1α,25-Dihydroxyvitamin D₃ and Platinum Drugs Act Synergistically to Inhibit the Growth of Prostate Cancer Cell Lines

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ABSTRACT

The majority of men who die from prostate cancer (PC) have hormone-refractory disease. To date, chemotherapeutic agents have had little or no impact on the survival of such patients. To explore a new approach for the treatment of hormone-refractory PC, we examined the combination effects of cis- or carboplatin with vitamin D. 1α,25-Dihydroxyvitamin D₃ (1α,25(OH)₂D₃) and its synthetic analogue, Ro 25-6760, have an antiproliferative effect on some prostate cancer cell lines. Consequently, the growth-inhibitory effects of the drugs were measured, both singularly and in combination with cis- or carboplatin, on PC cells. Our results show that although each of the drugs alone displayed antiproliferative activity, the growth inhibition of PC cells was further enhanced by the combination of 1α,25(OH)₂D₃ or Ro 25-6760 and either platinum agent. The greatest enhancement of inhibition occurred using smaller concentrations of the platinum compound in combination with higher concentrations of 1α,25(OH)₂D₃. Isobologram analysis revealed that 1α,25(OH)₂D₃ and platinum acted in a synergistic manner to inhibit the growth of PC cells. Our findings suggest that there is potential clinical value in combining 1α,25(OH)₂D₃ with platinum compounds for the treatment of advanced-stage human PC.

INTRODUCTION

In 1999, an estimated 179,300 new cases of PC will be diagnosed in American men (1). The incidence of the disease rose dramatically in the late 1980s with the widespread use of improved detection modalities including prostate-specific antigen and transrectal ultrasound (2). Consequently, there has been a significant shift in the stage of disease at diagnosis. More early stage (localized) carcinomas are being detected and treated, and more of these are being detected in younger men. However, in spite of these seeming successes, 37,000 American males are still expected to die from PC this year, making it the second leading cause of cancer death in men (1).

Androgens regulate the growth, differentiation, and rate of apoptosis in the prostate and its malignancies (3–5). Therapy for advanced-stage PC often involves systemic androgen withdrawal combined with an androgen receptor antagonist (combined androgen blockade). Although combined androgen blockade often results in initial tumor regression, it does little to alter the ultimate course of the disease, as androgen refractory progression ensues in an average of 17 months (6). The average survival duration for patients with metastatic PC is 2.5–3.5 years, and nearly all of the patients who die of PC succumb to this hormone-refractory form of the disease.

Curative treatments for patients with hormone-refractory disease are not presently available. Many studies of chemotherapy in hormone-refractory PC were performed in the 1970s and 1980s. Unfortunately, although a variety of agents have been tested, no single agent or combination regimen has demonstrated convincing evidence of benefit to patients (7). Better treatments for hormone-refractory disease are needed, and a large clinical research effort in the area is ongoing. A number of agents are coming to clinical trials, including differentiation agents, angiogenesis agents, signal transduction inhibitors, metalloproteinase inhibitors, bisphosphonates, dietary agents (such as pectin, selenium, and soy protein), and gene therapy. However, it is not yet possible to assess their impact on this disease.

It is now recognized that steroid hormones other than androgens influence benign and malignant prostatic epithelial cells. Of those that have been examined, one of the most active is the secosteroid 1α,25(OH)₂D₃. Although its role as the major regulator of calcium homeostasis in the body has been widely recognized for some time (8, 9), recent findings indicate that 1α,25(OH)₂D₃ is an important modulator of cellular proliferation and differentiation in a wide variety of benign and malignant cells. The primary actions of 1α,25(OH)₂D₃ are mediated through its nuclear vitamin D receptor, a member of the steroid/thyroid hormone superfamily of ligand-activated transcription factors (10, 11). Miller et al. (12) were the first to report the finding of saturable, high affinity binding sites for 1α,25(OH)₂D₃ in PC cells. Since then, all PC cell lines examined to date have been found to contain receptors for vitamin D (13). This is also true for primary cultures of epithelial and stromal cells derived from benign and malignant prostatic tissues (14). It is, therefore, widely accepted that 1α,25(OH)₂D₃ decreases the growth of PC cells in vitro (13–16).

To date, there have been two clinical trials that have begun to examine the utility of 1α,25(OH)₂D₃ in the treatment of PC. The first, by Osborn et al. (17), was a small Phase II trial in 13 men with hormone-refractory metastatic PC. Although this trial did not demonstrate significant activity for oral 1α,25(OH)₂D₃,
two patients had a significant reduction in serum prostate-specific antigen levels. The second, reported by Gross et al. (18) looked at the treatment of early recurrent PC with 1α,25(OH)₂D₃. Their findings showed that 1α,25(OH)₂D₃ slowed the rate of increase of serum prostate-specific antigen in a subset of men with early recurrent PC after primary therapy with radiation or surgery.

Unfortunately, the well-recognized hypercalcemic effects of 1α,25(OH)₂D₃ diminish its potential usefulness as a chemopreventive or chemotherapeutic agent. Therefore, intensive efforts have been directed at the development of synthetic vitamin D analogues that retain the antiproliferative and differentiating properties of 1α,25(OH)₂D₃ but have less calcemic activity. A number of these analogues have demonstrated higher efficacy and potency than the parent compound in their ability to inhibit the growth of PC cells in vitro (19–22). Our laboratory tested several of these analogues on PC cells and found that of the compounds examined, the Hoffman-La Roche analogue Ro 25-6760 was the most potent at reducing growth and promoting differentiation (13).

Cisplatin is one of the most widely used chemotherapeutic agents and has been shown to kill cancer cells through the formation of covalent, bifunctional adducts (23, 24). Although initial clinical trials of cisplatin in PC were not encouraging (25, 26), more recent trials have provided modest results (27, 28). Clinical evidence suggests that opportunities exist to enhance this modest effect. Cho et al. (29) discovered that 1α,25(OH)₂D₃ enhanced the effects of platinum agents in inhibiting the growth of the breast cancer cell line, MCF-7. Additional in vivo evidence for the positive interaction between 1α,25(OH)₂D₃ and platinum compounds was demonstrated using a murine squamous cell carcinoma model system (30). Therefore, this study was undertaken to determine whether 1α,25(OH)₂D₃ or its analogue Ro 25-6760 would enhance the effects of platinum agents in inhibiting the growth of PC cells in vitro, thereby providing the rationale for combination therapy of advanced-stage PC patients.

**MATERIALS AND METHODS**

**Cell Lines and Culture.** LNCaP (31) cells were obtained from Dr. J. Horoszewicz (Roswell Park Memorial Institute, Buffalo, NY) at the 14th passage and were used at approximately passage 20. The DU-145 (32) cell line was obtained from American Type Culture (Rockville, MD) at passage 57 and was used at approximately passage 140. The cell lines were maintained by serial passage in RPMI 1640 containing 10% FBS (HyClone Laboratories, Logan, UT) without antibiotics. The authenticity of each cell line was established by comparison to the original published karyotypes.

**Drugs.** 1α,25(OH)₂D₃ and its analogue Ro 25-6760 were a generous gift from Dr. M. Uskokovic (Hoffman-La Roche, Nutley, NJ). These compounds were kept dissolved in ethanol and stored as concentrated solutions at −20°C. 1α,25(OH)₂D₃ and Ro 25-6760 were freshly diluted in RPMI 1640 before each experiment. The ethanol concentrations in each test condition never exceeded 0.1%. Carboplatin and cisplatin were obtained from Sigma Chemical Co. (St. Louis, MO). Concentrated stocks of carboplatin and cisplatin were made using either sterile distilled water or dimethylformamide, respectively. Fresh stocks were made on the day of each experiment, and dilutions were made with RPMI 1640.

**Growth Inhibition Assays.** The cell lines were transferred into multiwell plates at the following densities: LNCaP, 1 × 10³/cm² and DU-145, 0.25 × 10³/cm². The cells were plated in triplicate in medium containing 10% FBS and were allowed to attach to the surface overnight. The plating medium was removed and replaced with medium containing the appropriate concentration of either vehicle(s), 1α,25(OH)₂D₃, Ro 25-6760, or cis- or carboplatin. Combined effects were evaluated by incubating LNCaP and DU-145 cells with 1α,25(OH)₂D₃ or its analogue, Ro 25-6760, with either cis- or carboplatin (see “Results”). Cells were allowed to grow for an additional 4 days. The total DNA content of the cells in each well was determined using the Hoechst 33258 assay, as described previously (33). Growth inhibition was calculated as the percentage of difference of the treated cells compared with vehicle controls. Each experiment was carried out in triplicate.

**Isobologram Analysis of Interactions between 1α,25(OH)₂D₃ and Platinum Drugs.** The isobologram method of analysis allows for stringent classification of the degree of interaction between two drugs into one of five categories within a given range of drug concentrations and molar ratios (34, 35). In clinical studies, improved median survival or disease-free interval with chemohormonal therapy has led to possibly erroneous conclusions that drugs have acted in an additive or synergistic fashion (36). However, the nonlinear response curve of most drugs makes it almost impossible to accurately demonstrate drug interactions, simply by comparing the sum of individual drug effects with the actual observed combined effect (37). Isobologram analysis alleviates this problem because it concisely evaluates drug interactions independent of the dose-response curve shapes. Additionally, isobologram analysis concisely categorizes the degree of interaction between two drugs, regardless of whether the drugs act through similar or different mechanisms (34). This method of analysis has been applied both to in vivo (34, 35) and in vitro (38, 39) studies.

We chose to evaluate antiproliferative effects using the ID₂₀ drug combination in our experiments. An ID₂₀ was chosen to maximize the combined growth-inhibitory effects seen at higher concentrations of 1α,25(OH)₂D₃ and lower concentrations of the platinum compounds. The ID₂₀ was determined experimentally, using the data from the dose-response experiments as a starting point. The data were graphed by plotting the ID₂₀ for drug I with 95% confidence limits on the y-axis, plotting the ID₂₀ for drug II plus 95% confidence limits on the y-axis, and connecting the paired points with a diagonal line. The degree of drug interaction was assessed by calculating the ID₂₀ of the combination of 1α,25(OH)₂D₃ and platinum and plotting the result along a fixed-drug ratio line that is determined by the chosen axis ranges and extends outward from the origin (34, 35). The interaction between two drugs is defined as “simple addition” when the effect of the combined drugs is the sum of the effects of the drugs administered separately. “Synergism” is defined in this instance as a mixture of drug I and drug II, giving a greater effect than the sum of their individual effects.
effects. It is manifested by the ID$_{20}$ of the combination being lower than those, which would be predicted, if the drugs acted in a simply additive fashion. When a binary mixture of drugs yields a response greater than that obtained with either drug alone but less than the response predicted on the basis of simple addition, the interaction is called “infra-additivity.” If the combination of drug I with drug II results in a response, which is not different from that of either drug alone, then this is termed “noninteraction.” If drug I causes drug II to elicit a lesser response than would be obtained by drug II alone, “antagonism” has occurred.

**Flow Cytometric Analysis of Cell Cycle.** LNCaP cells ($3.5 \times 10^5$) were plated in RPMI plus 10% FBS in T-25 tissue culture flasks. The medium was replaced with 5 ml of fresh RPMI with 10% FBS containing the appropriate concentrations of either 1α,25(OH)$_2$D$_3$, carboplatin, or a combination of the two drugs. The cells were incubated with the drugs for various amounts of time ranging from 6 to 96 h. The cells were harvested by pooling the floating cells with the trypsinized monolayers and were pelleted by centrifugation at 600 rpm for 5 min. The cells were resuspended in a solution containing 25 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO), 0.1 mM EDTA, 0.01 mg/ml DNase-free RNase, and 0.3% saponin in PBS (pH 7.4). The samples were incubated in this solution for 15 min at room temperature and then for at least 5 h at 4°C before cell cycle analysis on a Coulter XL flow cytometer (Coulter Corp., Hialeah, Fl). Doublet correction was achieved by gating on peak fluorescence versus integral fluorescence.
Statistics were performed on 20,000 events per sample using Modfit 5.2 software (Verity Software House, Inc., Topsham, ME).

**Statistical Analysis.** Comparisons of growth-inhibitory effects of combination drug treatment to singular treatments and analysis of the differences between subgroups of different drug conditions and lengths of treatments within selected phases of the cell cycle were performed using parametric one-way ANOVA. Differences between groups were interpreted as being statistically significant when P values were <0.05.

**RESULTS**

**Dose-Response of 1α,25(OH)2D3, Ro 25-6760, Carbo-**

**platin, and Cisplatin on LNCaP and DU-145 Cells.** Initial experiments were performed to determine the range of drug concentrations that would elicit partial responses in LNCaP and DU-145 cell growth inhibition. To determine optimal concentration for each drug, LNCaP (1.0 $\times\ 10^3$/cm$^2$) and DU-145 (0.25 $\times\ 10^3$/cm$^2$) cells were incubated with 0.1–10 nM 1α,25(OH)2D3, 0.1–10 nM Ro 25-6760, 0.2–200 μg/ml carboplatin, or 0.02–2.0 μg/ml cisplatin. The doses of 1α,25(OH)2D3 and Ro 25-6760 to be tested were selected on the basis of previous work from our laboratory (22). The doses of the platinum drugs to be tested were selected on the basis of a previous report that described the use of platinum drugs on malignant cells in vitro (29).

**Fig. 1,** A and B, shows that both 1α,25(OH)2D3 and Ro 25-6760 are able to inhibit the growth of LNCaP cells. 1α,25(OH)2D3 (1.0 and 10 nM) inhibited LNCaP growth by 15.25 ± 2.2% (mean ± SE) and 26.70 ± 1.7%, respectively. The same concentrations of Ro 25-6760 inhibited LNCaP growth by 44.6 ± 7.3% and 47.4 ± 4.5%, respectively. 1α,25(OH)2D3 was not capable of inhibiting growth at any of the chosen concentrations in the DU-145 cell line. However, Ro 25-6760 inhibited the growth of DU-145 cells at each concentration tested. Ro 25-6760 (1.0 and 10 nM) inhibited growth by 25 ± 0.5% and 21.5 ± 4.9%, respectively. The differences between control and treated groups were statistically significant (P < 0.05).

Cis- and carboplatin inhibited the growth of LNCaP and DU-145 cells in a dose-dependent manner as shown in Fig. 1, C and D. The degrees of growth inhibition induced by 0.2–200 μg/ml of carboplatin were similar to those induced by 0.02–2.0 μg/ml cisplatin. For example, in LNCaP cells, the highest dose of cisplatin (2.0 μg/ml) induced a 76.3 ± 5.7% growth inhibition, and the highest dose of carboplatin (200 μg/ml) gave a 77.3 ± 5.3% inhibition. The lower doses of each drug (0.2 μg/ml cisplatin and 2.0 μg/ml carboplatin) induced a 39.63 ± 0.0% and 35.3 ± 6.3% inhibition of growth, respectively. The differences between control and treated groups were statistically significant (P < 0.05).

**Fig. 2** Combined effects of 1α,25(OH)2D3 and cisplatin on the growth of LNCaP and DU145 cells. Cells were plated in medium containing 10% FBS and were allowed to attach to the surface overnight. The plating medium was removed and replaced with medium containing various concentrations of cisplatin in the presence of 0.1, 1.0, or 10 nM 1α,25(OH)2D3. The cells were allowed to grow for 4 days. The total DNA content of the cells in each well was determined using the Hoechst 33258 assay as described previously (33). Growth inhibition was calculated as the percentage of difference of the treated cells compared with vehicle controls. Each experiment was carried out in triplicate. Columns, mean of three wells; bars, SE. A, 0.1 nM 1α,25(OH)2D3 with cisplatin; B, 1.0 nM 1α,25(OH)2D3 with cisplatin; C, 10 nM 1α,25(OH)2D3 with cisplatin.

Statistics were performed on 20,000 events per sample using Modfit 5.2 software (Verity Software House, Inc., Topsham, ME).

**Synergistic PC Growth Inhibition by Vitamin D and Platinum**

Research.
Effects of Combination Treatment with Both 1α,25(OH)2D3 and Ro 25-6760 and Platinum. To assess the effects of combination treatment on the growth of LNCaP and DU-145 cells by 1α,25(OH)2D3 or Ro 25-6760 combined with either cis- or carboplatin, LNCaP (1.0 × 10³ cells/well) and DU-145 (0.25 × 10³/cm²) cells were incubated with various concentrations of the platinum drug in the presence or absence of 0.1, 1.0, and 10 nM 1α,25(OH)2D3 or Ro 25-6760 for 4 days and assayed.

Fig. 2 shows the growth inhibition of LNCaP and DU-145 cells after treatment with the combination of cisplatin and 1α,25(OH)2D3. In LNCaP cells, the increase in growth inhibition with the addition of 1.0 and 10 nM 1α,25(OH)2D3 over that of cisplatin by itself was statistically significant (P < 0.05) at all platinum concentrations except 2.0 µg/ml, where inhibition by cisplatin was already nearly 100%. At 1.0 nM 1α,25(OH)2D3, the enhancement ranged from 0% at 2.0 µg/ml to 75% at 0.02 µg/ml, and at 10 nM 1α,25(OH)2D3, the enhancement was nearly 200% at 0.02 µg/ml cisplatin. Although 1α,25(OH)2D3 was not able to inhibit the growth of DU-145 cells by itself, the growth inhibition obtained with the combination of 0.1 and 1.0 nM 1α,25(OH)2D3 and 0.02 µg/ml cisplatin over that of 0.02 µg/ml cisplatin alone was statistically significant (P < 0.05). Similar results for each cell line were obtained using carboplatin (data not shown).

Fig. 3 shows the growth inhibition of LNCaP and DU-145 cells after treatment with the combination of cisplatin and Ro 25-6760. For LNCaP cells, the increase in growth inhibition with the addition of 1.0 and 10 nM Ro 25-6760 over that of cisplatin by itself was statistically significant (P < 0.05) at 0.02 and 0.2 µg/ml cisplatin. At 1.0 nM Ro 25-6760, the enhancement ranged from 0% at 2.0 µg/ml to 147% at 0.02 µg/ml cisplatin. In DU-145 cells treated with 0.1, 1.0, or 10 nM Ro 25-6760 and 0.02 µg/ml cisplatin, the growth inhibition obtained over that of 0.02 µg/ml cisplatin by itself was significantly significant (P < 0.05). Similar results were obtained in both cell lines combining Ro 25-6760 with carboplatin. In view of these findings, isobologram analysis was performed.

Isobologram Analysis of the Effects of Combination Treatment with 1α,25(OH)2D3 and Platinum. The data from Fig. 2 showing that 1α,25(OH)2D3 and platinum compounds interacted most effectively at lower concentrations of platinum were used to design an isobologram experiment to determine whether the two drugs are capable of acting synergistically. Therefore, the following ranges were chosen: 0.2–1.2 nM 1α,25(OH)2D3; 0.005–0.025 µg/ml cisplatin; and 0.2–1.2 µg/ml carboplatin. Fig. 4A shows an isobologram for the interaction of 1α,25(OH)2D3 and cisplatin in the LNCaP cell line. The ID₅₀ and 95% confidence limits for 1α,25(OH)2D3 alone (1.0 ± 0.1 nM) and for cisplatin alone with vehicle controls. Each experiment was carried out in triplicate. Columns, mean of three wells; bars, SE. A, 0.1 nM Ro 25-6760 with cisplatin; B, 1.0 nM Ro 25-6760 with cisplatin; C, 10 nM Ro 25-6760 with cisplatin.

Fig. 3 Combined effects of Ro 25-6760 and cisplatin on the growth of LNCaP and DU-145 cells. Cells were plated in medium containing 10% FBS and were allowed to attach to the surface overnight. The plating medium was removed and replaced with medium containing various concentrations of cisplatin in the presence of 0.1, 1.0, or 10 nM Ro 25-6760. The cells were allowed to grow for 4 days. The total DNA content of the cells in each well was determined using the Hoechst 33258 assay as described previously (33). Growth inhibition was calculated as the percentage of difference of the treated cells compared

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Cells in each well was measured after 4 days using the Hoechst 33258 staining assay. The total DNA content of the cells in each well was measured after 4 days using the Hoechst 33258 assay (33). The final concentration that produced each ID$_{20}$ was determined with incubations of 0.0–2.0 nM $1\alpha,25$(OH)$_2$D$_3$ (shown on the x-axis of each plot), 0.0–0.05 μg/ml cisplatin (shown on the y-axis of A), and 0.0–1.5 μg/ml carboplatin (shown on the y-axis of B). Combined drug ID$_{20}$s were determined by incubating with multiple concentrations of $1\alpha,25$(OH)$_2$D$_3$ and cisplatin or $1\alpha,25$(OH)$_2$D$_3$ and carboplatin. The combined drug ID$_{20}$s and 95% confidence limits were plotted as a single data point along the fixed drug ratio line; bars, SE. A, isobologram with $1\alpha,25$(OH)$_2$D$_3$ and cisplatin; B, isobologram with $1\alpha,25$(OH)$_2$D$_3$ and carboplatin.

Fig. 4  Isobologram plots of the interactions between $1\alpha,25$(OH)$_2$D$_3$ and platinum compounds on LNCaP cells. Cells ($1 \times 10^6$/cm$^2$) were plated in medium containing 10% FBS and were allowed to attach to the surface overnight. The plating medium was removed and replaced with medium containing various concentrations of $1\alpha,25$(OH)$_2$D$_3$ and cisplatin or $1\alpha,25$(OH)$_2$D$_3$ and carboplatin. The total DNA content of the cells in each well was measured after 4 days using the Hoechst 33258 assay (33). The final concentration that produced each ID$_{20}$ was determined with incubations of 0.0–2.0 nM $1\alpha,25$(OH)$_2$D$_3$ (shown on the x-axis of each plot), 0.0–0.05 μg/ml cisplatin (shown on the y-axis of A), and 0.0–1.5 μg/ml carboplatin (shown on the y-axis of B). Combined drug ID$_{20}$s were determined by incubating with multiple concentrations of $1\alpha,25$(OH)$_2$D$_3$ and cisplatin or $1\alpha,25$(OH)$_2$D$_3$ and carboplatin. The combined drug ID$_{20}$s and 95% confidence limits were plotted as a single data point along the fixed drug ratio line; bars, SE. A, isobologram with $1\alpha,25$(OH)$_2$D$_3$ and cisplatin; B, isobologram with $1\alpha,25$(OH)$_2$D$_3$ and carboplatin.

(0.020 ± 0.002 μg/ml) are shown, connected by diagonal lines. The ID$_{20}$ and 95% confidence limits for the combined drugs were plotted along a line beginning at the origin and extending along a fixed-drug ratio of 50:1, where the dose of platinum is plotted on the x-axis and the dose of $1\alpha,25$(OH)$_2$D$_3$ is plotted on the y-axis. The ID$_{20}$ for the combined drugs (0.39 ± 0.02 nM $1\alpha,25$(OH)$_2$D$_3$ and 0.0075 ± 0.0007 μg/ml cisplatin) falls below the line connecting the single drug ID$_{20}$s, indicating that $1\alpha,25$(OH)$_2$D$_3$ and cisplatin interact synergistically at the given concentrations to inhibit the growth of LNCaP cells. Fig. 4B shows an isobologram for the interaction between $1\alpha,25$(OH)$_2$D$_3$ and carboplatin in the LNCaP cell line. The ID$_{20}$ for $1\alpha,25$(OH)$_2$D$_3$ by itself was 1.0 ± 0.1 nM, and the ID$_{20}$ for carboplatin by itself was 1.0 ± 0.1 μg/ml. When the two drugs were tested in combination, the ID$_{20}$ for $1\alpha,25$(OH)$_2$D$_3$ was 0.275 ± 0.02 nM, and the ID$_{20}$ for carboplatin was 0.25 ± 0.02 μg/ml. When plotted along the 1:1 fixed-drug ratio line, the combination of the two drugs again fell below the line connecting the single-drug ID$_{20}$s, indicating that $1\alpha,25$(OH)$_2$D$_3$ and carboplatin also act synergistically to inhibit the growth of LNCaP cells at the given concentrations.

Cell Cycle Analysis of the Combination Treatment of $1\alpha,25$(OH)$_2$D$_3$ and Carboplatin. To understand the growth-inhibitory effects of the combination treatment of $1\alpha,25$(OH)$_2$D$_3$ and carboplatin on PC cells, we assayed cell cycle distribution of LNCaP cells after treatment. Table 1 gives the percentage of cells that are in either the G$_1$ or the G$_2$ phase of the cell cycle after each treatment at the given time. Results from a representative experiment using flow cytometry of propidium iodide-stained cells are shown in Fig. 5.

Table 1 demonstrates that with increasing time, the combination of $1\alpha,25$(OH)$_2$D$_3$ and carboplatin significantly increases the percentage of cells in the G$_2$-M phase of the cell cycle. This trend correlates with an apparent decrease in the amount of cells in G$_1$. The percentage of cells in S phase does not change significantly. After 6 days of treatment with both 10 nM $1\alpha,25$(OH)$_2$D$_3$ and 2 μg/ml carboplatin, the percentage of cells in G$_2$ decreased from 4.83% in the vehicle control to 32.2%. Treatment with $1\alpha,25$(OH)$_2$D$_3$ by itself resulted in 8.7% G$_2$, and carboplatin by itself had 22.7% of the cells in G$_2$. These results indicate that the combination treatment of $1\alpha,25$(OH)$_2$D$_3$ and carboplatin on PC cells increases the amount of cells in the G$_2$-M phase of the cell cycle over that with either drug alone.

DISCUSSION

PC cells possess functional receptors for $1\alpha,25$(OH)$_2$D$_3$ and display a significant amount of growth inhibition in response to $1\alpha,25$(OH)$_2$D$_3$ treatment (12–16). These receptors are necessary for mediating the antiproliferative effects of $1\alpha,25$(OH)$_2$D$_3$ on prostatic carcinoma cells (40, 41). Recent evidence demonstrates that $1\alpha,25$(OH)$_2$D$_3$ is able to potentiate platinum antitumor activity in nonprostatic systems (29, 30). These data led to the present study to evaluate the potential interaction between $1\alpha,25$(OH)$_2$D$_3$ and platinum agents in inhibiting the growth of PC cells.

The two cell lines used in this study both represent advanced-stage PC. Although LNCaP was derived from an androgen-refractory patient (31), it retains some degree of androgen response, whereas DU-145 does not. LNCaP responds to $1\alpha,25$(OH)$_2$D$_3$ treatment (12–16). These receptors are necessary for mediating the antiproliferative effects of $1\alpha,25$(OH)$_2$D$_3$ and its synthetic analogues (19–22). However, the proliferation of DU-145 is inhibited to a greater degree by synthetic analogues than the parent compound (19–22). Together, therefore, these cell lines represent a range of responses that might be encountered in advanced clinical disease.
Platinum analogues have become the mainstay of treatment for many tumors including ovarian cancer, lung cancer, germ cell tumors, head and neck cancer, bladder cancer, and to a lesser degree breast cancer and gastric cancer (42). Cisplatin was followed clinically by carboplatin, a second-generation analogue with comparable antitumor activity to the original compound. However, carboplatin is less toxic to the kidneys, gastrointestinal tract, and peripheral nerves (42). Cisplatin responses in advanced stage PC range in the literature from a partial response of 43% for a median duration of 5.8 months in a weekly cisplatin study (25) to no objective response in another study in which patients were treated with a single dose of cisplatin every three weeks (26). Carboplatin therapy for advanced-stage PC has also been studied. In an Eastern Cooperative Oncology Group pilot study using standard dose carboplatin, no important activity in hormone-refractory PC was observed (27). However, The National Institute for Cancer Research in Genoa, Italy obtained better results using a weekly regime of carboplatin treatment. In their study, 17% partial response and 50% disease stabilization was reported in 25 evaluable patients (28). This is likely plausible because it has been recently found that patients with advanced-stage prostate cancer are frequently vitamin D deficient (43).

Platinum analogues are believed to exert their cytotoxic effects by forming covalent, bifunctional adducts between adjacent guanosine residues in DNA (23, 24). This alteration inhibits DNA replication and results in cell death. However, the precise mechanisms through which platinum inhibition of DNA replication leads to cell death remain poorly understood. Inhibition of DNA synthesis has been observed in cells after platinum treatment, leading to the conclusion that cells actively synthesizing DNA are most sensitive to platinum therapy (44). However, Donaldson et al. (45) determined that tumor cells or fibroblasts in G1-S, just prior to DNA replication, are the most sensitive to cisplatin cytotoxicity. Furthermore, the cells that are blocked in G1-S have been shown to remain maximally sensitized upon release with progression through the cell cycle. The reported ability of 1α,25(OH)2D3 to exert a G1-S phase block (46–49) in tumor cells and the potential of platinum-mediated tumor cell toxicity in relation to the cell cycle suggest the possibility of interaction between these two agents.

Comparison of inhibition of PC cell growth by platinum alone versus 1α,25(OH)2D3 or its analogue in combination with platinum compounds (Figs. 1–3) demonstrates a substantial enhancement of

Table 1  Cell cycle analysis of LNCaP cells after drug treatment

LNCaP cells were plated and treated with vehicle control, 10 nm D3, 2 μg/ml carboplatin, or 10 nm D3 + 2 μg/ml carboplatin for the indicated time. Cells were harvested and subjected to flow cytometric analysis. Data are presented as the mean %± SE from four independent experiments.

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* Differences in percentage of cells were found to be significant (P < 0.001) with respect to both time and drug combination.
growth inhibition at clinically relevant concentrations of cisplatin (0.02 to 2.0 μg/ml) and at the lower concentrations of carboplatin (0.2 to 2.0 μg/ml). At the highest concentrations of the platinum compounds, in excess of what would be attainable in patients, the platinum drugs were cytotoxic, and no enhancement of growth inhibition by 1α,25(OH)2D3 or Ro 25-6760 was observed. Furthermore, isobologram analysis of combination effects at clinically relevant concentrations of either platinum compound and 1α,25(OH)2D3 showed the drugs to act in a synergistic manner. Although synergism between the two compounds was observed at low concentrations of platinum, the same was not seen with higher concentrations of the platinum compounds. These results are consistent with other findings that show that the degree of drug interaction is dependent upon drug concentrations and drug ratios (35). Our data further support the concept that conclusions regarding drug interactions must be specific to the circumstances examined.

An alternative hypothesis for the mechanism of interaction between the two drugs was raised when the combination of 1α,25(OH)2D3 and cisplatin was examined using HL60 leukemic cells (50). That study showed that the binding of DNA and cisplatin was increased more than 10-fold by 100 nM 1α,25(OH)2D3, suggesting that 1α,25(OH)2D3 altered chromatin structure by making DNA more accessible to cisplatin, thereby enhancing cisplatin binding. Adducts formed in the DNA by platinum compounds may also disrupt gene expression by tetrating or “hijacking” transcription factors from normal sites of action (51). However, if the vitamin D receptor is “hijacked” in PC cells, this would diminish the antiproliferative effects of vitamin D. Possibly, this problem could be overcome by presenting a higher concentration of ligand to the cells. As mentioned previously, clinical data indicate that advanced-stage PC patients are often vitamin D deficient (43). It is likely, therefore, that for clinical synergism to occur, serum levels of 1α,25(OH)2D3 would have to be increased prior to initiating, and maintained during, platinum-based chemotherapy.

Our data demonstrate that the combination of 1α,25(OH)2D3 and platinum results in an increase in the number of cells in the G2-M phase of the cell cycle. Although it is believed that platinum treatment by itself causes an accumulation of cells in G2-M (52), the enhancement by 1α,25(OH)2D3 raises a new possibility regarding the mode of interaction between these two drugs. Additional experiments are necessary to determine the exact mechanism regarding the synergistic activity seen between platinum compounds and 1α,25(OH)2D3 in the growth inhibition of PC cells.

In conclusion, this study demonstrates that the combination of 1α,25(OH)2D3 and platinum compounds is capable of producing a synergistic effect in the growth inhibition of PC cells in vitro. These results provide a basis to investigate this therapeutic approach in patients with advanced-stage PC in the clinical setting. Further insight into the mechanism involved in the interaction between 1α,25(OH)2D3 and platinum compounds will optimize additional treatment designs and will expand the potential utility of 1α,25(OH)2D3 in the treatment of PC.

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REFERENCES


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