Progestins Inhibit Calcitriol-induced CYP24A1 and Synergistically Inhibit Ovarian Cancer Cell Viability: An Opportunity for Chemoprevention

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24 Key Words: ovarian cancer; chemoprevention; progestin; vitamin D; CYP24A1

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Abstract

Objectives. Previously we have shown in endometrial cells that progesterone (P4) and calcitriol (CAL, 1,25(OH)₂D₃) synergistically promote apoptosis and that progestins induce expression of the vitamin D receptor. In the current study we examined the progestin/vitamin D combination in ovarian cells and searched for other progestin-related effects on vitamin D metabolism that may underlie the novel interaction between progestins and vitamin D, including whether progestins inhibit *CYP24A1*, the enzyme that renders CAL inactive.

Methods. We investigated the impact of P4 on CAL-induced *CYP24A1* expression in cancer cell lines expressing progesterone receptors (PRs), [OVCAR-5, OVCAR-3-PGR (PR-transfected OVCAR-3 ovarian line), and T47D-WT, T47D-A and T47D-B (breast lines expressing PRs or individual PR isoforms)] or lines that do not express PRs (OVCAR-3 and T47D-Y). We examined *CYP24A1* expression using RT-PCR and western blotting, and apoptosis by TUNEL. We also investigated P4 inhibition of *Cyp24a1* in ovaries from CAL-treated mice.

Results. CAL treatment induced CYP24A1 expression. When co-treated with P4, cell lines expressing PRs showed marked inhibition of *CYP24A1* expression ($p < 0.001$), along with increased apoptosis ($p < 0.01$); cells not expressing PRs did not. Mouse ovaries showed a significant reduction in CAL-induced *Cyp24a1* mRNA ($p < 0.001$) and protein ($p < 0.01$) in response to P4.

Conclusions. We show for the first time that progestins and vitamin D synergistically reduce cell viability and induce apoptosis in ovarian cells and that progestins PR-dependently inhibit CAL-induced *CYP24A1*, thus extending CAL activity. The combination of progestins and vitamin D deserves further consideration as a strategy for inhibiting ovarian carcinogenesis.

1. Introduction

There is immense potential to decrease ovarian cancer incidence and mortality through prevention. Extensive epidemiological evidence has shown that routine use of the combination estrogen-progestin oral contraceptive pill (OC) confers a remarkable 30-50% reduced risk of ovarian cancer [1]. Based on our research findings, we believe the progestin component of the OC is functioning as a chemopreventive agent by activating potent and well-known molecular pathways such as apoptosis and transforming growth factor-beta (TGF- β) signaling in the genital tract [2, 3]. Our animal research findings are supported by human data demonstrating that progestin-potent OCs confer twice the protective effect against ovarian cancer as OCs containing weak progestins [4]. These human data suggest that enhancing progestin potency will confer enhanced ovarian cancer preventive efficacy.

Progestin potency can be enhanced by either increasing the dosage of progestin or by selecting a pharmacologically potent progestin. Both of these approaches, however, may increase side effects that would be undesirable for long-term chemoprevention. An alternative strategy would combine a progestin with a second preventive agent that is both non-toxic and enhances progestin potency. In this regard, there is epidemiological and laboratory evidence in support of vitamin D, which is non-toxic, for the prevention of malignancy including ovarian cancer, making vitamin D an attractive second agent.

The beneficial effects of vitamin D are due to the activity of its dihydroxylated metabolite, 1,25(OH)₂D₃ (“calcitriol”, CAL), the active form of the molecule. The human body obtains vitamin D (specifically vitamin D₃ or “cholecalciferol”, chole) through synthesis by skin exposed to sunlight or orally through the diet. Of these two sources, however, very little is supplied by the diet as few foods contain appreciable amounts of vitamin D. Through a series of

enzymatic reactions, vitamin D₃ is converted to 1,25(OH)₂D₃, which binds to the vitamin D receptor (VDR). Vitamin D-responsive genes then confer a number of chemopreventive effects, including cell cycle arrest, apoptosis and differentiation in a variety of cells, including prostate, breast, colon, and ovarian [5, 6]. Notably, the VDR is expressed ubiquitously throughout most epithelia. Additionally, via expression of the 1-alpha hydroxylase enzyme, many normal tissues convert circulating 25(OH)D₃ to 1,25(OH)₂D₃, which acts in an autocrine or paracrine fashion to regulate cell growth and biology. Once 1,25(OH)₂D₃ confers its local effect, it induces production of vitamin D-24-hydroxylase (CYP24A1), which catalyzes its conversion to inactive metabolites.

Worldwide, the geographic distribution of ovarian cancer shows increasing incidence commensurate with distance from the equator [7]. Similarly, in the U.S. a North-South gradient favors a higher ovarian cancer risk in Northern versus Southern latitudes, demonstrating a statistically significant inverse correlation between regional sunlight exposure and ovarian cancer mortality [8]. Given that sunlight induces production of pre-vitamin D₃ in the skin, it is interesting to speculate that vitamin D might confer protection against ovarian cancer via direct chemopreventive biologic effects in the non-malignant ovarian epithelium, similar to that induced by progestins. For example through induction of apoptosis and/or TGF- β (TGFB) in the ovarian epithelium, vitamin D may cause the selective removal of non-malignant, but genetically damaged ovarian epithelial cells [9, 10]. A small case-control study supports the notion that vitamin D confers ovarian cancer prevention, at dosages easy to achieve through the diet. Compared to a low dietary intake of vitamin D, a high dietary intake of vitamin D was associated with a 50% reduction in ovarian cancer risk [11].

Recently, we reported that progesterone (P4) and CAL have synergistic inhibitory effects on cell viability in cells derived from the endometrium, characterized by a marked increase in apoptosis [12]. We also demonstrated that progestin increases expression of the vitamin D receptor, thereby providing a potential mechanism underlying the novel interaction between the two agents. In this study, we sought to characterize the effect of progestins on vitamin D metabolism in cells derived from the ovarian epithelium, and we report that progestins inhibit *CYP24A1*, the enzyme that catalytically degrades vitamin D. We also report that vitamin D and progestins synergistically reduce ovarian cancer cell viability.

2. Materials and Methods

2.1 Cell lines and treatment

All culture media, sera and reagents were purchased from Invitrogen except insulin and CAL, which were purchased from Sigma. CB1089, a stable analog of CAL with a functional Kd of 3.4 nM (compared with 0.9 nM for CAL) [13], was kindly provided by Cougar Biotechnology, Inc. (now a division of Johnson and Johnson). The parental ovarian cancer cell line OVCAR-3 (purchased from ATCC) was grown in RPMI 1640 medium with glutamine supplemented with 10% heat-inactivated FBS, 10 µg/ml insulin and penicillin/streptomycin. The P4 receptor (PR, progesterone receptor *PGR*)-transfected cell line OVCAR-3-PGR, and the PRA-positive OVCAR-5 cell line (kindly provided by Dr Tom Hamilton, Fox Chase Cancer Center, Philadelphia, PA) were grown in the same medium as OVCAR-3 cells without the penicillin/streptomycin. The T47D-wild type breast cancer cell line known to express both PRA and PRB (purchased from ATCC) and transfected lines, T47D-YA (expresses only PRA), T47D-YB (expresses only PRB), and T47D-Y (expresses neither PR) were a gift from Dr. Javier

Menendez (Catalan Institute of Oncology, Girona, Spain). All T47D cell lines were grown in Iscove's Modified Eagle Medium and 10% FBS. T47D-YA and T47D-YB were routinely subjected to 200 µg/ml geneticin and tested for PRs by western blot. Cell lines were maintained in 5% CO₂ at 37°C.

2.2 Transfection with progesterone receptor (PGR) gene – OVCAR-3-PGR cells

OVCAR-3 cells were transfected with pCMV6-AC-GFP expressing PR (OriGene) using Lipofectamine 2000 (Life Technologies, Grand Island, NY). The plasmid was amplified in *Escherichia coli* (*E. coli*) and purified using a QIAfilter Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions.

The plasmid was then confirmed by endonuclease digestion using EcoRI/XhoI restriction enzymes prior to transfection. 100 µg/ml geneticin (Invitrogen) was used to select and maintain the OVCAR-3-transfected cells. The presence of PR in the transfected cells was confirmed and monitored by western blot.

2.3 Cell viability testing

Cells were seeded overnight in 96-well plates at 2500–5000 cells/well. The culture medium was replaced with fresh medium containing experimental agents (P4, CAL, or CB1089 in varying concentrations). Cells were treated for 72 hours, at which time untreated controls were nearly confluent. All treatments were performed in quadruplicate. Viability was tested using the formazan dye-based MTS assay (Promega, Madison, WI) according to the manufacturer's specifications. The plates were incubated at 37°C until the untreated wells exhibited an A₄₉₀ of 0.7-0.9. Wells containing medium alone were used as blanks. Viability was

expressed as a percentage of untreated controls. All experiments were repeated at least three times.

2.4 CYP24A1 siRNA transfection and cell viability

OVCAR-3-PGR cells were plated in 96-well plates for MTS assays and 6-well dishes for western blots. They were grown in RPMI medium with 10% heat-inactivated FBS, 1% glutamine and 1% insulin. After allowing the cells to settle for 24 hours, they were transfected with 40nM control siRNA (GE Dharmacon, Lafayette, CO; ON-TARGET control pool cat# D-001810-05) or 40nM CYP24 siRNA (Dharmacon, ON-TARGETplus SMART pool Human CYP24A1 siRNA, cat# L-009269-02), using Dharmfect 1 Transfection reagent (Dharmacon, cat# T-2001-01). Cells were transfected in OptiMEM medium (Life Technologies, cat# 31985-070) for 48 hours. Upon completion of the 48-hour transfection, cells were treated for 72 hours with 100 nM CAL and 30 μ M progesterone, alone or in combination, in RPMI 1640 medium (Life Technologies, cat# 11835-030), 10% charcoal-stripped serum, 1% glutamine and 1% insulin. Viability was tested using MTS assays as described above in section 2.3.

2.5 Detection of apoptosis by TUNEL assay

OVCAR-3 parental and OVCAR-3-PGR cells were seeded into 60 mm dishes at 2.8×10^5 cells/dish and incubated overnight. The culture medium was then replaced with fresh medium containing P4 with and without CAL, and incubated for 48 hours. Vehicle concentrations did not exceed 0.32% for ethanol. All cells, floating and adherent, were collected, washed, fixed and stained by deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) using the APO-Direct kit (BD Pharmingen, San Jose, CA), according to the manufacturers' protocols. 10,000 cells

were measured by FACS Calibur (BD Biosciences) and the data were analyzed using Cellquest 3.3 software. FITC fluorescence was collected through a 488 bandpass filter and forward and side scatter were analyzed on a linear scale. For the TUNEL assay, each sample was gated to include only a singlet population using dual parameters of DNA width (x-axis) and DNA area (y-axis). In assessing apoptosis, the resultant gates were used to generate dual parametric graphs of DNA area (x-axis) and FITC-dUTP (y-axis). Gates were based on increased dUTP-FITC labeling compared to the untreated controls. Results are expressed as fold-increase over controls. Experiments were repeated at least three times and/or run in triplicate.

2.6 Expression of mRNA

OVCAR-3, OVCAR-3-PGR, OVCAR-5 and the four types of T47D cells (T47D-WT, T47D-YA, T47D-YB and T47D-Y) were seeded as described for the TUNEL assay. The culture medium was then replaced with fresh medium containing P4 with and without CAL and incubated for 1, 4 or 6 hours. Total RNA was extracted from cell lines with Tri-Reagent (Molecular Research Center, Cincinnati, OH) and RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's suggested protocols. RNA was reverse transcribed using Taqman® Reverse Transcription Reagents (Life Technologies, Foster City, CA). The reverse transcription reaction consisted of a 10-min step at 25°C, followed by a 1-h incubation at 37°C and concluding with a 5-min step at 95°C. Real time QPCR was performed using Taqman® Assays for *CYP24A1*, *CYP27B1*, *VDR*, and the control gene *GAPDH*, and Taqman® Gene Expression Master Mix (Life Technologies). The Life Technologies assays for human genes were Hs00167999_m1 (*CYP24A1*); Hs01096154_m1 (*CYP27B1*); Hs00172113_m1 (*VDR*); and 402869 (*GAPDH*). These real time reactions were conducted on a Stratagene MX3000P Real

Time machine under the Life Technologies conditions for comparative QPCR. Gene expression was calculated using the $\Delta\Delta C_t$ method. Each experiment was repeated six times.

2.7 CYP24A1 protein assay

OVCAR-3, OVCAR-3-PGR, OVCAR-5 and the four types of T47D cells were seeded as described for the TUNEL assay. The culture medium was then replaced with fresh medium containing P4 with and without CAL and incubated for 6, 16 or 24 hours. In the inhibitor experiments, cells were incubated with the PR inhibitor RU486 for 2 h prior to the addition of P4 and CAL and then incubated for an additional 6 h. The cells were rinsed twice in PBS and lysed in buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.4% IGEPAL and 1:200 Protease Inhibitor Cocktail Set 2). All reagents were purchased from Sigma-Aldrich except the protease inhibitors (EMD Millipore). Cells were then incubated at 0°C for 30 min with frequent vortex mixing. Lysates were centrifuged at maximum speed for 15 min. Protein concentrations were determined by the DC Protein Assay Kit (Bio-Rad Laboratories). Equal amounts of protein from cell lysates were separated by 7.5% SDS-PAGE and then analyzed by western blotting. Blots were incubated overnight in CYP24A1 antibody (kindly donated by Cytochroma), 3 ug/ml PR antibody (PgR 1294, Dako, Carpinteria, CA), or in HRP-conjugated actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were purchased from Cell Signaling Technology and Luminol from Santa Cruz Biotechnology. Luminol was used for all antibody detection except for the PR antibody, which was detected by SuperSignal West Femto Chemiluminescent Substrate (Thermo-Fisher). Protein bands were quantified by densitometry (LabWorks) and normalized using actin as a loading control. Experiments were either repeated 3 times and/or run in triplicate.

2.8 Determination of synergy

Data were analyzed isobolographically using CalcuSyn software (Biosoft) to characterize the inhibitory effect of treatment agents on the cell lines. Raw data for each agent or combination was entered singly to generate a median effect plot. From this plot, the combination index (CI) equation was applied to determine whether the drug effects were additive, synergistic or antagonistic. CI values of <1 , $=1$ or >1 indicated synergy, additivity or antagonism, respectively.

2.9 Animals and treatments

Six-week-old, 18-22 gram, female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME), housed in 12-h light/dark cycles, and allowed free access to water and standard rodent chow. Animal care and the conduct of all experiments were approved by the NorthShore University HealthSystem Research Institute Animal Care and Use Committee.

Mice in the first experiment were randomized to four groups of 10 and were treated by intraperitoneal injection with P4 (1 mg/mouse), CAL (400 ng/mouse), combined P4 and CAL, or vehicle (corn oil). These doses were based on efficacy in mice of P4 on a biologic endpoint [14], and of CAL to induce CYP24A1 [15]. For the combination treatment, CAL was injected 30 min following P4 injection. All mice were euthanized 4 hours after the CAL treatment. Ovaries were collected from each mouse and frozen for molecular analysis. A second set of four groups of mice was treated the same way but tissues were collected 16 hours after the CAL treatment and frozen for analysis of *CYP24A1* mRNA and protein.

Total RNA was isolated from one ovary from each animal using the RNeasy Micro kit (#74004, Qiagen). *CYP24A1* mRNA was quantified by RT-PCR as described above in section 2.6. CYP24A1 protein was assayed by Western blot in one ovary from each mouse. Ovaries

weighing approximately 4 mg each were homogenized in 80 μ l RIPA Lysis Buffer System (Santa Cruz) using a Qiagen TissueLyser II for 30 seconds twice at 20 Hz. The homogenate was centrifuged twice at 13.2 x g and then 30 μ g protein was separated, blotted, and detected as described above in section 2.7.

2.8 Statistical analysis

Results are presented as mean $\pm$ SE and were compared statistically with $p < 0.05$ considered significant. Multiple group comparisons were made using an ANOVA with post hoc Newman-Keuls, and a Student's t-test was used when comparing two groups. All tests were two-tailed.

3. Results

3.1 Impact of vitamin D and progesterone on the viability of parental OVCAR-3 and PR-transfected (OVCAR-3-PGR) cell lines

OVCAR-3 parent cells do not express classical nuclear PRs (Fig. 1A), although they do express membrane progesterone receptors. Treatment with P4 alone only modestly reduced viability of the parental OVCAR-3 cells in a 3-day MTS assay, and only at the highest dose (Fig. 1B). In combination with CAL, viability was reduced dose-dependently at all doses of P4, demonstrating an inhibitory effect of P4 and vitamin D on cell viability of the parental OVCAR-3 cells, but viability remained at 60% or greater and the inhibitory effect with combined P4 and CAL did not exhibit synergy. Even in a 6-day MTS assay, the viability of the PR-negative OVCAR-3 parent cells remained at 50% or greater with P4 and CAL (Fig. 1C). The reduced viability by all doses of P4 in combination with CAL, as well as by P4 alone at 30 μ M, compared

to controls demonstrated only moderate synergy between CAL and 25 μ M P4 (IC=0.852) at this extended time.

In contrast, in OVCAR-3 cells transfected with PRs (OVCAR-3-PGR), synergistic effects of CAL and P4 on cell viability were clearly observed at 3 days (Fig. 1D). We stably transfected the parental OVCAR-3 cells with PRs and used this line (OVCAR-3-PGR) in MTS and CYP24A1 assays. Fig. 1A demonstrates the expression of both PRA and PRB in this cell line. P4 alone and in combination with CAL produced a dose-dependent reduction of cell viability to less than 70% at its lowest dose and to less than 30% at the highest dose (Fig. 1D). Isobolographic analysis indicated synergy between P4 and CAL at 30 and 40 μ M P4 (CI=0.711 and 0.695, respectively).

We tested the specificity of CYP24A1 inhibition on cell viability, using CYP24A1 siRNA transfection of OVCAR-3-PGR cells. The results show that CYP24A1 siRNA reduced the viability of CAL-treated cells by 20% ($p<0.001$), compared with scrambled (control) siRNA and CAL treatment. CYP24A1 siRNA transfection in cells treated with combined CAL and P4 reduced viability approximately 13% more ($p<0.001$) than the reduced viability in similarly treated cells transfected with scrambled siRNA (Fig. 2).

A synthetic analog of CAL, CB1089, has a greater stability and fewer calcemic side effects than does CAL and could provide an advantage for clinical use. We tested the comparative effects of CAL and CB1089, in combination with P4 on cell viability. In a P4 dose-response experiment, CAL exhibited significantly greater efficacy to reduce cell viability than did CB1089 (Supplement S1A). The effect of P4 in combination with CAL was dose-dependent only at 25 and 30 μ M showing equal efficacy at 30-40 μ M P4. Viability of OVCAR-3 cells with either of these compounds was not reduced below 60% with any of the treatment doses tested.

Similarly, in OVCAR-3-PGR cells, CB1089 was less effective than was CAL, reducing cell viability to approximately the same extent in OVCAR-3-PGR cells as it did in OVCAR-3 parental cells (Supplement S1B).

Finally, a TUNEL assay was conducted in cells with and without PRs. No significant changes in TUNEL-positivity were observed when the parental OVCAR-3 cells were treated with the vehicle, with P4, CB1089 or the combination of P4 and CB1089 (Supplement S1C). OVCAR-3-PGR cells, in contrast, exhibited a significant response to P4 with and without CB1089, and TUNEL-positive cells increased 2-2.5-fold compared with controls.

These initial assays at higher concentrations of P4 were conducted to determine the relative vulnerabilities of the cells to the treatments. Subsequent experiments to test the impact of the treatments on CYP24A1 expression were conducted at lower, more pharmacologic concentrations.

3.2 Progesterone receptor dependence of the effect of progesterone on vitamin D-induced CYP24A1 expression

3.2.1 CYP24A1 in progesterone receptor-negative parental OVCAR-3 cells

CAL induced CYP24A1 mRNA expression in the parental OVCAR-3 cells as expected. In these cells lacking PRs, calcitriol-induced CYP24A1 expression was not reduced in response to P4 administered in combination with CAL at any time tested (Fig. 3A). Similarly, western blots showed no difference in CYP24A1 protein production when P4 was combined with CAL in these cells (Fig. 3B).

3.2.2 CYP24A1 in progesterone receptor-positive OVCAR-3-PGR and OVCAR-5 cells

We then tested PR dependence of the effect of P4 on CAL-induced CYP24A1 in PR-

expressing OVCAR-3-PGR cells. P4 significantly reduced *CYP24A1* mRNA expression at 4 and 6 h in these cells (Fig. 4A). Western blots showed that CYP24A1 protein production was stimulated by CAL at all times through 24 h and was significantly reduced by P4 treatment at 6 h but was increased at 16 h and not different from controls at 24 h (Fig. 4B). We compared the response of *CYP24A1* expression to P4 and CAL treatment in OVCAR-5 cells, an ovarian cancer cell line that expresses the progesterone receptor A isoform (PRA). The response of the OVCAR-5 cells was very similar to that of OVCAR-3-PGR cells (Fig. 4C-D). The similarity of results in the ovarian cancer cell lines compared with breast cancer cell lines (below) indicates the tissue specificity of response to P4 and CAL treatments.

3.2.3 *CYP24A1* in progesterone receptor-positive T47D cells

In order to determine whether PRA, PRB or both PR isoforms may be necessary for an effect of P4 on CAL-induced *CYP24A1* expression, we used a series of breast cancer cell lines (T47D-YA, -YB, -WT, -Y) which stably express PRA, PRB, both receptors, and neither receptor, respectively. In cells expressing either or both PRs, P4 strongly and significantly reduced CAL-induced *CYP24A1* mRNA expression. In contrast, P4 did not affect expression of *CYP24A1* in the PR-negative T47D-Y cells (Fig. 5A-D). Western analysis revealed a time-dependent as well as receptor-dependent process (Fig. 5E-H). CYP24A1 protein production was significantly reduced at 6 and 16 h post-treatment when PRA or PRB was present. When both receptors were present, CYP24A1 protein was significantly reduced at all times through 24 h. The shorter-lived result in OVCAR-3-PGR cells could be due to the higher PRA:PRB ratio in these cells compared to T47D-WT cells (an approximate 2:1 ratio versus a 1:1 ratio, data not shown), since PRA can inhibit PRB [16] and may limit the duration of the response. Similarly, the limited response in OVCAR-5 cells could be due to the absence of PRB, a quasi “inhibition”

of this receptor. Progesterone receptor blockade with RU486 prevented the inhibition of CAL-induced CYP24A1 expression by P4 (Supplement S2). Taken together, these results demonstrate the PR-dependence of the reduction of CAL-induced CYP24A1 by P4.

3.2.4 Cyp24a1 in normal mouse ovaries.

We conducted assays for CYP24A1 in mouse ovaries to determine whether P4 and CAL would inhibit expression of this enzyme *in vivo* as it did *in vitro*. Figure 6 shows a highly significant inhibition of CAL-induced *Cyp24a1* mRNA expression at 4 hours post-treatment. Inhibition of CYP24A1 protein production was delayed by several hours and showed significant inhibition by combined P4 and CAL at 16 hours post-treatment.

4. Discussion

A growing body of human and animal evidence supports a robust cancer preventive role for progestins in the upper gynecologic tract, including a) evidence of a marked reduction in ovarian, fallopian tube and endometrial cancers associated with routine use of progestin-containing contraceptives [1, 17], b) evidence of progestin activation of surrogate endpoint biomarkers relevant to chemoprevention in the ovary and endometrium [2, 3, 18], c) evidence that progestins effectively reverse endometrial hyperplasias, the most common precursor to endometrial cancer [18, 19] and d) prospective evidence in the chicken ovarian cancer animal model of a marked reduction of ovarian and oviductal cancers in hens treated with progestins [20].

Although the biologic mechanism(s) underlying the chemopreventive effect of progestins in the genital tract have not been fully characterized, the finding that progestins markedly activate programmed cell death and regulate TGF- β in the ovarian and uterine epithelia suggest a

mechanism whereby carcinogenesis is not only arrested but likely reversed via the clearance of genetically damaged cells [2, 3]. The current study demonstrates an additional mechanism by which progestins may confer chemopreventive effects. We have found that progestins markedly inhibit expression of *CYP24A1*. The cytochrome p450 enzyme CYP24A1 catalytically degrades the active form of vitamin D (1,25(OH)₂D₃) thereby limiting its anti-tumorigenic signaling. Progestin inhibition of *CYP24A1* therefore potentially enhances the robustness of vitamin D by prolonging its active half-life.

Dysregulation of vitamin D metabolism, and in particular of *CYP24A1*, is a common derangement associated with the whole spectrum of carcinogenesis [21]. The dysregulation can be due to both genetic and epigenetic mechanisms. Polymorphisms in *CYP24A1* impact the influence of vitamin D supplementation on 25(OH)D₃ levels, the main measure of vitamin D stores [22]. Additionally, specific polymorphisms in *CYP24A1* have been shown to be associated with either an increase in cancer risk or to significantly influence the inverse impact of 25(OH)D₃ levels on cancer risk [23-25]. Moreover, increased expression of *CYP24A1* has been shown to be associated with a number of cancers including those of the breast, lung, prostate, colon, kidney and thyroid, as well as with a more aggressive cancer biology [26-28], leading to the suggestion that altered *CYP24A1* is an oncogene [29].

These accumulated results have prompted recent efforts to develop CYP24A1 inhibitors specifically to target this molecule for cancer prevention or treatment [21, 30, 31]. Proposals include RNAi targeting of CYP24A1 to improve the clinical responsiveness of prostate cancer to vitamin D therapy [28]. Additionally, selective inhibitors of CYP24A1 have been developed to increase the anti-tumor effects of vitamin D [32]. For example, a protein kinase-selective inhibitor that disrupts CYP24A1 promoter activity has been proposed to enhance vitamin D anti-

tumor effects [33]. Calcitriol in combination with CYP24A1 inhibition has also been shown to reduce proliferation and to promote caspase-independent apoptosis [34]. These results suggest that inhibition of CYP24A1 may improve the efficacy of cancer therapy, and further, may have the potential for cancer prevention via enhanced vitamin D activity to eliminate the earliest neoplastic lesions.

Selective inhibition of CYP24A1 to enhance and/or restore the protective benefits of vitamin D has been proposed for cancer treatment or prevention by nutritional means. In the prostate and colon, it has been proposed that the markedly lower cancer incidence in Asia may be due to a beneficial effect of isoflavones on vitamin D metabolism [35-37]. The isoflavone genistein has been shown to inhibit *CYP24A1* transcriptionally, markedly enhancing expression of vitamin D-related genes, and leading to synergistic effects with vitamin D on cell cycle arrest and apoptosis [36-38]. Additionally, in DU145 prostatic cancer cells that are resistant to the inhibitory effects of vitamin D, inhibition of *CYP24A1* by genistein has been shown to reverse vitamin D resistance [39]. It is thus possible that a beneficial effect of progestins on vitamin D metabolism, leading to enhanced vitamin D potency, may be a mechanism underlying the protective effects of contraceptive use and pregnancy. The finding that progestins inhibit vitamin D-induced CYP24A1 *in vivo* in the normal ovary not only validates a novel interaction between the two agents but further provides an additional rationale for investigation of this combination of agents for ovarian cancer chemoprevention.

Our study is the first to demonstrate that the combination of a progestin and vitamin D synergistically inhibits cell growth and induces apoptosis in ovarian epithelial cells. The underlying mechanism(s) responsible for this synergy remain to be elucidated. It is possible that progestins and vitamin D target apoptotic and/or other molecular endpoints that converge to

399 achieve enhanced apoptosis. Alternatively, the mechanism may involve progestin inhibition of
400 *CYP24A1*, leading to markedly enhanced vitamin D potency and downstream vitamin D-related
401 transcriptional events.

402 Similar to chemotherapeutic treatment of full-fledged cancers, using multiple agents
403 working via different mechanisms, it is likely that an optimal cancer preventive strategy can be
404 best achieved using a combination of chemopreventive agents. A combinatorial chemopreventive
405 strategy could achieve enhanced cancer preventive efficacy, while minimizing the risk of failure
406 due to dysplastic cells which are resistant to one or more preventive agents [40]. Fortunately,
407 compared to fully malignant cells, the dysplastic cells targeted by a chemopreventive approach
408 should have less cellular heterogeneity and be less likely to harbor multiple genetic alterations,
409 decreasing the likelihood for resistance as the molecular pathways underpinning the biologic
410 effects of chemopreventives are more likely to be intact. Importantly, if the combination of
411 preventive agents chosen has a synergistic effect on cancer preventive molecular pathways, and
412 non-overlapping toxicities, it may be possible to achieve increased cancer preventive efficacy
413 while reducing the dosage of each agent, thereby lessening the long term risks associated with
414 chemoprevention.

415 Previously we have shown that the inhibitory effect of progestins on cell viability
416 in ovarian epithelial cells can be synergistically enhanced with NSAIDs. Several-fold increases
417 in activated caspase-3 and TUNEL were shown with the NSAIDS sulindac sulfide and celecoxib
418 when combined with P4 in both the OVCAR-3 and OVCAR-5 ovarian cancer cell lines as well
419 as in normal ovarian epithelial cells and the HIO-118V immortalized ovarian epithelial cell
420 culture. The finding of synergy suggested the possibility that perhaps NSAIDs and progestins,
421 when combined, could be used at modest dosages that would limit side effects while maintaining

robust chemopreventive potency [41]. Although short term use of NSAIDS is well tolerated, long-term use, however, can be associated with gastrointestinal bleeding and other serious cardiovascular adverse effects [42], limiting their use for prevention in women who are well. In contrast, vitamin D is a much more attractive agent since it is extremely safe, and when administered as cholecalciferol, is essentially devoid of any serious adverse effects. After hydroxylation in the liver to 25(OH)D₃, the active hormone, 1,25(OH)₂D₃, can be produced at the ovarian tissue level via the 1- α hydroxylase enzyme, both limiting risk from systemic 1,25(OH)₂D₃ while at the same time synergizing with progestins to clear genetically damaged cells.

In conclusion, we demonstrate that vitamin D acts in combination with P4 synergistically to inhibit cell viability and also to promote apoptosis in ovarian epithelial cells. Additionally, we find that P4 beneficially impacts vitamin D metabolism by inhibiting *CYP24A1*, thereby potentially increasing the activity of 1,25(OH)₂D₃, the active hormone. These data support further investigation of combined progestin and vitamin D for the chemoprevention of ovarian cancer.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgements

The authors are grateful to Dr. Weidong Xu for his expertise with the transfection of OVCAR-3 cells with PR to produce the OVCAR-3-PGR line; and to Ms. Dan Li for her excellent technical assistance with some of the molecular biological procedures reported herein.

This work was supported by Grant #DAMD17-00-1-0570 from the Department of

Defense, and also by an award from the United States Army Medical Research and Material Command (PI: Chad A. Hamilton), W81XWH-11-2-0131. Additional support also provided by Bears Care, The Matthews Family Foundation, and the Auxiliary of NorthShore.

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Figure Legends

Fig. 1.

Progesterone receptors in the cell lines and the responses of OVCAR-3 and OVCAR-3-PGR cells to calcitriol (CAL) and P4. **(A)** Western blot showing the presence of P4 receptor A (PRA) in OVCAR-5 cells, receptors A and B (PRA and PRB) in OVCAR-3-PGR (PGR-transfected stable line) and T47D-WT cells and their absence in parental OVCAR-3 cells. OVCAR-3 parent cell lanes, at 5 μ g and 40 μ g protein, showed no PR. OVCAR-3-PGR and T47D-WT lanes, using 5 μ g protein, exhibit prominent bands for both PRA and PRB. OVCAR-5 cells (in first lane) respond to progesterone similarly as do OVCAR-3-PGR cells in spite of their relatively low production of PRA. **(B)** Three-day dose-response to CAL, P4, or the combination. One-way ANOVA results: *significantly different from control ($p<0.001$) as well as between P4 and CAL+P4 groups ($p<0.001$) at same P4 dose; $^{\&}p<0.001$ compared to CAL alone; $^{\dagger}p<0.001$ among all P4-alone groups (except $p<0.05$ for 20 μ M P4 versus 30 μ M P4); $^{\ddagger}p<0.05$ among all CAL+P4 groups (except $p<0.001$ for CAL+20 μ M P4 versus CAL+40 μ M P4). No synergy was observed between P4 and CAL when the treatments were combined. **(C)** Six-day dose-response to CAL, P4, or the combination. One-way ANOVA results: *significantly different from control ($p<0.001$) as well as between P4 and CAL+P4 groups ($p<0.001$) at same P4 dose; $^{\circ}p<0.01$ compared to control; $^{\&}p<0.001$ compared to CAL alone; $^{\dagger}p<0.001$ compared to 20 μ M P4, $p<0.01$ compared to 25 μ M P4, and $p<0.05$ compared to CAL alone; $^{\ddagger}p<0.05$ compared to CAL+25 μ M P4 and $p<0.01$ compared to CAL+30 μ M P4. Isobolographic analysis showed moderate synergy with combined P4 and CAL treatments at 25 μ M P4 ($IC=0.852$). **(D)** Three-day MTS assay showing the impact of CAL and P4 on viability of OVCAR-3-PGR cells. One-way ANOVA results: * $p<0.001$ compared to control group; $^{\&}p<0.001$ (except $p<0.01$ for

CAL+20 μ M P4) compared to CAL alone; [°]significantly different between same-dose P4 and CAL+P4 groups ($p<0.01$ for 30 μ M P4; $p<0.001$ for 40 μ M P4); [†] $p<0.001$ between 20 μ M P4 and 40 μ M P4 groups; $p<0.01$ between 30 μ M P4 and 40 μ M P4 groups; [‡] $p<0.001$ between CAL+20 μ M P4 and CAL+40 μ M groups; $p<0.01$ between CAL+30 μ M P4 and 40 μ M P4 groups. Isobolographic analysis demonstrated synergy with combined P4 and CAL treatments at 30 and 40 μ M P4 (IC=0.711 and 0.695, respectively).

Fig. 2.

Viability of OVCAR-3-PGR cells, in a 3-day MTS assay, transfected with *CYP24A1* siRNA or scrambled (control) siRNA and then treated with 100 nM CAL and 30 μ M P4. The Western blot image shows >50% inhibition of CYP24A1 protein production by *CYP24A1* siRNA. Actin is the gel loading control. *CYP24A1* siRNA reduced viability in CAL-treated cells by approximately 20%. *CYP24A1* siRNA reduced viability in cells treated with both CAL and P4 by 13% compared to scrambled siRNA-transfected cells treated with the same agents. * $p<0.05$, *** $p<0.001$ compared with the respective controls or between groups marked by the horizontal lines above the chart bars, by ANOVA.

Fig. 3.

CYP24A1 expression in the parental OVCAR-3 cells in response to calcitriol (CAL) and P4. *CYP24A1* mRNA expression (**A**) and protein production (**B**) were both strongly stimulated by 100 nM CAL and neither was reduced by 100 nM P4 treatment in these cells.

Fig. 4.

CYP24A1 expression in OVCAR-3-PGR and OVCAR-5 cells in response to calcitriol (CAL) and

P4. (A) *CYP24A1* mRNA expression (measured by RT-PCR) was stimulated by 100 nM CAL and was significantly inhibited by 100 nM P4 in PR-expressing OVCAR-3-PGR cells (** $p < 0.001$ versus each untreated group and between treated groups within each respective 4-h and 6-h experiment). (B) *CYP24A1* protein production by OVCAR-3-PGR cells (determined by western blot densitometry) also demonstrated stimulation by 100 nM CAL and significant reduction by 100 nM P4 in the presence of CAL (** $p < 0.01$, *** $p < 0.0001$ between CAL and CAL+P4 treatment groups; $\&p < 0.001$ among 6-, 16-, and 24-h CAL groups; $\#p < 0.001$ compared to other CAL+P4 groups). (C) *CYP24A1* mRNA expression was stimulated by 100 nM CAL and was significantly inhibited by 100 nM P4 in PRA-expressing OVCAR-5 cells (** $p < 0.001$ versus each respective untreated group; $*p < 0.05$ versus untreated group at 1 h; $\&p < 0.001$ between treated groups at 1 h; $\#p < 0.01$ between treated groups at 4 and 6 h). (D) *CYP24A1* protein production by OVCAR-5 cells demonstrated stimulation by 100 nM CAL and significant reduction by 100 nM P4 in the presence of CAL (** $p < 0.0001$ between CAL and CAL+P4 treatment groups).

Fig. 5.

CYP24A1 expression in T47D cells in response to calcitriol (CAL) and P4. (A-D) *CYP24A1* mRNA expression (measured by RT-PCR) was stimulated by 100 nM CAL and was significantly inhibited by 100 nM P4 in cells containing PRA (T47D-YA) or PRB (T47D-YB) or both (T47D-WT), but not in cells lacking both PRs (T47D-Y). (E-H) *CYP24A1* protein production (western blot densitometry) was stimulated by 100 nM CAL and was significantly inhibited by 100 nM P4 in the presence of CAL in cells containing PRA or PRB or both, but not in cells lacking both PRs. $*p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to CAL alone (by

643 ANOVA).

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646 **Fig. 6.**

647 CYP24A1 in normal mouse ovaries. **(A)** Ovaries from mice treated with 1 mg P4, 400 ng CAL,
648 or combined P4 and CAL show significant inhibition of CAL-induced *Cyp24a1* mRNA

649 expression in response to P4 at 4 hours after treatment. **(B)** Ovaries from similarly treated mice

650 show significant reduction of CYP24A1 protein production at 16 hours after treatment with P4

651 and CAL. ** $p < 0.01$, *** $p < 0.001$ between groups marked by horizontal lines above the chart

652 bars **(A)**, or compared with other groups **(B)**, by ANOVA.

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Figure 1

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