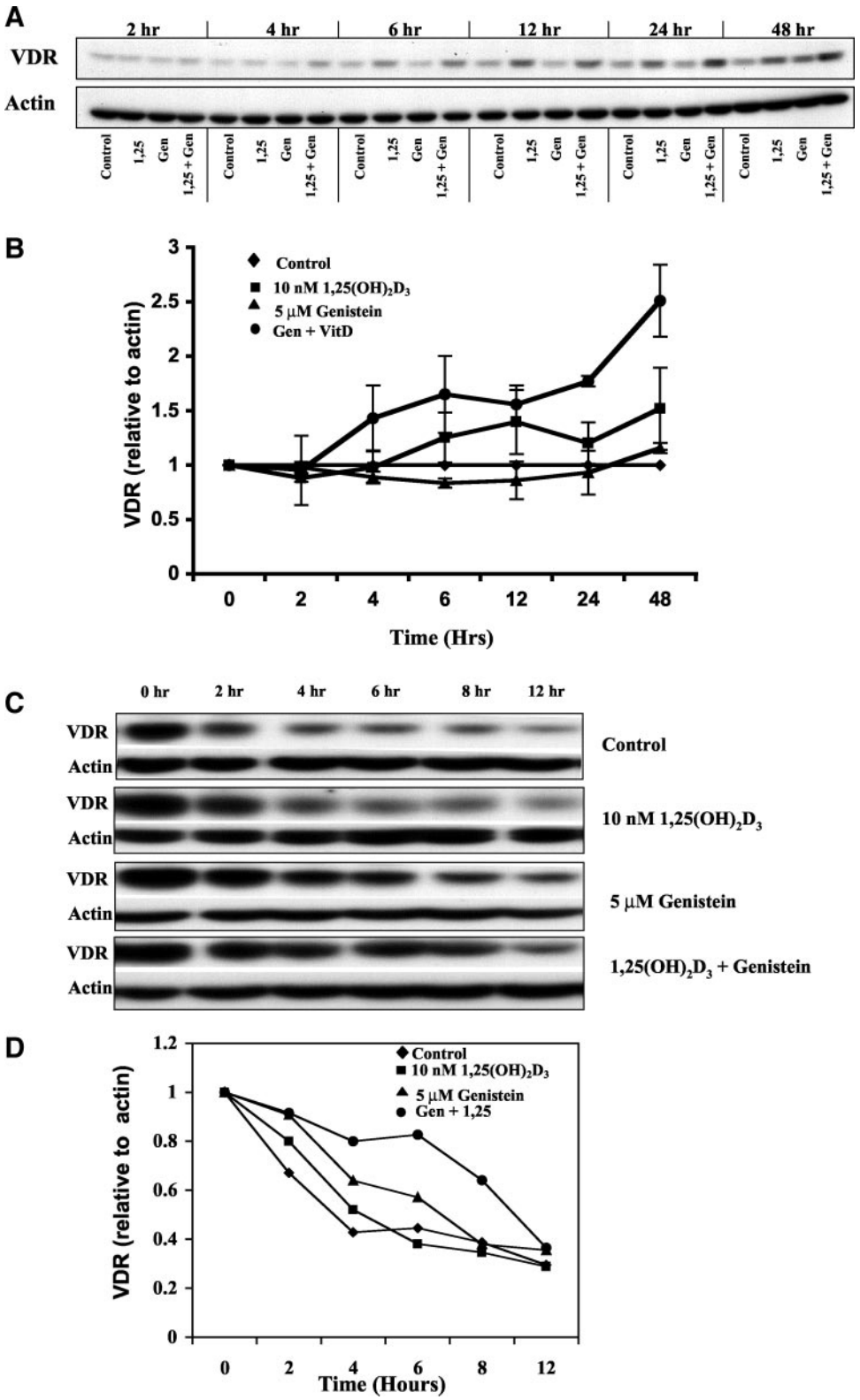

Fig. 1. Up-regulation of vitamin D receptor (VDR) in LNCaP cells by 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and genistein from 2 to 48 h. *A*, representative immunoblot for VDR expression. LNCaP cells were treated with vehicle control, 10 nM $1,25(OH)_2D_3$, 5 μM genistein, or the combination of both agents. Cells were harvested at 2, 4, 6, 12, 24, and 48 h, and cell lysates were prepared as described in "Materials and Methods." Proteins were separated by electrophoresis on a 10% gel, and the blot was probed for VDR and actin. *B*, induction of VDR in LNCaP cells after treatment with vehicle control, 10 nM $1,25(OH)_2D_3$, 5 μM genistein, or the combination of both agents, normalized to actin. Data are the means ± SE of two independent experiments. *C*, LNCaP cells were treated with vehicle control, 10 nM $1,25(OH)_2D_3$, 5 μM genistein, or the combination of both agents in the presence of 10 μM cycloheximide, and blots were probed for VDR and actin. *D*, half-life of VDR protein in LNCaP cells treated with vehicle control, 10 nM $1,25(OH)_2D_3$, 5 μM genistein, or the combination of both agents with 10 μM cycloheximide. VDR levels were normalized to actin.

was a 1.4-fold up-regulation, whereas at 48 h, the up-regulation was 2.5-fold over control. This potentiation effect of genistein on $1,25(OH)_2D_3$ regulation of VDR levels continued for 120 h after initial treatment, at which time there was a 2.2-fold increase over control levels (data not shown).

Real-time PCR showed that there was no effect of $1,25(OH)_2D_3$ or genistein treatments, either alone or in combination, on VDR mRNA (data not shown). To determine whether genistein could alter the stability of the VDR, we carried out experiments with cycloheximide. As seen in Fig. 1, *C* and *D*, treatment with 10 nM $1,25(OH)_2D_3$ alone increased VDR $T_{1/2}$ (half life) by 1 h [3 h for control *versus* 4 h for $1,25(OH)_2D_3$-treated cells], 5 μM genistein alone increased $T_{1/2}$ by about 3.5 h when compared with control ($T_{1/2}$ of about 6 h and 30 min), whereas the combination of the two increased $T_{1/2}$ by 6.5 h ($T_{1/2}$ is approximately 10 h). Two additional repeats of this experiment showed similar results (data not shown).

Induction of p21 in LNCaP Cells by $1,25(OH)_2D_3$ and Genistein. The cyclin-dependent kinase inhibitors p21 and p27 have both been implicated as downstream mediators of the growth-inhibitory actions of $1,25(OH)_2D_3$, and there is a functional vitamin D response element in the p21 promoter. Therefore, we examined the ability of $1,25(OH)_2D_3$ and genistein to induce p21 and p27 in LNCaP cells. As seen in Fig. 2, *A* and *B*, 10 nM $1,25(OH)_2D_3$ up-regulated p21 expression at 12 and 24 h after initial treatment (1.5- and 1.3-fold up-regulation over control levels, respectively). Five μM genistein alone up-regulated p21 levels at 6 and 12 h after treatment (1.3- and 2.3-fold increase over control levels, respectively). By 24 h, the levels of p21 in the genistein-treated samples were equivalent to that of control. When LNCaP cells were treated with a combination of 10 nM $1,25(OH)_2D_3$ and 5 μM genistein, there was a robust increase in the up-regulation of p21 levels at 4 and 6 h after initial treatment (1.4- and 2.9-fold increase over control levels, respectively). Subsequent levels were either equivalent or intermediate to either treatment alone. There was no effect of any treatment on p27 expression (data not shown).

p21 Is Essential for the Action of $1,25(OH)_2D_3$ and Genistein in LNCaP Cells. We suppressed p21 expression using siRNA to determine whether p21 was necessary for the growth-inhibitory actions of $1,25(OH)_2D_3$ and genistein, both alone and in combination. The ability of the siRNA construct to specifically decrease p21 expression was demonstrated by comparing p21 *versus* p27 levels over a 5-day time period. Fig. 3*A* shows that p21 levels were suppressed 60–70% within 24 h after transfection and remained suppressed over the 5-day period. In contrast, cells treated with the p21 siRNA exhibited an elevation of p27 levels 24 h after transfection but were unchanged compared with control cells at other time points.

We next tested the growth responsiveness of cultures treated with p21 siRNA. For these experiments, we used higher individual and combined doses of $1,25(OH)_2D_3$ and genistein than the doses used for the cooperative effects on VDR and p21 presented in Figs. 1 and 2. This was by design to increase the growth-inhibitory effects on the cultures treated with either agent alone so that we could make statements about the individual effects of these agents. Cultures transfected with control siRNA and treated with 10 μM genistein, 100 nM $1,25(OH)_2D_3$, or the combination for 72 h exhibited growth reductions of 37%, 33%, and 44%, respectively, which were statistically significantly different from cells treated with vehicle (ANOVA, $P < 0.05$). These are similar to results reported previously by us (13). In contrast, there was no significant growth inhibition in response to 100 nM $1,25(OH)_2D_3$, 10 μM genistein, or the combination in cells transfected with p21 siRNA. Although there was a trend toward growth suppression in the combination treatment in the presence of the p21 siRNA, this difference was not statistically significantly different from control (ANOVA, $P > 0.05$).

DISCUSSION

We have previously demonstrated synergistic growth inhibition between $1,25(OH)_2D_3$ and genistein (13). In this study we examined potential mechanisms of this synergy between $1,25(OH)_2D_3$ and genistein, using LNCaP cells as a model system. The major findings from these studies are that $1,25(OH)_2D_3$ and genistein cooperatively increase expression of the VDR and the cell cycle inhibitor p21 and that p21 is required for the growth-inhibitory actions of both $1,25(OH)_2D_3$ and genistein.

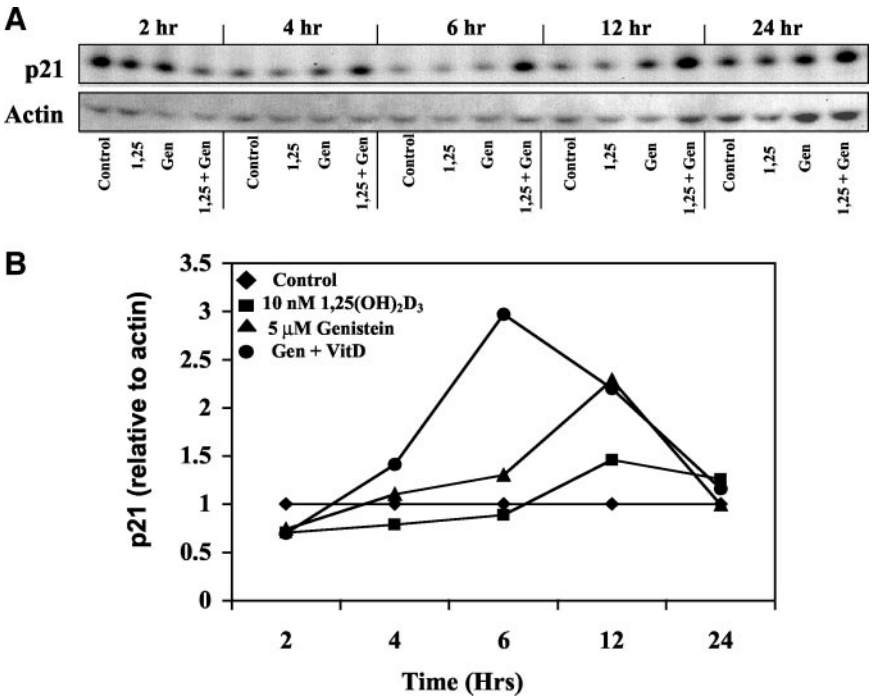

Fig. 2. Induction of p21 in LNCaP cells by 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and genistein. *A*, LNCaP cells were treated with vehicle control, 10 nM $1,25(OH)_2D_3$, 5 μM genistein, or the combination of both agents for the indicated times, and cell lysates were prepared as described in "Materials and Methods." The blot was probed for p21 and actin. *B*, induction of p21 in LNCaP cells on treatment with vehicle control, 10 nM $1,25(OH)_2D_3$, 5 μM genistein, or the combination of both agents, normalized to actin.

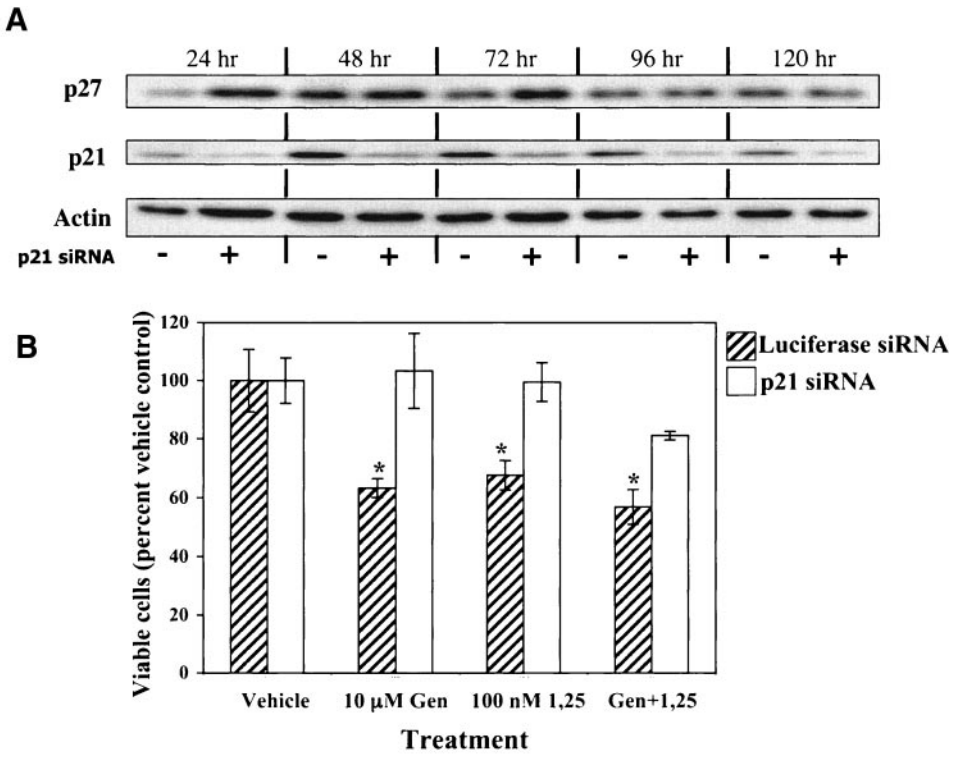

Fig. 3. p21 is essential for the growth-inhibitory actions of genistein, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], and the combination in LNCaP cells *A*, p21 small interfering RNA (siRNA) decreases p21 expression in LNCaP cells. LNCaP cells were mock transfected or transfected with p21 siRNA as described in "Materials and Methods." At the indicated time points, total protein was harvested, and blots were prepared and probed for p21, p27, and actin. +, cells transfected with p21 siRNA, −; mock-transfected cells. *B*, LNCaP cells were transfected with control siRNA against luciferase (▨) or p21 siRNA (□) as described in "Materials and Methods." Cells were then treated with vehicle control, 10 μM genistein, 100 nM $1,25(OH)_2D_3$, or the combination. The number of viable cells was assessed after 72 h by trypan blue exclusion. Values significantly less then vehicle control are indicated by an *asterisk*. $P < 0.05$ was considered significant.

These findings provide new information regarding genistein regulation of the VDR, which is known to be required for the growth-inhibitory effects of $1,25(OH)_2D_3$ (7). Both *in vivo* and *in vitro* studies support the concept that increases in VDR as modest as 2-fold can enhance cellular sensitivity to $1,25(OH)_2D_3$ (15–17). In a previous study with MCF-7 cells, Jensen *et al.* (18) examined VDR induction in two clones that were hypersensitive and two clones that were more resistant to the growth-inhibitory actions of $1,25(OH)_2D_3$. VDR was increased by $1,25(OH)_2D_3$ treatment only in hypersensitive clones, suggesting that VDR induction correlates with sensitivity to $1,25(OH)_2D_3$ (18). Therefore, it is possible that the cooperative increase in VDR protein caused by genistein and $1,25(OH)_2D_3$ makes LNCaP cells more sensitive to $1,25(OH)_2D_3$-mediated growth inhibition.

In contrast to studies in breast cancer cells (17), we found no evidence that the up-regulation of VDR protein in $1,25(OH)_2D_3$- or genistein-treated LNCaP cells was the result of enhanced transcription of the VDR gene. Instead, our data indicate that genistein increased VDR stability and that VDR stability was highest in cells treated with the combination of $1,25(OH)_2D_3$ and genistein. Additional studies are necessary to define the mechanism by which genistein modulates VDR turnover. However, our observations supporting a role for genistein in enhancing VDR signaling are consistent with work of Farhan *et al.* (19). They reported inhibition of CYP24, the vitamin D catabolic enzyme, in genistein-treated prostate cancer cells. Thus, genistein appears to enhance vitamin D signaling in cancer cells through multiple mechanisms.

The cyclin-dependent kinase inhibitors p21 and p27 play an important role in progression through the cell cycle by inhibiting cyclin-cyclin-dependent kinase activity and causing G_0-G_1 arrest (20, 21). We show here that p21, not p27, is up-regulated in LNCaP by either $1,25(OH)_2D_3$ or genistein. This is consistent with reports from other groups (10). However, these are the first studies to show a cooperative up-regulation of p21 by the combination of $1,25(OH)_2D_3$ and genistein. It is probable that a modest induction of p21 at low levels of either $1,25(OH)_2D_3$ or genistein may cause a small percentage of cells to arrest in G_0-G_1. However, when $1,25(OH)_2D_3$ and genistein are used together, the higher levels of p21 may cause a larger proportion of cells to undergo G_0-G_1 arrest. Previous flow cytometry data from our laboratory support this idea (13). Here, the cooperative increase in p21 was only observed at 4 and 6 h; however, the total area under the curve was greatly enhanced in the treatment with both agents combined. Because even a short 2-h exposure to $1,25(OH)_2D_3$ results in irreversible growth inhibition of prostatic cells (22), we hypothesize that the early, transient, but cooperative increase in p21 causes a greater proportion of cells to undergo a G_0-G_1 arrest when treated with the combination of $1,25(OH)_2D_3$ and genistein.

In this study, we demonstrate that the presence of p21 is essential for the growth-inhibitory actions of $1,25(OH)_2D_3$ and genistein, both alone and in combination. The importance of p21 for $1,25(OH)_2D_3$ action has been demonstrated previously using ALVA-31 human prostate cancer cells. In those studies, inhibiting p21 expression by stable transfection with antisense p21 cDNA resulted in significantly less growth inhibition on treatment with $1,25(OH)_2D_3$ when compared with cells transfected with a control vector (11). Although previous studies have demonstrated that genistein increases p21, ours are the first data to demonstrate the requirement for p21 in genistein-mediated growth inhibition of any cell type. Interestingly, the lack of significant growth inhibition by the combination of $1,25(OH)_2D_3$ and genistein in the absence of p21 demonstrates the central role of p21 in growth inhibition. However, treatment of p21 siRNA-transfected LNCaP cells with the combination did cause a trend (19%) toward growth inhibition compared with control. Whereas this was not statistically significant, the growth inhibition was far greater then that observed in cells treated with 10 μM genistein or 100 nM $1,25(OH)_2D_3$ alone. Because siRNA technology causes a "knock-down" but not a complete lack of p21 expression, it is possible that residual p21 expression was sufficient to cause the growth inhibition when the two compounds are used together. For instance, if the combination of $1,25(OH)_2D_3$ and genistein has effects on p21 protein stability, then a

small amount of residual p21 mRNA expression may translate into a large increase in p21 protein in the presence of both agents. Alternatively, treatment with both genistein and $1,25(OH)_2D_3$ may activate both p21-dependent and p21-independent pathways to inhibit growth, resulting in the synergistic growth inhibition observed.

In summary, we have identified the VDR signaling pathway through p21 as a potential target of genistein that contributes to synergistic growth inhibition with $1,25(OH)_2D_3$.

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