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Progestins inhibit calcitriol-induced CYP24A1 and synergistically inhibit ovarian cancer cell viability: An opportunity for chemoprevention

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HIGHLIGHTS

- Progestins and vitamin D synergistically reduce viability in ovarian cancer cells.
- Progestins inhibit *CYP24A1*, extending vitamin D activity in ovarian cancer cells.
- Combination progestin and vitamin D may inhibit ovarian carcinogenesis.

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ABSTRACT

Objectives. Previously we have shown in endometrial cells that progesterone (P4) and calcitriol (CAL, 1,25 $(OH)_2D_3$) 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

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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, $1,25(OH)_2D_3$ ("calcitriol", CAL), the active form of the molecule. The human body obtains vitamin D (specifically vitamin D_3 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 D_3 is converted to $1,25(OH)_2D_3$, 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 $25(OH)D_3$ to $1,25(OH)_2D_3$, which acts in an autocrine or paracrine fashion to regulate cell growth and biology. Once $1,25(OH)_2D_3$ confers its local effect, it induces production of vitamin D-24-hydroyxlase (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 D_3 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 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, RU486 (mifepristone) 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% CO_2 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 *Eco*RI/*Xho*I 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 h, 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 A_{490} 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 h, they were transfected with 40 nM control siRNA (GE Dharmacon, Lafayette, CO; ON-TARGET control pool cat# D-001810-05) or 40 nM 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 h. Upon completion of the 48-hour transfection, cells were treated for 72 h with 100 nM CAL and 30 μM progesterone, alone or in