Genistein potentiates the growth inhibitory effects of 1,25-dihydroxyvitamin D₃ in DU145 human prostate cancer cells: Role of the direct inhibition of CYP24 enzyme activity

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Abstract

In a search for improved therapies for prostate cancer, we investigated the effect of genistein in combination with 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], on the growth of DU145 human prostate cancer cells. DU145 cells were very resistant to the growth inhibitory action of 1,25(OH)₂D₃ or genistein when administered individually. However, the combination caused a significant growth inhibition seen at lower concentrations of both agents. 1,25(OH)₂D₃ induces the expression of the CYP24 gene, which codes for the enzyme that initiates the catabolism of 1,25(OH)₂D₃. We showed for the first time that genistein at low doses (50–100 nM) directly inhibited CYP24 at the enzyme level. Addition of genistein to mitochondrial preparations inhibited CYP24 enzyme activity in a noncompetitive manner. CYP24 inhibition by genistein increased the half-life of 1,25(OH)₂D₃ thereby augmenting the homologous up-regulation of the vitamin D receptor (VDR) both at the mRNA and protein levels. Genistein co-treatment enhanced 1,25(OH)₂D₃-mediated transactivation of the vitamin D responsive reporters OC-Luc and OP-Luc transfected into DU145 cells. Consistent with the growth inhibition due to the combination treatment, significant changes in the expression of genes involved in growth arrest and apoptosis were seen. We conclude that genistein potentiates the antiproliferative actions of 1,25(OH)₂D₃ in DU145 cells by two mechanisms: (i) an increase in the half-life of 1,25(OH)₂D₃ due to the direct inhibition of CYP24 enzyme activity and (ii) an amplification of the homologous up-regulation of VDR. Together these two effects lead to a substantial enhancement of the cellular responses to the growth inhibitory and pro-apoptotic signaling by 1,25(OH)₂D₃.

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1. Introduction

Prostate cancer (PCa) is the most common cancer next to skin cancer and the second leading cause of cancer death among men in the US (Hellerstedt and Pienta, 2002). Initially it is characterized by androgen-dependent growth and androgen deprivation therapy (ADT) remains the most important therapy for advanced PCa (Feldman and Feldman, 2001; Hellerstedt and Pienta, 2002; Nelson et al., 2003; Santos et al., 2004). However, as the disease progresses, ADT eventually fails leading to the development of androgen-independent prostate cancer (AIPC) (Feldman and Feldman, 2001). One of the goals of current research has been the identification of new strategies for the prevention and/or treatment of PCa including AIPC. 1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃], the active form of vitamin D, is the major regulator of bone and calcium homeostasis in the body. The two key enzymes involved in the metabolism of 1,25(OH)₂D₃ are 25-hydroxyvitamin D₃-1α-hydroxylase (CYP27B1) which is the rate limiting enzyme in the synthesis of 1,25(OH)₂D₃, and the 25-hydroxyvitamin D₃-24-hydroxylase (CYP24) which initiates the catabolism of 1,25(OH)₂D₃ converting it to less active metabolites (Feldman et al., 2001). In addition to its role in calcium homeostasis, 1,25(OH)₂D₃ has also been shown to be a potent inhibitor of a wide variety of cancers (van Leeuwen and Pols, 2005). Extensive laboratory and clinical studies have demonstrated that 1,25(OH)₂D₃ exerts antipro-
liferative effects in PCa (Gross et al., 1997; Blutt and Weigel, 1999; Beer et al., 2003; Krishnan et al., 2003; Pechtl et al., 2003; Trump et al., 2004). However, the sensitivity of various prostate cancer cells to 1,25(OH)₂D₃ is varied (Miller et al., 1995). While the LNCaP human PCa cells are very sensitive to the growth inhibitory actions of 1,25(OH)₂D₃, the DU145 human PCa cells are very resistant to the effects of 1,25(OH)₂D₃ (Skowronski et al., 1993; Miller et al., 1995). The growth inhibitory effects of 1,25(OH)₂D₃ in PCa cells appear to correlate inversely to the inducible expression of CYP24 in these cells (Miller et al., 1995). The high levels of basal and 1,25(OH)₂D₃-inducible CYP24 activity in DU145 cells are thought to be responsible for this resistance (Skowronski et al., 1993; Miller et al., 1995). Previous studies from our laboratory have shown that DU145 cells and primary prostate cancer cells were inhibited by 1,25(OH)₂D₃ when used in combination with the potent P-450 enzyme inhibitors liarozole and ketoconazole (Ly et al., 1999; Pechtl et al., 2002). These imidazole compounds inhibit CYP24 enzyme activity thereby increasing the bio-availability of 1,25(OH)₂D₃. Thus combination therapy with a CYP24 inhibitor is a means of increasing the sensitivity of cancer cells to the actions of 1,25(OH)₂D₃.

Traditional Asian diets rich in soy products may have cancer-protective effects (Adlercreutz et al., 1995). Genistein, an isoflavone with some structural resemblance to estradiol, is a major component of the soy diet (Severson et al., 1989; Adlercreutz et al., 1995; Coward et al., 1996). Genistein inhibits the growth of several types of cancers including prostate, breast, colon, leukemia, lymphoma, melanoma and lung cancer (Messina et al., 1994; Spinozzi et al., 1994; Barnes et al., 1995; Constantinou and Huberman, 1995). In vivo studies suggest that genistein may be useful in the treatment (Axelson et al., 1999) as well as chemoprevention (Mentor-Marcel et al., 2001; Wang et al., 2002) of PCa.

Several in vitro studies have shown that genistein inhibits the growth of PCa cells in culture (Davis et al., 1998; Knowles et al., 2000; Shen et al., 2000; Ihabia and Agarwal, 2001; Rao et al., 2002, 2004; Xiang et al., 2002; Hedlund et al., 2003). However, the sensitivity of PCa cells to genistein has been shown to vary considerably. While LNCaP cells were more sensitive and were significantly inhibited by lower concentrations of genistein (<10 μM), concentrations exceeding those typically achieved in vivo were necessary to inhibit the growth of PC-3 and DU145 cells (>100 μM) (Davis et al., 1998). A recent study on genistein administration to healthy men suggests that the highest genistein levels in serum achievable by ingesting a diet rich in soy products ranges from 1 to 5 μM (Busby et al., 2002). However, Farhan et al. (2002) showed that the concentrations of genistein achieved within the prostatic tissue following genistein ingestion in mice is about 10-fold higher when compared to the plasma levels. As genistein has been shown to decrease the expression of enzymes involved in vitamin D metabolism (both CYP24 and CYP27B1) (Farhan and Cross, 2002; Farhan et al., 2002, 2003), genistein co-treatment is expected to enhance vitamin D effects. Recent studies (Rao et al., 2002, 2004) have demonstrated a synergism between genistein and vitamin D in inhibiting the growth of LNCaP cells which are sensitive to growth inhibition by 1,25(OH)₂D₃.

In the current study we have examined the antiproliferative effects of genistein, both alone and in combination with 1,25(OH)₂D₃, in DU145 human PCa cells. These cells are insensitive to the growth inhibitory actions of 1,25(OH)₂D₃ alone (Skowronski et al., 1993; Miller et al., 1995). Although genistein alone fails to exhibit antiproliferative effects at concentrations below 10 μM, in combination with 1,25(OH)₂D₃, genistein causes significant growth inhibition seen at lower concentrations of both the agents. Our data show that genistein sensitizes the cells to the effects of low concentrations of 1,25(OH)₂D₃ by inhibiting CYP24 activity and extending 1,25(OH)₂D₃ half-life. We provide evidence for the first time that genistein directly inhibits mitochondrial CYP24 enzyme activity in a noncompetitive manner at concentrations that are achievable in vivo (Busby et al., 2002). Genistein co-treatment also enhances the homologous up-regulation of the VDR. The increases in the bio-availability of the active hormone 1,25(OH)₂D₃ as well as augmented VDR levels sensitize DU145 cells to the growth inhibitory actions of 1,25(OH)₂D₃.

2. Materials and methods

2.1. Materials

25-Hydroxy-[³H]-vitamin D₃ (SA 30 Ci/mmol), [³H]-1,25(OH)₂D₃ (SA 98 Ci/mmol) and [³H]-thymidine (SA 20 Ci/mmol) were obtained from Amersham Life Sciences (Arlington Heights, IL). 1,25(OH)₂D₃ was a gift from Dr. M. Uskokovic (Hoffmann-LaRoche Inc., Nutley, NJ). DU145 cell line was obtained from American Type Culture Collection (Rockville, MD). Tissue culture media and other supplements were obtained from Mediatech (Herndon, VA). All other reagents, except where indicated, were purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Methods

2.2.1. Cell culture

DU145 human prostate cancer cells were routinely cultured at 37 °C in a humidified atmosphere of 5% CO₂. They were maintained in RPMI 1640 medium containing 5% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml) and were routinely sub-cultured every 5–7 days. The primary prostatic epithelial cells derived from normal prostate tissue were established in culture and propagated as described before (Pechtl et al., 1994).

2.2.2. [³H]-Thymidine incorporation

Cell proliferation was determined by measuring [³H]-thymidine incorporation at the end of 6 days as described...
cultures and incubated for 2 h at 37 °C to attach for 24 h. Cells were then treated with 1,25(OH)2 D3, and/or genistein at varying concentrations in the growth medium (RPMI + 5% FBS). At the end of 6 days, cells were incubated with 0.15 μCi/ml [3H] thymidine in the culture medium for an hour at 37 °C in a 5% CO2 atmosphere, followed by a 5 min incubation in phosphate-buffered saline (PBS) containing unlabeled thymidine and processed as described before (Krishnan and Feldman, 1992). [3H] thymidine incorporation was expressed as dpm/well.

2.2.3. Mitochondrial preparation
Mitochondria were isolated from 1,25(OH)2D3-treated DU145 cells by the method previously described (Nomn et al., 2003; Ma et al., 2004) with slight modifications. Briefly, cells were harvested and washed once with ice-cold PBS and resuspended and incubated in a hypotonic buffer (buffer A, 10 mM Tris, 10 mM NaCl, 3 mM MgCl2, 1 mM EDTA and 1 mM EGTA) for 30 min on ice. After addition of sucrose to buffer A (0.2 M final concentration), cells were lysed by 30 strokes with a tight-fitting dounce homogenizer. Nuclei and unbroken cells were pelleted by centrifugation, at 600 × g for 15 min at 4°C. The supernatant was transferred to a fresh tube and mitochondria were pelleted by centrifugation at 14,000 × g at 4°C. The mitochondrial pellet was resuspended in the assay buffer (buffer B, 25 mM Tris-HCl, 0.5 mM EDTA, 0.125 M sucrose, 20 mM succinate, 0.7 mM 1 mM EGTA, and 0.125 M sucrose) for 30 min on ice. After addition of sucrose to buffer A (0.2 M final concentration), cells were lysed by 30 strokes with a tight-fitting dounce homogenizer. Nuclei and unbroken cells were pelleted by centrifugation, at 600 × g for 15 min at 4°C. The supernatant was transferred to a fresh tube and mitochondria were pelleted by centrifugation at 14,000 × g at 4°C. The mitochondrial pellet was resuspended in the assay buffer (buffer B, 25 mM Tris-HCl, 0.5 mM EDTA, 0.125 M sucrose, 20 mM succinate, 0.7 mM CaCl2 and 5 mM MgCl2, pH 7.4) and disrupted by sonication on ice for 15 s. Mitochondrial protein was quantified by the Bradford method (Bradford, 1976).

2.2.4. CYP24 activity assays
CYP24 enzyme activity was assayed in intact cells, cell homogenates as well as mitochondrial preparations as described before (Chen et al., 1986; Ly et al., 1999) with slight modifications. The various experimental manipulations are described as follows.

2.2.4.1. CYP24 activity after genistein treatment of DU145 cell cultures. In some assays DU145 cells were treated with various concentrations of genistein in the presence or absence of 10 nM 1,25(OH)2 D3 for 48 h in standard culture medium. The cultures were then rinsed with PBS and incubated for 30 min with culture medium alone at 37°C in a humidified atmosphere of 5% CO2 to remove 1,25(OH)2D3. The substrate 5 nM [3H]-25(OH)D3 (30 Ci/mmol) and 1 μM 25(OH)D3) was added in fresh culture medium to the cell cultures and incubated for 2 h at 37°C. At the end of the incubation, cells were gently scraped into the incubation medium and extracted with methanol/chloroform (2:1, v/v) for further analysis of metabolites.

2.2.4.2. CYP24 activity after genistein addition to cell homogenates. In experiments designed to test the direct effect of genistein on CYP24 enzyme activity, cell cultures were treated with vehicle or 10 nM 1,25(OH)2D3 for 30 min, rinsed with PBS and incubated with culture medium to remove 1,25(OH)2D3. Cells were then detached by gentle trypsinization, counted and homogenized in a defined volume of the culture medium using a dounce homogenizer. Aliquots of the homogenates were incubated with the substrate 25(OH)D3 as described above in the presence of varying concentrations of genistein (100 nM–50 μM) for 2 h at 37°C.

2.2.4.3. CYP24 activity after genistein addition to mitochondrial preparations. To test the effect of genistein on mitochondrial CYP24 activity, aliquots of the mitochondrial preparations (30–50 μg protein) were incubated in buffer B containing 1 mM NADPH, 10 mM glucose-6-phosphate and 0.3 unit of glucose-6-phosphate dehydrogenase along with 4 nM [3H]-25(OH)D3 and varying concentrations of radiometric 25(OH)D3 (0.25–4 μM) in the presence of genistein (0–200 nM) and the antioxidant, N,N′-diphenyl-p-phenyleneediamine (10 μM) for 1 h at 37°C. Auto-oxidation of the substrate 25(OH)D3 was assessed in parallel assay tubes incubated with all the components except for the mitochondrial enzyme preparation. The units of CYP24 activity were derived after correcting for auto-oxidation of the substrate.

2.2.4.4. Extraction, separation and quantitation of metabolites. At the end of the incubations the enzymatic reactions were terminated by the addition of methanol/chloroform (2:1, v/v). The vitamin D metabolites were extracted three times with methanol/chloroform as described before (Chen et al., 1986; Ly et al., 1999). The organic phases were combined, dried and reconstituted in 100 μl of hexane/isopropanol (90:10, v/v) mixture. Aliquots of the extract were separated by TLC on silica gel/aluminum foil strips using a methylene chloride/ethyl acetate (1:1, v/v) solvent system as described previously (Chen et al., 1986; Ly et al., 1999). Authentic 25(OH)D3, 1,25(OH)2D3 and 24,25(OH)2D3 (Roche, NJ) were used as standards to validate the assay and identify the metabolites. After separation the TLC strips were cut into fractions and the incorporation of radioactivity into the metabolites was determined by liquid scintillation counting. CYP24 enzyme activity was expressed as picomols of 24,25(OH)2 D3 formed/106 cells/30 min.

2.2.5. 1,25(OH)2D3 half-life studies
Semi-confluent cultures of DU145 cells were exposed to 1,25(OH)2D3 (10 nM radiometric 1,25(OH)2D3, and 0.5 nM [3H]-25(OH)2D3) added to the culture medium in the presence of 0.1% ethanol or 10 μM genistein. The half-life of 1,25(OH)2D3 was determined by measuring residual nonmetabolized [3H]-25(OH)2D3 over time as described in detail previously (Chen et al., 1986; Ly et al., 1999). Aliquots (200 μl) of the conditioned media were removed at various time intervals following [3H]-1,25(OH)2D3 addition and extracted three times with 750 μl of methanol/chloroform (2:1, v/v). The organic phases were pooled, dried down and
plates were used in transactivation assays. Two different
the relative fold changes in mRNA expression.

values of control and treated samples was used to arrive at
jected to TLC as described for CYP24 enzyme assay using
reconstituted in hexane:isopropanol (90:10, v/v) and sub-
alyzed by electrophoresis on agarose gels to confirm size
harvested and high salt extracts were made as described pre-
viously (Malloy et al., 1989). The protein concentration in the
samples and the expression of the gene of interest was normalized
binding protein (TBP) mRNA were determined in all the sam-
follows: initial denaturation at 72
◦C for 5 min, followed by 40 cycles each of 94
◦C for 20 s, 58
◦C for 15 s and 72
◦C for 20 s. The PCR products were analyzed by electrophoresis on agarose gels to confirm size of the amplified product. As a control the levels of TARAS box binding protein (TBPA) mRNA were determined in all the samples and the expression of the gene of interest was normalized to TBPA mRNA. The comparative Ct (ΔΔCt) method (Livak and Schmittgen, 2001), which compares differences in Ct values of control and treated samples was used to arrive at the relative fold changes in mRNA expression.

vitamin D responsive reporter plasmids namely the human osteocalcin-VDRE-luciferase (OC-Luc) and the mouse osteopontin-VDRE-luciferase (OP-Luc) along with the human VDR expression plasmid were transiently transfected into the cells using LipofectAMINE as described before (Gross et al., 1998). The pRL-CMV renilla luciferase plasmid (Promega, Madison, WI) was included in the transfections to control for transfection efficiency. After overnight incubation with the DNA–lipid mixture, cells were treated with 10 μM genistein or 10 nM 1,25(OH)2D3 or a combination of both in RPMI + 5% FBS for 24 h. The cells were harvested and lysates were prepared in passive lysis buffer (Promega) and luciferase activity was determined using the Dual Luciferase Reporter System Assay (Promega) in a Turner Designs 20/20 luminometer (Sunnyvale, CA). Reporter activity was expressed as the ratio of reporter luciferase to renilla luciferase.

2.3. Statistical analysis

Data were analyzed by ANOVA using the StatView 5.0 software (SAS Institute, Cary, NC). p < 0.05 was considered significant.

3. Results

3.1. Effects of genistein and 1,25(OH)2D3 on DU145 cell growth

We studied the effect of 1,25(OH)2D3, genistein and combinations of both on DU145 cell growth in dose-response and time-course experiments. Fig. 1A shows the dose-response to genistein as determined by [3H]-thymidine incorporation. DU145 cells were not inhibited to any significant degree by concentrations of genistein up to 10 μM. However, significant inhibition of growth was seen when higher concentrations (>10 μM) of genistein were used. Similarly, the effect of 1,25(OH)2D3 alone on DU145 cell growth was also minimal and growth inhibition was not seen even when pharmacological concentrations of 1,25(OH)2D3 (1-5 μM) were used

Fig. 1. Dose-response effect of 1,25(OH)2D3, genistein and the combination on the proliferation of DU145 cells. DU145 cells were seeded in six-well dishes (100,000 cells/well) in RPMI 1640 medium containing 5% FBS. After 24 h fresh media containing 0.1% ethanol vehicle (control) or varying concentrations of 1,25(OH)2D3 or genistein individually or in combination were added to cells. Fresh medium and hormones were replenished every other day and [3H]-thymidine incorporation was measured at the end of 6 days. Results are expressed as a percentage of control value set at 100% and was equal to 46,197 ± 11,060 dpm/well. (A) Dose-response to genistein (open circles); (B) Dose-response to 1,25(OH)2D3 (open squares); and the vitamin D analog RO 25–6760 (open diamonds). (C) Dose-response to 1,25(OH)2D3 in the presence of 0.1% ethanol vehicle (control, open triangles); 100 nM genistein (closed circles) or 10 μM genistein (closed triangles). All values are given as mean ± SE (n=9). * p<0.05, ** p<0.01 and *** p<0.001 when the hormone-treated samples were compared to the corresponding controls.
However, cell growth was significantly inhibited by the vitamin D analog RO 25–6760 \([1\alpha, 25(OH)_2-16\text{-ene}-23\text{-yne-26,27-hexafluoro-19-nor-D}_3]\) with structural modifications that protect the molecule from catabolism into inactive metabolites by the C-24 oxidation pathway (Uskokovic et al., 2001). Interestingly when genistein was combined with 1,25(OH)_2D_3 significant growth inhibition was detected at all the 1,25(OH)_2D_3 concentrations tested (0.1 nM–100 nM) (Fig. 1C). The growth inhibition was also dependent on the dose of genistein used in the combination. Similar results were seen when DNA content was determined as a measure of cell growth (data not shown).

Time-course experiments using more sparsely seeded cultures showed similar results (Fig. 2). No differences in growth were detected between cells treated with ethanol vehicle (control) and those treated with either 1,25(OH)_2D_3 (10 nM) or genistein (10 μM) alone except at a single time point on day 4 when a small decrease was detected. However, at the end of the time-course experiment growth was similar in control, 1,25(OH)_2D_3- or genistein-treated cells. In contrast, in cells treated with a combination of genistein and 1,25(OH)_2D_3 significant growth inhibition was apparent beginning 3 days after seeding (50% inhibition) and continuing throughout the time-course.

3.2. Effect of genistein on basal and 1,25(OH)_2D_3-induced CYP24 activity

CYP24 is the key enzyme initiating the degradation of 1,25(OH)_2D_3 by hydroxylation at the C-24 position of the side chain. In target cells 1,25(OH)_2D_3 induces the expression of CYP24 thereby causing auto-regulation of its levels and biological activities (Feldman et al., 2001). We studied...
Fig. 3. (A) Effect of genistein treatment on CYP24 enzyme activity in DU145 cells. DU145 cells were exposed to 0.1% ethanol (control) or 10 nM 1,25(OH)\textsubscript{2}D\textsubscript{3} with and without co-treatment with 0.1 or 10 μM genistein for 48 h. After washes the cell cultures were incubated with the substrate for 2 h at 37°C. The vitamin D metabolites were then extracted and quantitated as described in Section 2. CYP24 activity is expressed as picomols of 24,25(OH)\textsubscript{2}D\textsubscript{3} formed/10\textsuperscript{6} cells/30 min and values represent mean±S.E. (n=6). ***p<0.001 when compared to control and +++p<0.001 when compared to cells treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} alone. (B) Direct inhibition of CYP24 enzyme activity by genistein added to cell homogenates. Semi-confluent cultures of DU145 cells were treated with 0.1% ethanol or 10 nM 1,25(OH)\textsubscript{2}D\textsubscript{3} for 30 min, harvested and homogenized to make a cell extract as described in Section 2. Aliquots of the cell homogenates were then incubated with the substrate in the presence of various concentrations of genistein (0.1–50 μM) and CYP24 enzyme activity was measured as described in Section 2. CYP24 activity is expressed as picomols of 24,25(OH)\textsubscript{2}D\textsubscript{3} formed/10\textsuperscript{6} cells/30 min and values represent mean±S.E. (n=6). ***p<0.001 when compared to control and +p<0.05 when compared to cells treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} alone.

We also examined the effect of adding genistein (0–200 nM) to mitochondrial preparations from 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated DU145 cells on CYP24 enzyme activity using various concentrations of the substrate. As suggested by the broken cell data (Fig. 3B) we observed significant inhibition of mitochondrial CYP24 activity at all the concentrations of genistein tested. An analysis of a Lineweaver–Burke plot of these data (Fig. 4) showed that genistein decreased the \( V_{\max} \) of the enzyme reaction in a concentration-dependent manner while the slopes of the double reciprocal plots increased with increasing genistein concentrations. This pattern is consistent with a noncompetitive inhibition of the enzyme by genistein and the apparent \( K_i \) for genistein was 3.61±0.70 μM.

3.3. Effect of genistein on 1,25(OH)\textsubscript{2}D\textsubscript{3} half-life

CYP24 is the key enzyme initiating the catabolism of 1,25(OH)\textsubscript{2}D\textsubscript{3} and we expected that the inhibition of CYP24 activity by genistein would lead to the prolongation of the half-life of 1,25(OH)\textsubscript{2}D\textsubscript{3}. This was indeed the case. As shown in Fig. 5, treatment of DU145 cells with 10 μM genistein appeared to prolong the half-life of 1,25(OH)\textsubscript{2}D\textsubscript{3} and higher concentrations of 1,25(OH)\textsubscript{2}D\textsubscript{3} remained in the culture medium over several hours when the cells were co-treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} and genistein. The concentrations of [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} remaining in the culture medium at the end of 48 h was twice as much in the cells exposed to genistein (3.1 pmols/ml) as compared to vehicle-treated controls (1.5 pmols/ml).
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Fig. 4. Inhibition of mitochondrial CYP24 activity in the presence of genistein. Mitochondrial fractions isolated from DU145 cells treated with 10 nM 1,25(OH)₂D₃ for 16–18 h were incubated with varying concentrations of the substrate 25(OH)D₃ in the absence (open squares) or presence of 50 nM (closed circles), 100 nM (closed triangles) and 200 nM (open diamonds) genistein and CYP24 activity was determined as described in Section 2. A representative Lineweaver–Burke plot of the data is shown. The analysis was repeated three times and the \( K_i \) of genistein was estimated to be 3.31 ± 0.52 nM. Estimate of \( K_i \) is expressed as mean ± S.E.

3.4. Effect of genistein on basal and 1,25(OH)₂D₃-induced CYP24 mRNA expression

In target cells 1,25(OH)₂D₃ induces the transcription of the CYP24 gene and thereby increases CYP24 mRNA levels (Feldman et al., 2001). We examined the effect of genistein on basal as well as 1,25(OH)₂D₃-induced expression of CYP24 mRNA by real-time RT-PCR. After 24 h of treatment, 1,25(OH)₂D₃ caused a 10-fold increase in CYP24 mRNA levels when compared to the control (Fig. 6). Genis-
tein (10 μM) alone did not cause any significant change in the basal CYP24 mRNA levels. A combination of 1,25(OH)2 D3 and genistein showed a further increase (~25 fold) in CYP24 mRNA suggesting that genistein enhanced the effect of 1,25(OH)2 D3 to induce CYP24 mRNA.

3.5. Effect of genistein and 1,25(OH)2 D3 on VDR protein and mRNA expression

We and others have shown that 1,25(OH)2 D3 and its analogs induce homologous up-regulation of the vitamin D receptor (VDR) in a number of cells (Costa et al., 1985; Krishnan and Feldman, 1997). As the abundance of VDR in target cells is an important determinant of the magnitude of responses to 1,25(OH)2 D3 (Krishnan and Feldman, 1992, 1997), we studied the effects of genistein, 1,25(OH)2 D3 and their combination on VDR levels in DU145 cells. On Western blots the VDR could be visualized as a 47 KDa immunoreactive band (Fig. 7A). Densitometric analysis of Western blots revealed that 1,25(OH)2 D3 or genistein individually caused an increase in VDR protein of about two-fold over the control after normalizing VDR levels to actin expression (data not shown). The combination of 1,25(OH)2 D3 and genistein caused a further increase resulting in a ~four-fold higher VDR concentration. VDR levels as determined by an ELISA did not show an increase due to genistein treatment alone. However, significant increases in VDR levels (~3–3.5 fold) were seen due to the combination treatment (Fig. 7B). We also tested the effect of the combination on VDR levels by ELISA at the end of 6 days, a time point when maximal growth inhibition was recorded (Fig. 2). Our data showed the VDR levels in cells subjected to the combination treatment remained elevated (32 ± 4.1 fmol/mg protein) as compared to vehicle treated controls (7.0 ± 4.1 fmol/mg protein) at the end of 6 days. Real-time RT-PCR analysis revealed that both 1,25(OH)2 D3 and genistein when added alone did not change VDR mRNA levels after a 24 h treatment period (Fig. 7C). The combination of genistein and 1,25(OH)2 D3 however, caused significant increases in VDR mRNA abundance.

3.6. Effect of genistein on 1,25(OH)2 D3-mediated transactivation of reporter genes

We examined the effect of genistein on 1,25(OH)2 D3-mediated transactivation responses. DU145 cells transiently transfected with the vitamin D responsive OC-Luc or OP-Luc reporter constructs responded to 1,25(OH)2 D3 with a two- to three-fold increase in reporter activity (Fig. 8). Genistein by itself had very minimal effect on reporter activity. However, genistein enhanced the effect of 1,25(OH)2 D3. A further two-fold increase in OC-Luc reporter activity was seen with the combination treatment when compared to 1,25(OH)2 D3 alone whereas OP-Luc enhancement was more modest.
Fig. 8. Effect of genistein on 1,25(OH)₂D₃-mediated transactivation. The vitamin D responsive reporter plasmids OC-Luc and OP-Luc as well as a VDR expression plasmid were transiently transfected into DU145 cells as described in Section 2. Following transfections, the cells were treated with 0.1% ethanol (C), 10 nM 1,25(OH)₂D₃ (D), 10 μM genistein (G) or a combination of both (D + G) for 24 h and luciferase activities were assayed using the dual luciferase system (Promega, WI). Values are expressed as a ratio of the reporter luciferase to renilla luciferase activity and are shown as mean ± S.E. (n = 9). **p < 0.01 when compared to control and +p < 0.05 ++p < 0.01 when compared to cells treated with 1,25(OH)₂D₃ alone.

3.7. Effect of 1,25(OH)₂D₃ and genistein on the expression of genes involved in proliferation and apoptosis

1,25(OH)₂D₃ exerts antiproliferative and pro-apoptotic effects on many PCa cells through the regulation of the expression of several genes involved in growth control and apoptosis (Krishnan et al., 2003). Our data showed an enhanced inhibition of DU145 cell growth when 1,25(OH)₂D₃ was combined with genistein (Fig. 1). We therefore examined the changes in the expression of some of the key genes involved in growth control following the treatment of cells with 1,25(OH)₂D₃ and genistein individually and in combination. Fig. 9A shows the changes in the expression of p21, cyclin D1 and IGFBP-3 genes. 1,25(OH)₂D₃ did not significantly affect the expression of these genes when added alone. Similarly genistein by itself did not significantly affect the expression of these genes except in the case of cyclin D1 whose mRNA levels registered a small but significant decrease due to genistein treatment. Interestingly, the effect of the combination of 1,25(OH)₂D₃ and genistein was synergistic on p21 and IGFBP-3 mRNA expression showing highly significant increases compared to control. The decrease in cyclin D1 mRNA levels due to the combination treatment was more modest suggestive of an additive effect. As seen in Fig. 9B, 1,25(OH)₂D₃, when used alone caused a modest but significant decrease in the mRNA levels of the antiapoptotic gene bel2 and significantly increased the expression of...
the pro-apoptotic gene bax. Genistein by itself had no effect on bcl2 or bax. However, the fold changes in the expression of these genes were much more pronounced with the combination treatment indicating synergistic effects. None of the treatments significantly altered bcl2L1 mRNA levels.

4. Discussion

In the present study we investigated the effects of 1,25(OH)2 D3 in combination with genistein on the growth of the highly aggressive DU145 human PCa cells. DU145 cells are very resistant to the growth inhibitory effects of 1,25(OH)2 D3 (Skowronski et al., 1993; Miller et al., 1995) and very high concentrations of genistein (>10 μM) are required to inhibit the growth of these cells (Hempstock et al., 1998; Bhatia and Agarwal, 2001). Our study shows that the combination of 1,25(OH)2 D3 and genistein causes significant inhibition of the growth of DU145 cells, apparent at concentrations of 1,25(OH)2 D3 as low as 0.1 nM and importantly at levels of genistein achievable in vivo (100 nM). When tested alone genistein inhibited cell growth only at concentrations greater than 10 μM. At concentrations >50 μM genistein exhibited a cytotoxic effect as reported previously (Bhatia and Agarwal, 2001). Similar synergistic effects of 1,25(OH)2 D3 and genistein have been reported in the case of LNCaP cells which are very responsive to growth inhibition by 1,25(OH)2 D3 (Rao et al., 2002).

Among the well-studied human PCa cell lines, DU145 cells show the highest basal levels of CYP24 enzyme activity (Skowronski et al., 1993; Miller et al., 1995). Previous studies from our lab have shown that inhibition of CYP24 enzyme activity by the potent 17β-HSD inhibitors liarazole and ketoconazole, renders different prostate cells responsive to growth inhibition by 1,25(OH)2 D3 (Ly et al., 1999; Pechel et al., 2002). The current data show that similar to the imidazole compounds, genistein inhibits CYP24 enzyme activity sensitizing DU145 cells to the growth inhibitory actions of 1,25(OH)2 D3.

Genistein appears to regulate CYP24 gene expression by a genomic mechanism causing changes in CYP24 mRNA levels. Cross and co-workers have shown that genistein inhibits basal CYP24 mRNA levels at concentrations 25 μM or greater but not at 5 μM or lower (Farhan and Cross, 2002; Farhan et al., 2002, 2003; Kallay et al., 2002). We did not detect any inhibition of the basal CYP24 mRNA expression at 10 μM genistein. Recent reports by Farhan et al. (Farhan and Cross, 2002; Farhan et al., 2002, 2003) show that genistein reduces the basal steady-state levels of CYP24 mRNA in DU145 cells and 1,25(OH)2 D3-induced CYP24 mRNA in PC-3 cells but at a five-fold higher concentration (50 μM). We limited the concentration of genistein in our studies to 10 μM or less to correlate with genistein concentrations achievable in serum in vivo. In our study 10 μM genistein did not reduce either the basal or 1,25(OH)2 D3-induced CYP24 mRNA suggesting a lack of a genomic effect at this lower genistein concentration. However, we saw an increase in 1,25(OH)2 D3-induced CYP24 mRNA with genistein co-treatment. Our interpretation of these data is as follows. Genistein causes a significant direct inhibition of CYP24 enzyme activity. The inhibition of CYP24 enzyme activity prolongs the half-life of 1,25(OH)2 D3 which in turn results in an increase in the homologous up-regulation of VDR. These increases in the concentrations of both 1,25(OH)2 D3 (the ligand) as well as VDR (the receptor) enhances the genomic actions of the 1,25(OH)2 D3-VDR endocrine system to induce many target genes including CYP24 gene itself as measured by its increased mRNA expression. However, the enzymatic activity of the increased CYP24 protein is blocked by genistein by a direct noncompetitive mechanism.

In the studies reported previously, PCa cell cultures were treated with high concentrations of genistein (>10 μM) and CYP24 inhibition at the genomic level resulted in decreased mRNA expression and decreased protein expression measured by enzyme activity (Farhan and Cross, 2002; Farhan et al., 2002, 2003). Interestingly our study shows an inhibition of CYP24 activity at the enzymatic level by genistein through a nongenomic mechanism, which causes a direct, noncompetitive inhibition of the enzyme activity. This enzymatic inhibition occurs at concentrations of genistein as low as 100 nM when added directly to mitochondrial preparations from 1,25(OH)2 D3-treated DU145 cells. Several studies have demonstrated the inhibitory effect of phytoestrogens, including genistein, on the activities of many enzymes involved in steroid metabolism such as 17β-HSD (Le Bail et al., 2000), 3β-HSD (Ohno et al., 2002), aromatase (Pelissero et al., 1996; Whitehead and Lacey, 2003) and the steroidogenic P450 enzyme 21-hydroxylase (CYP21) (Ohno et al., 2002). The phytoestrogen effect on 3β-HSD and CYP21 has been shown to be a direct competitive inhibition at the enzyme level with the inhibitory concentrations ranging from 1 to 25 μM (Ohno et al., 2002). Our study is the first demonstration of a direct inhibition by genistein of the P450 enzyme CYP24 involved in the vitamin D pathway. In this case the inhibition appears to be noncompetitive with a Ki of 3.61 ± 0.70 μM for genistein.

The biological actions of 1,25(OH)2 D3 are mediated via the VDR, a steroid receptor belonging to the nuclear receptor superfamily. Regulating the abundance of VDR is an important mechanism for modulating target cell responsiveness to 1,25(OH)2 D3 (Krishnan and Feldman, 1997). 1,25(OH)2 D3 causes homologous up-regulation of VDR by a combination of effects on VDR mRNA and protein levels that is different in different target cells (Costa et al., 1985; Costa and Feldman, 1987; Krishnan and Feldman, 1997). We expected that the prolongation of 1,25(OH)2 D3 half-life by genistein would enhance homologous VDR up-regulation (Ly et al., 1999). This was confirmed by our Western blot and VDR ELISA data demonstrating increases in VDR protein concentrations with the combined treatment. Similar enhancement of the homologous up-regulation of VDR by genistein has been reported in LNCaP cells by Rao et al. (2004). Although Wietzke and
Welsh (2003) found an increase in VDR levels by Western blot following genistein treatment in T47D human breast cancer cells, in our study genistein by itself did not increase VDR content in DU145 cells by ELISA determinations. Our data showed that 1,25(OH)2D3 alone did not significantly affect VDR mRNA in DU145 cells. However, the combination of genistein and 1,25(OH)2D3 increased VDR mRNA significantly.

The increase in the half-life of 1,25(OH)2D3 (the hormone) and VDR levels (the receptor) due to genistein treatment resulted in significant enhancement of 1,25(OH)2D3-mediated functional responses as measured by the transactivation of the vitamin D-responsive reporters. We achieved significant growth inhibition with 1,25(OH)2D3, and genistein combination whereas the individual drugs at these concentrations failed to inhibit growth. Consistent with the growth data, the expression of genes associated with growth arrest and apoptosis showed minimal or no change with the individual drugs but registered significant changes due to the combination treatment. The mRNA levels of the cyclin-dependent kinase inhibitor p21/WAF1, whose expression is associated with G1/S or G1/G0 cell cycle arrest, significantly increased following the combination treatment. Similar cooperation between 1,25(OH)2D3 and genistein to up-regulate p21 protein expression has been demonstrated in LNCaP cells (Rao et al., 2004). Cyclin D is the prime integrator of mitogenic signals to initiate the progression of cells through the early G1 phase (Swanton, 2004) and increased cyclin D1 expression has been reported in several human cancers (Hirama and Koeffler, 1995). The decrease in cyclin D1 mRNA seen with the combination treatment is reflective of the significant growth inhibition achieved. Similarly the expression of IGFBP-3 gene, which we have shown to play an important role in vitamin D-mediated growth inhibition of LNCaP cells (Boyle et al., 2001) revealed a synergistic increase with the combination treatment. Our data also show that 1,25(OH)2D3 by itself caused a significant decrease in the expression of the antiapoptotic gene bcl2 while increasing that of the pro-apoptotic gene bax. Genistein co-treatment further enhanced these effects. The ratio of bcl2 to bax has been shown to influence tumor apoptosis rate (Olivai and Korsmeyer, 1994) and is predictive of DNA fragmentation due to apoptosis (Huang et al., 1997). Our data suggest that genistein by prolonging 1,25(OH)2D3 half-life and increasing VDR levels sensitizes the cells to the pro-apoptotic actions of 1,25(OH)2D3. The action of genistein to enhance the growth inhibitory effects of 1,25(OH)2D3 has also been demonstrated in LNCaP cells (Rao et al., 2004).

In addition, we have examined the effect of the combination in primary prostate epithelial cells derived from nonmalignant prostate tissue and found an enhancement of the regulation of growth control genes such as p21 and cyclin D1 due to genistein co-treatment. Rao et al (Rao et al., 2004) similarly found synergistic growth inhibition in primary prostate cells. These data show that the combination is also effective in inhibiting the growth of nonmalignant prostate cells.

In many cells genistein exhibits a weak estrogenic activity by binding to and activating the estrogen receptors α and β (Kuijer et al., 1997). Therefore the question whether estrogenic molecules, including other isoflavones, also inhibit CYP24 and related enzymes naturally arises. Farhan et al. (2003) have studied the effect of other flavanoid and estrogenic molecules on CYP24 as well as CYP2B1 activities in DU145 cells treated with these compounds. While genistein and its natural metabolite dihydrogenistein inhibit both the enzymes, daidzein, a structurally related isoflavone does not do so. 17β-Estradiol does not inhibit either estrogen. However, antiestrogen ICI 182780 causes a significant inhibition of both enzymes (Farhan et al., 2003). In all the cases, the effects are due to the genomic action of these molecules to regulate CYP24 and CYP2B1 expression and their direct effects at the enzymatic levels have not been examined.

In conclusion, our study demonstrates that the sensitivity of DU145 cells to the growth inhibitory actions of 1,25(OH)2D3 is increased by co-treatment with genistein. Lower and biologically achievable concentrations of both compounds are sufficient to cause significant growth inhibition of the DU145 cells. Our data are supportive of a direct noncompetitive inhibition of mitochondrial CYP24 enzyme activity by genistein. Genistein potentiates the action of 1,25(OH)2D3 by two mechanisms: (i) it directly inhibits CYP24 enzyme activity and increases the half-life of 1,25(OH)2D3, and (ii) as a result of the prolongation of 1,25(OH)2D3 half-life the homologous up-regulation of cellular VDR levels is increased. This dual action of genistein leads to enhanced vitamin D-mediated responses and target gene activation rendering the cells more sensitive to the growth inhibitory and pro-apoptotic signals of 1,25(OH)2D3.

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