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PRINCIPAL INVESTIGATOR: David M. Gershenson, M.D.

CONTRACTING ORGANIZATION: The University of Texas
M. D. Anderson Cancer Center
Houston, TX 77030

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14. **ABSTRACT**
The overarching hypothesis of this program project is that 4-HPR (a synthetic vitamin A) and oral contraceptives (OCP) induce apoptosis, possibly through induction of TGFß production by stromal cells, as well as by direct interaction with the surface epithelial cells, and these two cell types may act synergistically. In Project 1, 18 adult Rhesus monkeys were given 4-HPR, OCP, the combination, or no medication for 3 months. There were consistent differences in the absolute fluorescence intensities and relative contributions noted between pre- and post-drug measurements in each drug group. A second study involving 30 Cynomolgus macaques and using a crossover design has been completed; immunohistochemical analysis of several biomarkers and analysis of the fluorescence spectroscopy data are ongoing. Project 2 has been transferred to the University of Arizona with the relocation of Dr. Molly Brewer. One patient has completed the study on the OCP arm, and 4 other patients are in line to start on the study pending approval of their surgery. In Project 3, we have focused on understanding the mechanism of action of 4-HPR in tissue culture using both normal and immortalized epithelial cells. Studies are now complete.

15. SUBJECT TERMS
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INTRODUCTION

Ovarian cancer is the leading cause of death from gynecologic cancer in the United States. In 2005, there will be approximately 22,000 new cases in the United States (3% of all cancer diagnoses in women) and approximately 16,000 women will die of this disease (American Cancer Society). In the United States, approximately 1 woman in 58 will develop ovarian cancer in her lifetime. Despite changes in treatment strategies and the introduction of new chemotherapeutic agents, the 5-year survival rate has not improved substantially over the past three decades.

No effective screening tool exists. Consequently, over 70% of cases are diagnosed after the cancer has already spread beyond the ovary. For women with stage III epithelial ovarian cancer—the most common stage—the 5-year survival rate is no higher than 20%. Clearly, early detection and prevention of this disease are critical issues. The overall goals of this program project are: 1) to develop innovative strategies for prevention of ovarian cancer through the assessment of the potential effect of oral contraceptives (OCP) and retinoids (Vitamin A derivatives) on the ovary and identification of molecular markers and mechanisms associated with the chemopreventive activity of these compounds, and 2) to assemble a multidisciplinary team that will become a world leader in the field of ovarian cancer prevention. A large body of epidemiologic evidence supports the fact that OCP can reduce a woman's risk of ovarian cancer as much as 50%. Similarly, preliminary data from a large Italian randomized chemoprevention trial for secondary breast cancers suggests that retinoids

may have preventive activity against ovarian cancer. In addition, retinoids have been shown to induce apoptosis in normal ovarian surface epithelial cells in the laboratory. The major overarching hypothesis of this program project is that 4-HPR and OCP induce both growth inhibition and apoptosis. Data on 4-HPR suggests this activity is mitochondrial mediated which can be assessed using fluorescence spectroscopy which is sensitive to changes in NADH and FAD, both electron carriers active in the mitochondria. Changes in mitochondrial permeability are thought to be one of the changes that occurs in a cell in response to 4-HPR. Evaluating gene expression is another way of understanding the action of these molecules and the combination of these results should help us develop better methods of prevention of ovarian cancer.

BODY

PROJECT 1

Chemoprevention of Ovarian Cancer Using a Rhesus Primate Model

The second study, using 30 cynomolgus monkeys in a cross over design has been completed and is being analyzed; the increased numbers of monkeys should overcome the limitations of the numbers in the first study. This protocol was approved by the Animal Care Use Committee at The University of Texas M.D. Anderson Cancer Center and was conducted at the Department of Veterinary Sciences in Bastrop, Texas, where all animals were caged separately. The animals were given 4-HPR (4 monkeys), OCP (5 monkeys), the combination of 4-HPR+OCP (5 monkeys), or no medication (4 monkeys) daily for 3 months. Doses of 4-HPR and OCP were calculated by allometric scaling (1). The OCP used was Ortho-Novum 1/35, a medium-dose oral contraceptive with 1 mg norethindrone and 35 ug ethinyl estradiol in each pill. The 4-HPR dose was calculated in the same manner from the accepted human dose of 200 mg daily. Prior to starting medication and following 90 days of medication, monkeys underwent laparotomy, spectroscopy, and ovarian biopsies. Following the first 3 months of treatment, the group receiving 4-HPR crossed over to the OCP arm, the monkeys receiving OCP crossed over to the 4-HPR arm and the control monkeys crossed over to the combination group. There was a 1 month washout period between crossovers. Following completion of the initial portion of this study, the 9 monkeys that were

negative on Herpes (Monkey B) serology were continued on OCP. A study by Gus Rodriquez has shown that monkeys receiving OCP and progestin for 2 years showed a 5 fold increase in the rate of apoptosis of the ovarian surface epithelial cells over the controls suggesting that the chemopreventive activity of the OCP may be induction of apoptosis of the surface epithelial cells. However, this rate of apoptosis was not observed in our study. We have continued the OCP, changing to the Tri-phasil which was used in the Rodriquez study hypothesizing that the increase in progestin in the third week may have caused the increased apoptosis. Monkeys have undergone ovarian biopsy every 6 months to assess the cumulative response to the OCP. This study was completed in December 2002 and 2 manuscripts have been submitted for publication.

Immunohistochemical markers have been analyzed that suggest that the combination of the OCP and 4HPR have more activity than either alone. ERα was negative as has been reported in other papers. However, ERβ was positive in the OCP and 4-HPR groups but strongly positive in the combination group suggesting that these 2 drugs have synergistic activity. We saw the same synergism in the results of our fluorescence spectroscopy in the combination group. These findings suggest that these drugs may have activity via activation of the ERβ receptor as well as an effect on mitochondrial metabolism. RAR α, β, γ evaluation suggests that RAR α and γ are upregulated with the combination but ERβ is not. There has been much work, particularly in cell lines, evaluating the effect of retinoids on the RAR's. 4-HPR has been suggested to be receptor independent but induce apoptosis and growth inhibition through changes in the mitochondria. However, this data from monkeys suggests that some of the activity of

this drug may be mediated through the nuclear receptors. Interestingly, OCP seems to increase this activity, again suggesting a synergistic response between the 2 drugs. This paper is also being prepared for publication. A second paper has been submitted on the fluorescence signature that resulted from each drug.

PROJECT 2

Chemoprevention of Ovarian Cancer: Modulation of Biomarkers in Women at Low- and High-Risk for Ovarian Cancer Using Fenretinide (4-HPR) and Oral Contraceptives

The clinical trial in Project 2 has been activated at the University of Arizona following approval by both the University of Arizona IRB and the Human Subjects Protection at the Department of Defense. We have received the 4-HPR from the NCI. The FDA has approved release of the drug with an investigator approved IND. The protocol was modified by the FDA and has been approved by the DOD. We have recruited one patient and have 5 others that are waiting for insurance approval prior to going on study. We have applied for and been approved for a no-cost extension due to the extreme difficulty in gaining the FDA and IRB approvals needed to start this project. One patient has completed the study on the OCP arm, and 4 other patients are in line to start on the study pending approval of their surgery.

PROJECT 3

Chemoprevention of Ovarian Cancer: Molecular Mechanisms and Markers Laboratory Investigations of 4-HPR and OCP

Growth inhibitory effects of 4-HPR in NOE, IOSE and ovarian cancer cells. The goal in this project has been to understand the mechanism of action of primarily 4-HPR in cell culture. Normal ovarian surface epithelial cells (NOE), immortalized ovarian surface epithelial cells (IOSE), and the three ovarian cancer cell lines were grown in monolayer cultures and treated with different concentrations (1-10 μM) of 4-HPR for 5 days. SKOV3, OVCA420 and OCC-1 were studied to determine which ovarian cancer cell line would serve as the best model for comparison between the normal, premalignant and malignant models. OVCA420 was chosen because it had the most sensitivity to 4-HPR and, thus, could be evaluated most comparably with the NOE and IOSE cell lines. We first compared low concentrations from 1-5 μM 4-HPR in NOE, IOSE and OVCA420. Concentrations from 2-10 μM 4-HPR were used on the ovarian cancer cells; 10 μM 4-HPR had greater growth inhibitory effects than the lower doses on the cancer cell lines. OVCA420 cells were the most sensitive cell line compared with other two cell lines. The morphologic changes of 1-10 μM 4-HPR in normal and ovarian cancer cells are shown in Fig 1. The

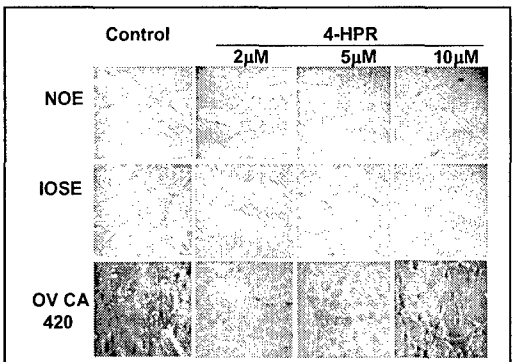


Fig 1. Effect of 4-HPR on the growth inhibition of NOE, IOSE and ovarian cancer cells. Cells were grown in the absence (control) or presence of 1, 2, 5 or 10 μM 4-HPR for 5 days, and stained with crystal violet for analysis of growth inhibition.. NOE* (average from 6 different NOE cells), IOSE and OVCA420 cells were treated with 1, 2 or 5 μM 4-HPR. b. ovarian cancer cells, SKOV3, OCC-1 and OVCA420 cells were treated with 2, 5 or 10 μM 4-HPR. c. Morphologic change of 2, 5 or 10 μM 4-HPR in normal and ovarian cancer cells.

NOE and IOSE cells were very sensitive to 10 μM 4-HPR; almost all cells were killed by day 3 (Fig 1).

Apoptosis induction by 4-HPR. To assess possible mechanisms of 4-HPR in ovarian cells, we analyzed the effects of 4-HPR on the induction of apoptosis in primary culture and cell lines by TdT labeling and flow cytometry. NOE and IOSE cells were treated with 5 μM 4-HPR, and DNA content and apoptosis induction were analyzed (Fig 2). The results showed that the apoptotic cell population did not change significantly at this concentration, but a population of cells with hypodiploid (HD) DNA content increased 2- to 8-fold with increasing time of incubation with 5 μM 4-HPR in these cells (Fig 2). The increase in HD cell population was shown as a population shift to the left (Fig 2). NOE, IOSE and OVCA420 cells were treated with 2, 5 and 10 μM 4-HPR. Apoptosis induction was analyzed at different concentrations and different time points (Fig 3a and 3b). Results demonstrated that 4-HPR-induced apoptosis was dose dependent in the NOE and OVCA420 cells (Fig 3a);

Fig 2.

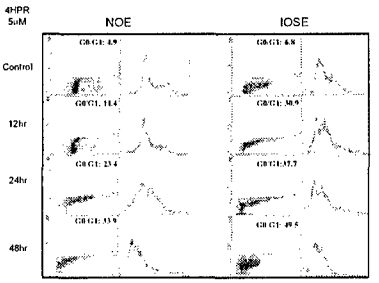
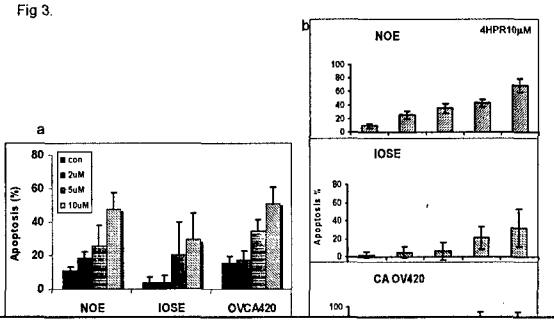


Figure 2Effect of 5 μM 4-HPR in NOE and IOSE cells. Cells were treated with 5 μM 4-HPR for 12, 24 and 48 hrs. The cells were harvested and then stained with propidium iodide for DNA content analysis and with fluorescein-labeled dUTP to label DNA fragments by the TUNEL method.. The fluorescence of cells labeled by the TUNEL method is presented in the left columns, which fluorescence of viable and apoptotic cells represented by the dark dots below and above a demarcation line determined by a standard cell line.

Fig 3.

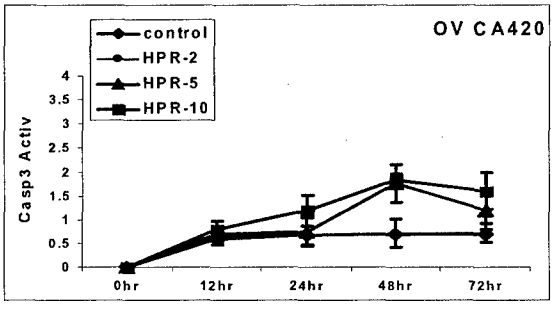


Effect of 4-HPR on apoptosis induction in NOE, IOSE and OVCA420 cells. a. Dose response of 4-HPR in apoptosis induction. Effect of different concentrations of 4-HPR on ovarian cells. Cells were treated with 2, 5 and 10 μM 4-HPR for 3 days. The apoptosis were determined by the TUNEL assay. The percentage of apoptotic cells is indicated in the right corner of each treatmentanalysis. b. Time-course of 10 μM 4-HPR on apoptosis induction. Cells were treated with 10 μM 4-HPR for 12, 24, 48 and 72 hrs. The percentage of apoptotic cells was determined by the TUNEL assay.

apoptosis was less marked in the IOSE cells than in the NOE and OVCA420 cells (Fig 3a). A time course of apoptosis induction was carried out in the NOE, IOSE and OVCA420 cells when treated with 10 μM 4-HPR (Fig 3b). 4-HPR induced apoptosis was time dependent in ovarian cells with maximal apoptosis occurring at 72 hours.

Effect of 4-HPR on caspase-3 activity. The 4-HPR effect on caspase-3 activity at different concentrations and different lengths of incubation was measured in NOE, IOSE and OVCA420 cells (Fig 4).

Fig4



Effect of 4-HPR on caspase-3 activity in NOE, IOSE and OVCA420 cells. Cells were grown in 96- well plates in the absence (control) or presence of 4-HPR (2, 5 and 10 μM) for 12, 24, 48 and 72 hrs, and incubated in caspase-3 activity assay buffer, as described in Materials and Methods. The intensity of staining was read using a fluorescent plate reader at 400 nm excitation, 505 nm emission, immediately after adding caspase-3 fluorescent substrate conjugate.

Cells were treated with 2, 5, and 10 μM 4-HPR and caspase-3 activity was measured at 12, 24, 48 and 72 hrs after treatment. The patterns of caspase-3 activity were different in the three cell types, and 2 μM 4-HPR had little effect on caspase-3 activity in any of the cells. After 24 hours of treatment, an increase in caspase-3 activity was detected in IOSE and OVCA420 cells treated with 10 μM 4-HPR. Caspase-3 activity was slightly increased when NOE cells were incubated for 3 days with 10 μM 4-HPR (Fig 4).

Effect of 4-HPR on mitochondrial permeability transition. Mitochondrial permeability transition (MPT) changes are associated with apoptosis. To investigate the mechanism of 4-HPR-induced apoptosis in ovarian cancer cells, experiments were carried out to

determine the effect of 4-HPR on MPT in NOE, IOSE and OVCA420 cells. OVCA420 cells had a greater change in MPT than did the NOE and IOSE cells after treatment with 4-HPR (Fig 5). 4-HPR decreased mitochondrial inner-membrane potential, which increased MPT in these cells (Fig 5). An inverse relationship in mitochondrial potential correlated in a dose-dependent manner with both increase in apoptosis and growth inhibition by 4-HPR in the 3 cell types

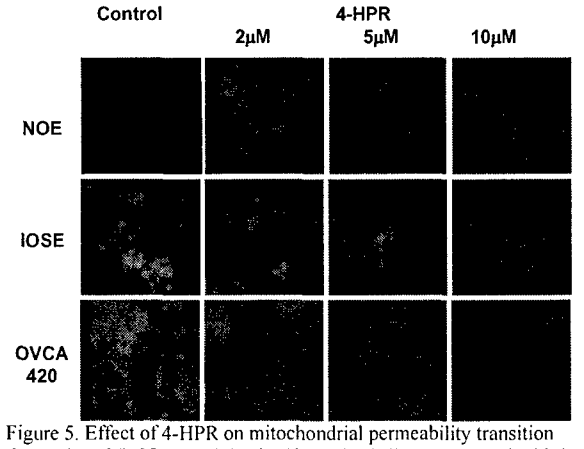


Figure 5. Effect of 4-HPR on mitochondrial permeability transition (MPT) in NOE, IOSE and OVCA420 cells. Cells were treated with 2, 5 and 10 μM 4-HPR for 3 days and then resuspended in 40 nM MitoFluor medium.

Modulation of p53 and other gene expressions by 4-HPR in NOE, IOSE and OVCA420 cells. The effect of 4-HPR on the expression of the apoptosis-associated genes, p53, p21 and p16, was examined in NOE, IOSE and OVCA420 cells; p53 expression was detected in the NOE, IOSE and OVCA420 cells. 4-HPR increased p53 expression in the NOE cells in a dose dependent manner but did not alter the expression in IOSE and OVCA420 cells (Fig 6). The expression of the p21 gene was increased in NOE and IOSE cells but was not detectable in OVCA420 cells.

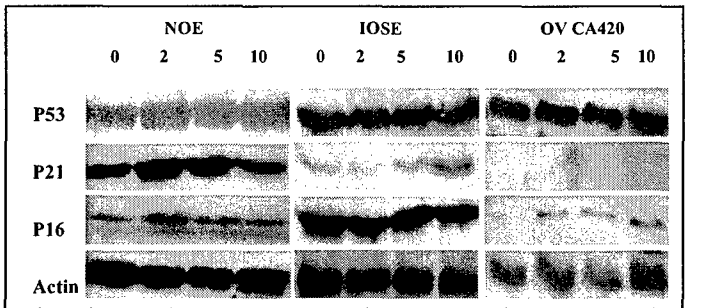


Figure 6 Effect of 4-HPR on p53, p21 and p16 expression in NOE, IOSE and OVCA420 cells. Nuclear proteins were extracted from cells treated with 2, 5, and 10 μM 4-HPR for 3 days. Eighty microgram/lane of nuclear proteins were subjected to SDS-PAGE. The p53, p21 and p16 proteins were identified by blotting with monoclonal antibodies.

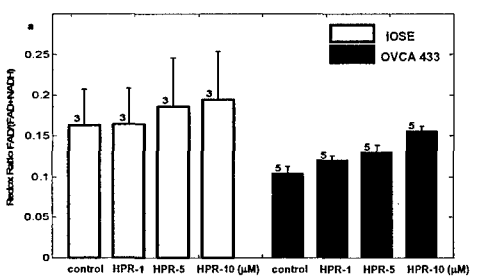
The expression of p16 was modulated by 4-HPR in the NOE and OVCA420 cells (Fig 6).

Redox Ratio Changed by 4-HPR Detected by Optical Spectroscopic Analysis

As shown in Fig 7, the IOSE cell line exhibited a highly variable redox related fluorescence ratio compared to the OVCA433 cell line in which the estimated redox increased in a linear fashion.

The OVCA433 cells demonstrated a strong sensitivity to 4-HPR treatment with dose dependence as a linear increase with a slope of 0.0059 / μM 4-HPR ($p<0.001$). An increased redox ratio suggests less oxidative metabolism indicating the cells may be entering quiescence. When considering the relative ratios to untreated cells, the OVCA433 cells had a higher value at each drug dosage. At higher concentrations of 4-HPR the redox related fluorescence ratio increased for the IOSE cells but never reached the level of the OVCA433 cells. This is consistent with the result that the IOSE cell line was variable in response to 4-HPR treatment and most of time was not as sensitive to 4-HPR as the OVCA433 cell line.

Fig. 7



b

	Relative Redox (Concentration/Control)		
	1 μM/Control	5 μM/Control	10 μM/Control
IOSE	1.01	1.14	1.19
OVCA 433	1.16	1.26	1.6

Figure 7 **a.** Estimated fluorescence redox ratios are presented from the OVCA433 and IOSE cells with increasing concentrations of 4-HPR. Standard error bars are shown and the value above each bar represents the number of times the treatment group was measured. **b.** Comparisons relative redox fluorescence ratio between the control and treatment measurements for both cell lines. The mean ratios for the treatment groups are normalized to the control group's mean ratio.

Administrative Core

During this reporting period, there have been no changes in key personnel. Dr. David Gershenson, PI, continues to meet with the administrative assistant on a weekly basis. The administrative assistant coordinates and schedules all grant-related meetings and conference calls, facilitating interactions and communications between investigators. Conference calls are conducted once a month to 1) review research activities and discuss scientific issues related to grant activities and 2) identify any problems or barriers associated with research and to assure that all goals are being met within realistic time and budget constraints.

Financial accounts have been established for the University of Arizona's subcontract with Dr. Molly Brewer and all projects and cores at The University of Texas M. D. Anderson Cancer Center. The administrative assistant monitors each account on a monthly basis to ensure that there are no problems or discrepancies.

The administrative assistant is also responsible for timely submission of all reports to the Department of Defense.

Histopathology Core

The purpose of the Histopathology Core is to provide central and uniform histopathologic, immunohistochemical, in-situ hybridization, and apoptosis assay support to the projects in this grant. Histopathologic evaluation, immunohistochemistry, in-situ hybridization, and evaluation of apoptosis have a central role in the design of these projects. The Histopathology Core, in using one central lab for this purpose, will promote uniformity of results by controlling variables associated with specimen handling, and with the technical performance and interpretations of these tests.

For Project 1, biopsies from 18 Rhesus monkeys before treatment and after treatment (for a total of 53 specimens) were fixed, processed, and embedded in paraffin blocks. These blocks were sectioned and stained with hematoxylin and eosin for histologic evaluation. A pathologist associated with the core reviewed these H&E slides. Immunohistochemical staining was then performed on all of the specimens. The markers included BAX, BCL-X, EGFR, ER, Her-2, Ki-67, p21, p53, PR, TGF beta, TGF beta-RI, TGF beta-RII. The 636 immunohistochemical stains were then evaluated and quantified. These results were then given to the investigator. Next the 53 specimens were evaluated for apoptosis using APO-TAG. These assays were reviewed and evaluated with the results forwarded to the investigator. In-situ hybridization for RAR-beta on these specimens is pending. An additional 30 primates have undergone three

surgeries each with two biopsies being obtained at each surgery (180 specimens). These specimens have been processed and the immunohistochemical stains are being completed.

As Project 2 has just started recruiting patients, no specimens have been processed by the Histopathology Core.

In support of the Idea Grant, immunohistochemical staining was reviewed and evaluated on seven cell lines. These cell lines included NOE 71, 72, 78, 79, 80, 83, and 86. Cytospins and smears from each of these lines were stained with AE1/AE3 and vimentin immunohistochemistry. The stains were reviewed and evaluated with the results forwarded to the investigator.

Please refer to the individual project summaries for the incorporation of the results of these assays and their implications.

KEY RESEARCH ACCOMPLISHMENTS

- Molly Brewer, Karen Johnson, Michele Follen, David Gershenson, Robert Bast. Prevention of Ovarian Cancer: Intraepithelial Neoplasia. Clinical Cancer Research 9:20-30, 2003.
- Molly Brewer, J Taylor Wharton, Jian Wang, David Gershenson, Robert Bast, Changping Zou. *In Vitro* Model of Normal, Immortalized Ovarian Surface Epithelial and Ovarian Cancer Cells for Chemoprevention of Ovarian Cancer. Gynecologic Oncology, In Press, 2005.
- Vengadesan Nammalvar, Urs Utzinger, Kimberly Hsu, Frans W.J. van den Berg, Janelle Bender, William Satterfield, Molly Brewer, Rebekah Drezek. Characterization of Chemopreventive Drug Activity in a Non-human Cynomolgus (Macaca fascicularis) Primate Model Using Fluorescence Spectroscopy and Chemometric Analysis. Submitted to Journal of Biomedical Optics, 2005.

- Molly Brewer, Nathaniel D. Kirkpatrick, J. Taylor Wharton, Jian Wang, Kenneth Hatch, David Gershenson, Nelly Auersperg, Robert Bast, Urs Utzinger, Changping Zou. 4-HPR Modulates Gene Expression in Ovarian Cells. International Journal of Cancer, In Press, 2005.

REPORTABLE OUTCOMES

- Not Applicable

CONCLUSIONS

Project 1:

The Statement of Work for this project has been completed. However, we have completed a second study involving 30 cynomolgus monkeys in a crossover study design. The results of this study have been submitted for publication.

Project 2:

This project was transferred to the University of Arizona, where Dr. Molly Brewer relocated. The protocol was subsequently approved by both the University of Arizona IRB and the Human Subjects Protection at the Department of Defense. The 4-HPR supply has been received from the National Cancer Institute. Because of regulatory issues that required clarification, there was a rather lengthy delay in moving forward with final approval. However, the study has been approved by both the University of Arizona IRB and the HSSR. We are recruiting patients to the study now.

Project 3:

The Statement of Work for this project has been completed. One manuscript has been published in the Journal of Gynecologic Oncology and another manuscript is in press in the International Journal of Cancer.

REFERENCES

- Not Applicable

APPENDIX

1. Molly Brewer, Karen Johnson, Michele Follen, David Gershenson, Robert Bast. Prevention of Ovarian Cancer: Intraepithelial Neoplasia. *Clinical Cancer Research* 9:20-30, 2003.

2. Molly Brewer, J Taylor Wharton, Jian Wang, David Gershenson, Robert Bast, Changping Zou. *In Vitro* Model of Normal, Immortalized Ovarian Surface Epithelial and Ovarian Cancer Cells for Chemoprevention of Ovarian Cancer. *Gynecologic Oncol*ogy, In Press, 2005.

3. Vengadesan Nammalvar, Urs Utzinger, Kimberly Hsu, Frans W.J. van den Berg, Janelle Bender, William Satterfield, Molly Brewer, Rebekah Drezek. Characterization of Chemopreventive Drug Activity in a Non-human Cynomolgus (Macaca fascicularis) Primate Model Using Fluorescence Spectroscopy and Chemometric Analysis. Submitted to *Journal of Biomedical Optics*, 2005.

4. Molly Brewer, Nathaniel D. Kirkpatrick, J. Taylor Wharton, Jian Wang, Kenneth Hatch, David Gershenson, Nelly Auersperg, Robert Bast, Urs Utzinger, Changping Zou. 4-HPR Modulates Gene Expression in Ovarian Cells. *International Journal of Cancer*, In Press, 2005.

Characterization of Chemopreventive Drug Activity in a Non-human Cynomolgus (Macaca fascicularis) Primate Model Using Fluorescence Spectroscopy and Chemometric Analysis

Vengadesan Nammalvar, Ph.D[1]. Urs Utzinger, Ph.D[2, 4]*. Kimberly Hsu, B.S[1]. Frans W.J. Van den Berg Ph.D [3]. Janelle Bender, B.S[1]. William Satterfield, DVM[5]. Molly Brewer, DVM, MD, MS [2,4]*. Rebekah Drezek, Ph.D [1*].

* Corresponding Authors

Rebekah Drezek
Bioengineering Department, PO Box 1892 – MS 142,
Rice University, Houston, TX 77251
Phone: (713) 348-3011, fax: (713) 348-5877
email: drezek@rice.edu

Urs Utzinger
Division of Gynecologic Oncology, University of Arizona,
Arizona Health Science Center 8319
1501 N Campbell Ave
Tucson AZ, 85724
Phone: (520) 626-9281, fax: (520) 626-9287
Email: uutzinger@azcc.arizona.edu

Molly Brewer
University of Arizona, Arizona Cancer Center,
Rm. 1968G, P.O. Box 245024, 1515 N. Campbell Ave
Tucson, Arizona 85724
Phone: (520) 626-9283, fax: (520) 626-9287
E-Mail: mbrewer@azcc.arizona.edu

[1]Bioengineering Department, Rice University, Houston, TX 77251
[2,4]ARL Division of Biomedical Engineering, University of Arizona, Tucson, AZ 85724
[4,2]Division of Gynecologic Oncology, University of Arizona, Tucson, AZ 85724
[3]The Royal Veterinary and Agricultural University, DK-1958 Frederiksberg-C, Denmark
[5]Department of Veterinary Sciences Park, The University of Texas M. D. Anderson Cancer Center, Houston, TX, 77030;

Abstract:

The steady state fluorescence emission spectra from 290 to 480 nm excitation, were collected from the ovaries of 30 adult female cynomolgus (Macaca fascicularis) monkeys receiving fenretinide (4HPR), oral contraceptive pills (OCP), combination of 4HPR and OCP or no medication. Each chemopreventive drug was shown to have unique effects on the optical signature, as seen through distinct variations in the emission spectra. The spectral changes between control and post-drug measurements due to the chemopreventive administration may be related to the alteration of three distinct molecules tyrptophan, collagen, and NAD(P)H. The spectral changes between control and drug-induced epithelial tissues were extracted by chemometric analysis using Bilinear Partial Least Squares analysis (Bi-PLS) followed by linear discrimination. We found that control against combination groups (4HPR+OCP, 4HPR after OCP and OCP after 4HPR) yielded higher accuracy rates than control against individual groups (4HPR or OCP) suggesting a synergistic effect of both drugs. Further, it was observed that the fluorescence emission spectra at the excitation wavelengths of 290, 300 nm and 320 as well as 360 nm yielded high accuracy rates than other excitation wavelengths. The cynomolgus monkey model facilitates the development of a non-human primate model for chemopreventive drug studies for ovarian cancer.

Key words: chemopreventive drugs, ovarian tissue, primate model, partial least squares analysis, fluorophores

1. Introduction

Ovarian cancer is the most common cause of death from a gynecologic malignancy in the United States. An estimated 25,580 new cases are expected in the United States in 2004. Ovarian cancer accounts for nearly 4% of all cancer among woman and ranks second among gynecological cancers, following cancer of the uterine corpus. From 1989 to 1999, the ovarian cancer incidence rate declined 0.7% per year. Even still, an estimated 16,090 deaths are expected in 2004, which is more than any cancer of the female reproductive system. If ovarian cancer is diagnosed and treated prior to metastasis, the 5-year survival rate is raised to 95%; however only about 25% of all cases are detected at the localized stages, which is the rationale for prevention of disease.[1] Primary prevention of cancer is one of the key approaches in the control of cancer. It includes (i) avoiding exposure to known cancer-causing agents, (ii) enhancement of the host defense mechanism, (iii) lifestyle modifications, (iv) early detection and (v) chemoprevention[2].

Cancer chemoprevention is a rapidly growing area of research because of the possibility to prevent disease and to restore cancer–suppressing cellular functions. Chemoprevention is defined as the use of specific agents to suppress or reverse carcinogenesis and thereby to prevent the development of cancer. Fundamental elements for chemoprevention studies include (a) a suitable cohort of patients with sufficient incidence to establish an acceptable risk to benefit ratio, (b) appropriate agents that are safe and whose use is supported by both epidemiological and mechanistic data, (c) measurable biomarkers that are likely to be affected by the agent and whose modulation

is predictive of the postulated chemopreventive activity, and (d) a suitable animal model[3,4].

There have been many studies with experimental results that indicate the effectiveness of specific agents on chemoprevention; however the number of agents actually proven to be effective by clinical trials is very small. In this regard, two classes of drugs – retinoids[5,6,7,8] and oral contraceptive pills (OCP)[9,10] – have received attention as agents that can prevent ovarian cancer. Preliminary results of a recent clinical study by Veronesi [11] showed that retinoids, particularly N-(4-hydroxyphenyl) retinamide (4HPR), did not prevent the development of second primary cancers in a group of patients with localized breast cancer, but appeared to protect women against the development of ovarian cancer[11]. After surgical treatment for breast cancer in this study, 2972 patients were assigned to treatment with 4HPR (1422 people) or placebo (1427 people) to prevent development of new primary breast cancers. After a median follow-up of 51.9 months, no overall difference in the development of new primary breast cancers was evident. However, there was a significant difference in the numbers of ovarian cancers that developed. During the treatment period, six new cases of ovarian cancer were diagnosed in the placebo group whereas none had appeared in the 4HPR group. After cessation of treatment, there were four additional cancers in the control group and six in the 4HPR group, suggesting that the effect was not durable [8].

Experimental studies have demonstrated that the effect of 4HPR may result from its ability to induce apoptosis (programmed cell death), which is thought to be an important mechanism in cancer prevention. Preliminary data shows that 4HPR induces a rate of 40–95% apoptosis in immortalized ovarian surface epithelial cells and 30–90% in

normal ovarian surface epithelial cells[12], compared with the baseline rate of 1–3% in untreated cells. The probability of developing a neoplasm decreases with an increase in apoptosis. Retinoids, particularly 4HPR, have been shown to increase aerobic glycolysis by increasing mitochondrial permeability to the co-enzymes NAD(P)H and FAD, as well as by increasing the activity of the electron transport chain through an increase in reactive oxygen species and cytochrome oxidase [6,13,14].

OCP is also being investigated to reduce the risk of developing ovarian cancer. Use of OCP for 5 years decreases the risk of ovarian cancer by 50%[6,9,10] but reduces the number of ovulatory cycles by approximately 15%. Consequently, there is no linear correlation between the duration of OCP use and the impact on ovarian oncogenesis, suggesting that more complex mechanisms than ovulation suppression may be at work in the chemopreventive activity of OCP. One effect of OCP use is inhibition of gonadotropin release from the pituitary, and that or other unknown effects of estrogens and progestins may play a role in this chemopreventive activity. In monkey studies using both progestins and OCP[15], the progestin arm displayed more apoptosis of the ovarian surface epithelial (OSE) cells than did the OCP arm, suggesting that progestin may be the active component in OCP. However, variances in those studies were large, and median values were used because of the large variability. Reanalysis by Schildkraut, et.al.[16] examined the strength of OCP components taken by 390 women diagnosed with ovarian cancer and indicated the greatest reduction in ovarian cancer risk was associated with the highest progestin potency. This body of evidence makes OCP, and potentially its progestin components, an excellent candidate as a chemopreventive agent for ovarian

cancer; however, more research is warranted to determine the mechanism of its protective effect.[3]

For over three decades, optical spectroscopy has been utilized to characterize various physical and chemical changes to tissues and cells for the discrimination of normal and pathological conditions for various organs[17,18,19]. Optical spectroscopy of tissues is generally carried out using a singular glass fiber or a bundle of fibers used to collect remitted light that is scattered back from the tissue. Tissue fluorescence originates from several endogenous fluorescent biomarkers, or fluorophores, including structural proteins (collagen, elastin), amino acids (tyrosine, tryptophan, phenylalanine), coenzyme factors (NAD(P)H, FAD), and endogenous porphyrin.[20,21] Pathologic, physiologic, and metabolic transformations result in associated modification on the molecular level, including the deposition and/or alteration of native fluorophores. Fluorophore concentration changes as tissue progresses from normal to pre-neoplastic state to cancer. The complete fluorescence pattern of a tissue reflects the relative contribution of individual fluorescence features and can be correlated to the biochemical composition of the tissues and the associated pathological and physiological conditions.

In our previous study, we utilized fluorescence spectroscopy as a marker for a drug activity in ovarian rhesus monkey tissues. Statistical analysis was conducted for fluorescence spectra of control and post-drug measurements based on the changes of fluorescence signatures of NAD(P)H and FAD in response to intake of 4HPR, OCP, and 4HPR+OCP.[6,12] That study involved univariate analysis related to fluorescence intensity of NAD(P)H and FAD molecules. However, analysis based on fluorescence intensity provides better information; thus there may be information hidden in the spectra that was

not extracted by univariate analysis. In order to effectively utilize more of the available information and characterize tryptophan, collagen, and NAD(P)H in response to various post-drug measurement groups, a chemometric approach[22,23,24] is used to obtain qualitative and quantitative information from the complete spectral data. Chemometric methods are mathematical and statistical techniques which decompose complex multivariate data into simple and easily interpretable structures that can improve the understanding of biological information. In this study, bilinear chemometric method – partial least square analysis (PLS)[25,26] followed by linear discrimination analysis [27,28] – were applied to the fluorescence spectra of chemopreventive drug-administrated and control ovarian monkey tissues

4HPR and OCP are drugs whose activity is potentially amenable to surveillance with fluorescence spectroscopy. However, it is difficult to study the ovary because its intraperitoneal location makes access difficult; therefore, the development of an animal model to study these drugs is appealing. Small animal models (mice, chicken, and guinea pigs) are being developed for ovarian cancer and are useful for understanding basic mechanisms, but the reproductive features of these animals differ from the human reproductive system. In contrast to small animal models, non-human primates more closely resemble humans in reproductive function and anatomy, as well as in hormonal and menstrual patterns.[29,30,31] In particular, histochemical analyses and hormone activity[30] of the non-human primate ovary indicate substantial similarities. Primates were chosen to be the patients in our current study to provide an *in vivo* model that can bridge the gap for human clinical studies, due to similarities in reproductive function between primates and women. This study evaluates the potential of combining Bi-PLS chemometric analysis

followed by linear discrimination with fluorescence spectroscopy to serve as a biomarker of chemopreventative drug activity.

2. Materials and Methods

2.1. Experimental Design

Thirty female adult *M. mulatta* participated in the studies, and each was measured on three separate occasions. A cross-over study was designed and is illustrated in Table 1. The cross over design was implemented to increase our sample size. There was a 3 week wash out period between administration of each drug to allow adequate time for the drug and potentially for the effect of the drug to be eliminated. The 30 monkeys were divided into three groups of ten each, and the treatment scheme was the same within each group. Before each treatment and after 3 months of treatment, optical measurements were conducted on 3 to 5 sites per animal resulting in 346 measurements.

This protocol was approved by the Animal Care Use Committee at The University of Texas M.D. Anderson Cancer Center and was conducted at the Department of Veterinary Sciences in Bastrop, Texas, where all animals were caged separately. The animals were given 4HPR, OCP, the combination of 4HPR+OCP, 4HPR after OCP, OCP after 4HPR, or no medication daily for three months. Doses of 4HPR and OCP were calculated by allometric scaling and given orally. Ortho-Novum 1/35, a medium-dose oral contraceptive with 1 mg norethindrone and 35 μg ethinyl estradiol per pill, was used as the oral contraceptive. The 4HPR dose was calculated from the accepted human dose of 200 mg daily. The detailed calculation of doses is explained in other publications.[12,32]

Prior to starting medication and after 90 days of medication, monkeys underwent laparotomy, spectroscopy, and ovarian biopsies. Two to three 2-mm samples were taken from the left ovary at the time of the first surgery and from the right ovary at the time of the second surgery for molecular analyses (not reported here). Fluorescence measurements were taken from each site before biopsy. Biopsy and healing of the ovary was hypothesized to interfere with the histochemical assessment and the spectroscopic signature because of the increased collagen associated with healing. Thus, to avoid this bias, one ovary was measured and biopsied on the first surgery and the other ovary was measured and biopsied on the second surgery.

2.2. Fluorescence measurements

Fluorescence excitation-emission matrices (EEMs), which contain the fluorescence intensity as a function of both excitation and emission wavelengths, were measured. The spectroscopic system for *in vivo* use records EEMs in less than 1.5 min and consists of a xenon arc lamp coupled to a scanning spectrometer that provides excitation light.[33] A fiber optic probe directs excitation light to the tissue, collects emitted fluorescence light, and delivers it to an imaging spectrograph and charge coupled device camera. The interrogated tissue area was 2 mm in diameter. Fluorescence emission spectra ranging from 320 to 850 nm were collected sequentially at 20 excitation wavelengths ranging from 290 to 480 nm in 10 nm steps. Before assembling the data into fluorescence EEMs, the system-dependent response and background signals were removed. Tissue exposure to broadband UV radiation from this device is below the total exposure limits developed by the American Conference of Governmental Industrial Hygienists (ACGIH) for epithelial tissues. Baseline fluorescence measurements were

collected from the ovary contralateral to the biopsied one. The data was visually inspected to identify measurements that appeared to be tainted from handling mistakes or device related issues. For this purpose, a routine was created that displays the EEMs in succession. A total of 19 suspicious measurements and group six were excluded which resulted in 288 total measurements available for further analysis. The distribution of the measurement for each measurement group is listed in Table 1 with numbers between parentheses.

2.3. Data Analysis

Average emission spectra from each group were calculated and visually inspected. Intensity differences between the control and post treatment groups were statistically analyzed. This first order analysis showed that although fluorescence emission was collected from 290 to 480 nm excitation, the range from 400 to 480 nm excitation showed no considerable spectral differences between the mean of the groups and no statistical significance between control and post drug measurement. Therefore, an in depth statistical analysis was conducted only between 290 to 390 nm excitation.

2.3.1. Statistical Algorithm

Partial least squares analysis and linear discrimination were performed for control and drug-administrated fluorescence spectra. Figure 1 shows the algorithm that was developed to analyze control and post-drug measurements. Two different types of input data sets were used for the analysis: the original and maximum normalized spectral data. The primary sources of variance in the original spectra were intensity and spectral line shape. The primary source of variance in the normalized spectra was attributed to the spectral line shape.[34]

Table 1 shows the number of control and various groups of chemopreventive drug-induced primate ovarian tissues. Table 2 shows the control versus the different categories of post-drug measurements. In the statistical analysis, control groups 1, 2, and 3 were combined and considered to be a single control group. For post drug measurements, individual drug groups (4HPR or OCP) and the combination of drug groups (4HPR+OCP, 4HPR after OCP, or OCP after 4HPR) were considered. The different categories of control versus post drug measurements were: control versus 4HPR (category I), control versus OCP (Category II), control versus 4HPR + OCP (Category III) , control versus 4HPR after OCP (Category IV) and control versus OCP after 4HPR(Category V) are shown in Table 2.

2.3.2 Partial least squares Analysis

There are several different multivariate calibration methods available that can be used for spectral analysis. Principal component analysis (PCA) and partial least squares analysis (PLS) are two powerful chemometrics methods[23,24,25,26,34], which decompose the data into ordered orthogonal basis sets. An increased order is associated with a decreased contribution to variance and an increased likelihood of representing noise components. Data is represented by a linear combination of the basis sets. Because PCA creates an ordered list of principal components with the first components accounting for the most variations of the spectra, these components can be selected for a subsequent regression or discriminant analysis. In PCA, the components are chosen for maximal explained variance, whereas PLS also takes into account the dependent variables (control or post-drug measurements) by optimizing variation within independent and covariation with dependent variables simultaneously. By adopting the information of the independent and

dependent variables simultaneously, PLS can reduce the impact of large but irrelevant variations of the independent variables in the calibration modeling. In the current study, the PLS method was chosen for the analysis of fluorescence spectra .[25,26]

For each calibration method, we collected a set of calibration data (X,Y), where the X matrix of independent variables contained the fluorescence data for each sample and the dependent variables data matrix (Y) contained the control or post-drug measurements that depend on the X matrix. This can be expressed as a matrix notation,

$$Y = Xb + e$$

where b is the regression coefficient that is determined during the calibration step and e is the residual matrix (model error, noise, etc.).

PLS transforms the original matrix into product of two lower rank matrices T and P,

$$X = TP' + E$$

where T is a sample-score matrix, P is a variable-loading matrix, and E contains the residual of un-modeled part of X. The loading matrix is transposed, as indicated by the apostrophe. The PLS scores are found in a different way than the PCA ones, taking into account the variation in Y during decomposition of X, and thus maximizing the covariance between X and Y. The goal of PLS is to find the components in X that closely describe the relevant variances in the spectra while having maximal correlation with the histopathology in Y, but without including noise or irrelevant variations. When performing the PLS decomposition, the controls were assigned a y-value of 0 and post-drug measurements were assigned the value of 1. The obtained score was then used for

linear discriminate analysis to calculate the probability of the tissues being pre-drug or tissues exposed to drug.

2.3.3. Validation

Model validation is of great importance in chemometrics in order to provide information on possible outliers and the number of components included in the model. Test-set validation and cross-validation are the two validation methods most applied.[25,35] Test-set validation requires two independent data sets representative of the sample population, where the model is built on one data set and tested on another. In the absence of a test-set, cross-validation resampling can be applied, where several sub calibrations are made with single samples (full cross-validation) or segments of samples (segmented cross-validation). Each sample is kept out of the calibration alternatively and predicted from a calibration model consisting of the rest of the samples, until all samples have been kept out once. The samples kept out are then used for validation. Such methods provide a good estimation of the expected predictor error for future data. The measure of the model performance is usually given by the correlation coefficient, which is the correlation between the measured reference, ($\hat{y}_i$), and the predicted reference, (y_i). This is given by the prediction error, root mean square error of cross validation (RMSECV),

$$RMSECV = \sqrt{\frac{\sum_{i=1}^{N} (y_i - \hat{y}_i)^2}{N}}$$

where N is the total number of samples. The calibration in this study was validated by cross-validation, due to the limited number of samples.

2.3.4. Linear Discriminant Analysis

Classical linear discriminant analysis was used to develop discriminant rules for the control and post-drug fluorescence measurements listed as categories in table 2. In short, this analysis finds the linear discrimination function of PLS scores (T) that maximizes among-group distances relative to within-group distances.[27,28] The selected PLS components from leave-one-out cross-validation method were used as the variables and in formulating a classification model using linear discriminate analysis. The dependent variables were one of two numeric values: 0 for control and 1 for post-drug measurements. After determination of the classification model, the control and post-drug measurements were used to determine the model's sensitivity and specificity. A probability membership was calculated for each spectral classification by the model. The probability of membership represents the classification confidence level. The final results are given in Tables 3 and 4. PLS analysis was performed using the Matlab® 6.5 (Mathworks Inc., Natick, MA) software package and tool box,[36] and linear discriminant analysis was performed using the SPSS/PC+12.0 software package.

3. Results

3.1. Fluorescence spectral signature of control and post-drug fluorescence measurements

Figure 2 shows the average fluorescence excitation-emission matrices (EEMs) of (a) the control group [control groups 1, 2 and 3; (n = 115)] in comparison with the post drug measurements for (b) 4HPR (n = 35). (c) OCP (n = 46), the combination of (d) 4HPR + OCP (n = 31), (e) 4HPR after OCP (n = 30), (f) OCP after 4HPR (n = 31) for excitation wavelengths from 290 to 480 nm. For all groups there were distinct peaks

around the excitation-emission wavelengths pairs of 290/350 and 340/450 nm, and small peaks were evident around 330/410 and 450/520 nm. These peaks may be attributed to the presence of tryptophan, collagen, NAD(P)H and FAD respectively.

When the individual spectra for excitation wavelengths from 290 to 390 nm were analyzed, the following features were observed. The fluorescence emission spectra for excitation wavelengths of 290 (Figure 3a), 300, 360 (Figure 3c) and 370 nm in the control group showed higher emission intensity than the post drug measurements of the same wavelengths. Higher differences in the absolute fluorescence intensities were noted between control and post-drug measurements for 290 and 300 nm excitations. The higher peak difference between control and post drug measurement was statistically significant ($p=0.001$). For all excitation wavelengths other than the aforementioned, the control group indicated higher emission intensity when compared to the individual groups (4HPR and OCP) and lower intensity when compared to the combination groups (4HPR + OCP, 4HPR after OCP and OCP after 4HPR). In addition it was observed that the fluorescence emission intensity for the 4HPR group was lower than that for all other groups at all excitation wavelengths.

3.2 Statistical Results

3.2.1. Statistical algorithm

A classification algorithm, which combined bilinear PLS analysis and linear discrimination, was developed using the fluorescence emission spectra from 290 to 480 nm excitation wavelengths. However, little discriminatory power was observed between 400 and 480 nm excitation wavelengths. For further analyses we considered spectra from 290 to 390 nm excitation wavelengths only. For each excitation wavelength, one-out

cross-validation method was performed, and the PLS scores obtained from the calibration set were used to develop the linear discriminant model. The selected parameters were used to estimate the probability of membership for the sample in the prediction set belonging to the control or any one of the post-drug measurements. Tables 3a-e show the results of PLS analysis followed by a stepwise linear discriminant analysis for original and normalized fluorescence spectra. In addition, we combined both individual groups (4 and 5) and the three combination groups (7, 8 and 9); and analyzed against control groups. The results are shown in the table 4.

3.2.2. Control versus 4HPR group (Category I)

Table 3a shows the specificity, sensitivity and accuracy rate for control against the 4HPR drug group and the number of PLS components selected for this category. From the table it was observed that the normalized fluorescence emission spectra for all excitation wavelengths have higher specificity than the original spectra for the same excitation wavelengths. However, normalized fluorescence spectra for 290, 300, 310, 330, 340, 370 nm excitations have lower sensitivity than the original, and it has an equal sensitivity to that of the original spectra at 380 nm. Normalized fluorescence spectra at all except 300 and 310 nm excitation wavelengths yielded higher accuracy rates than those for original spectra. Additionally, fewer PLS components were selected for the original spectra than the normalized spectra for all excitation wavelengths except 350 and 380 nm.

3.2.3. Control versus OCP group (Category II)

Table 3b shows the specificity, sensitivity and accuracy rate for control against the OCP group and the number of PLS components selected. From the table, it was

observed that normalized fluorescence spectra for all except 370 nm excitation wavelengths yielded higher specificity than that the original spectra. Normalized fluorescence emission spectra at 310 and 340-390 nm excitations yielded higher sensitivities than the original spectra. In addition, normalized fluorescence emission spectra at all but 370 nm excitation wavelengths had higher accuracy rates than the original spectra. There were more PLS components selected for the normalized spectra at the excitation wavelengths of 300, 340, 350, 360 and 380 nm than for the original spectra at corresponding wavelengths; the same numbers of components were selected for both spectra at the 290, 310, 320, 330 and 370 nm excitation wavelengths.

3.2.4. Control versus (4HPR+OCP) group (Category III)

Table 3c indicates the results for the control against the combination of the 4HPR + OCP group. From the table it was observed that the normalized fluorescence spectra at 290, 300, 310, 330, 360 and 390 nm excitations had higher specificities than those of the original spectra at the same excitation wavelengths. At the excitation of 350 nm, the specificities of the normalized and original spectra were the same. Normalized fluorescence spectra yielded higher sensitivities than the original spectra at all but 380 nm excitation wavelengths. The same sensitivities were observed for normalized and original spectra at 290, 300 and 370 nm excitation wavelengths. Also, the normalized fluorescence spectra at all but 370 and 380 nm excitation wavelengths yielded higher accuracy rates than the original spectra. There were more PLS components selected for normalized fluorescence emission spectra at the 310, 320, 330, 340, 360 and 390 nm excitations than the original spectra at corresponding excitations, while the same numbers

of components for both spectra are selected at 290, 300, and 350 nm excitation wavelengths.

3.3.5. Control versus 4HPR after OCP group (Category IV)

From the Table 3d, 100% accuracies were found for both normalized and original fluorescence emission spectra at 290 and 300 nm excitation wavelengths. The normalized fluorescence emission at 370 and 390 nm excitation wavelengths had higher specificity than the original spectra and the specificities at 310 and 350 nm excitation wavelengths yielded lesser than the original spectra. The specificities at 320, 330, 340, 360 and 380 nm were equal between the normalized and original spectra. Next to 290 and 300 nm excitation, normalized fluorescence spectra for all excitation wavelengths yielded higher sensitivities and accuracy rates than the original spectra, with the exception of the accuracy rate for 310 nm excitation. Note that in normalized spectra the accuracy rate was 100% for the normalized spectra at excitation wavelengths from 290 to 340 nm excluding 310 nm. There were more components selected for the normalized spectra at the 300, 330, 340, 360, 370 and 390 nm excitation, and the same numbers of components were selected for the 310 and 380 nm excitation wavelength.

3.3.6. Control versus OCP after 4HPR group (Category V)

From Table 3e, it was observed that both normalized and original spectra yielded higher specificities, except the original spectra at 370 and the normalized spectra at 310 nm excitation wavelengths. In addition, normalized spectra for all excitation wavelengths provided higher sensitivities and accuracy rates that the original spectra, excluding the accuracy rate for the original spectra at 310 nm; at 290 and 300 nm excitations, the sensitivities of both spectra are the same. There were more PLS components selected for

the normalized fluorescence spectra at 290, 330, 340, 360, 370, 380 and 390 nm than for the original spectra and lesser number of components selected for 300 and 320 nm. At 320 and 350 nm excitations, the same numbers of components were selected for both original and normalized spectra.

3.3.7. Comparison of performance of each category and individual versus combinational groups

It was observed that the fluorescence spectra for most of the excitation wavelengths in table 3b (control versus OCP group) showed higher accuracy rates than those in table 3a (control versus 4HPR group). In addition, table 3c-e (combination groups) showed higher accuracy rate than those in table 3a and 3b (individual groups). To verify the accuracy rates between the individual and combinational groups, we combined both individual groups (4 and 5) and the three combination groups (7, 8 and 9); and analyzed "control vs. individual" group and "control vs. combinational" groups. The results are shown in table 4 and it was observed that fluorescence spectra for the combination group at almost all excitation wavelengths yield higher accuracy rates than spectra for the individual groups. This observation affirms the previous results (table 3a - 3e).

4. Discussion

Epithelial ovarian cancer has the highest mortality rate of any of the gynecological cancers in the United States. In this paper we show that strategies proposed for early detection show promise as surrogate biomarkers for chemopreventive studies. There are many improvements that can be made regarding chemoprevention of ovarian cancer, mainly because there is a lack of understanding of the link among current

epidemiological, biological, and pathological data for ovarian cancer and there is not an accepted animal model to test agent activity or mechanism of action. Suitable endpoint biomarkers have not been consistently identified, and it has been difficult to test promising chemoprevention agents in patients at risk for the disease[3,4].

Using humans as experimental models is difficult because of ethical limitations, cost limitations, and lack of volunteers. A reliable animal model is invaluable in providing optimal flexibility for examining mechanisms, dose response relationships, and comparative efficacy of treatments. Under current circumstances, the opportunity to test the many hypotheses being generated by a growing list of potential agents with activity against targets implicated by the new molecular technologies is limited. Although several rodent models have been developed, they are not ideal for chemopreventive studies for several reasons: these animals differ from humans in their reproductive function they tend to develop stromal and germ cell rather than epithelial ovarian carcinoma[37,38], and they do not spontaneously develop ovarian tumors at a rate high enough to support chemopreventive studies.[39,40] Many researchers have addressed these limitations by using ovarian tumor induction in rodents via intraeperitoneal injection of transformed ovarian cancer cells[41]. However, even these models are limited because the induced tumors may differ biologically and histologically from spontaneous tumors. Moreover, these rodent models often require the use of animals with compromised immune systems. Because the immune system may play an important role in ovarian carcinogenesis, recent efforts have focused on developing rodent models with intact immune systems. These models may prove to be more suitable for studying chemopreventive agents than those lacking full immune function[4].

Next to the rodent model, the mostly widely accepted animal model for ovarian cancer preventative agents is the domestic white Leghorn chicken[42]. The potential utility of immunohistochemical markers in the chicken (*Gallus domesticus*) has been investigated as a model for spontaneous ovarian carcinoma. The chicken model has an extremely high rate of Müllerian cancer, of which 30-50% is oviductal in origin. The spontaneous rate of ovarian cancer is approximately 19% in hens ranging from 2-7 years old and may be difficult to differentiate from an oviductal primary cancer. In addition, chickens have less reproductive similarity to humans than do primates, thus limiting the potential applicability of this model. In contrast to the small animal model, non-human primates more closely resemble humans in reproductive function and anatomy, as well as in hormonal and menstrual pattern.[29,30,31] In particular, histochemical analysis and hormone activity[30] of the non-human primitive ovary indicates substantial similarities to humans. Moreover, the microanatomy is almost identical among primates. These factors support non-human primates as an excellent model to study ovarian chemopreventive agents. However, primates are costly to obtain and maintain, their development time is slow, their reproductive times are longer than for rodents, and the spontaneous ovarian cancer rate is relatively low in primates. Primates were chosen to be studied because of their close similarity to humans. However, the absence of tumor formation is a drawback,.[6,12] : nonetheless, from this study it was observed that the non-human primate is an excellent model to test drug effects on the ovarian surface and subsurface.

In the past three decades, optical spectroscopy has been considered one of the most exciting techniques for an early marker of cancer in many organs.[17,18, 19] Among

various optical spectroscopic techniques, fluorescence spectroscopy is being used to detect cancers non-invasively in many organ systems using fiber optic probes that can interrogate the tissue[20]. Tissue fluorescence originates from various fluorophores, such as amino acids (tyrosine, tryptophan, and phenylalanine), structural protein (collagen, elastin), coenzymes (NAD(P)H, FAD) and endogenous porphyrin.[18,19,21] Of the various fluorophores, structural proteins and aromatic amino acids are associated with the structural arrangement of cells and tissues. NAD(P)H, FAD, and endogenous porphyrin are related to metabolic processes or can be connected to the onset of pathological conditions. Among these fluorophores, tryptophan, collagen, and NAD(P)H have been studied by many researchers interested in the characterization of normal and pathological tissues. Previous researchers[6,43,44] have utilized the fluorescence technique to the redox potential, which is calculated by (FAD/FAD+NAD(P)H) and is, in part, a measure of the relative hypoxia of the tissue. FAD and NAD(P) are reduced in the citric acid cycle (anaerobic glycolysis) to FADH and NAD(P)H, which are used as coenzymes in the electron transport chain. In tumors, these coenzymes will accumulate in their reduced states (NAD(P)H and FADH) and produce a unique fluorescence signatures as a result of alteration in blood flow, decreases in tissue pH, and abnormalities in the mitochondria and in the transport of electron carrier molecules into the mitochondria.[43,44] Unlike in cancer, where redox potentials are reduced, redox potentials are increased in response to both OCP and 4HPR[6, 43]. Changes in fluorescence signatures are hypothesized to be due to a decrease in NAD(P)H and an increase in FAD in response to the drugs. These agents also produced an increase in the redox-related potential of the target organ, suggesting

that hypoxia was less extensive and that the system was more quiescent. Thus, optical spectroscopic signatures may serve as early markers of drug activity.

To gather more information about the spectral origin of our fluorescence emission spectra, data was compared to published emission spectra of various compounds. The observed spectral changes between control and post drug measurement may be due to the alteration of three distinct molecules tryptophan, collagen and NAD(P)H.

Fluorescence emission spectra for 290 (Figure 3a) and 300 nm excitation showed the primary emission peak at 350 and 360 nm, respectively, which was most likely due to excitation of tryptophan residues with some contribution of tyrosine and phenylalanine.

Tryptophan has a higher absorption cross section compared to the other amino acids, and the resonance energy transfer from proximal phenylalanine to tyrosine and from tyrosine to tryptophan most likely causes the emission spectrum of tissues containing the three residues to mainly resemble that of tryptophan.[45] The primary peak intensity difference at 290 and 300 nm excitation showed very high significance ($p < 0.001$) between control and post-drug measurements. The changes in the spectral signature of tryptophan with regard to chemoprevention drugs are poorly understood. Although there is information in earlier literature discussing the relationship between tumor tissues and tryptophan, there was little evidence in the available literature regarding the changes chemopreventative drugs have on tryptophan. In our recent study on ovarian surface epithelial cells in suspension, we observed that the fluorescence spectra at 270 nm excitation decreased with increased 4HPR concentration[46].

For fluorescence emission spectra at 320 (Figure 3b), 330, 340 and 350 nm excitations, it was found that the primary peak was around 390 nm with a secondary peak

at 460 nm. The primary peak may be attributed to the presence of the structural protein collagen and its cross link residue hydroxypyridinium. The intensity of the control group at this primary peak was comparable with the one of individual groups and lower than that of the combination groups. The emission of the control and individual drug groups were similar at the primary emission peak: these chemopreventive drugs would not be expected to affect collagen[6] because the OCP is thought to be receptor mediated and 4HPR is thought to be both nuclear receptor mediated and receptor independent. However, the primary peak intensity for combination groups was higher than that of the control group, suggesting a synergistic effect of 4HPR and OCP on collagen cross link residues related emission which can not be achieved with either drug alone. Other publications have observed that the majority of the normal tissue organs indicated higher collagen peak intensity for normal tissue than cancerous or precancerous tissues.[47,48] Our data suggest that these drugs in combination produce an even higher than normal tissue peak in the wavelengths attributed to collagen. The secondary peak around 460 nm may be attributed to NAD(P)H. It appears as emission at this peak is reduced after treatment which is consistent with changes in the signature of NAD(P)H in response to drug treatment as suggested by a previous studies[6,21] Nevertheless, differences in spectral line shape at the excitation wavelengths between 320 and 350 nm indicate that the individual groups and combination groups represent unique optical signatures that might be explained by differences in cellular metabolism as well as collagen contribution.

Fluorescence emission spectra at 360 to 390 nm excitation (Figure 3c) present with a peak around 470 nm for both control and post-drug measurements. The peak location was consistent with NAD(P)H emission and earlier literature effectively

reviewed changes of NAD(P)H related with tumor tissues.[43,44] It was observed that the control group had a higher emission intensity than individual and combined drug groups. Interestingly, differences were smaller between the control group and the combined treatment groups than with the individual drugs. Additionally the 4HPR group was affected more strongly than OCP group, again indicating a complex interaction between the groups suggesting that each drug group as well as the combination of both drug groups have a unique effect on cellular metabolism.

To quantify the spectral difference between control and post-drug measurements, original and normalized fluorescence emission spectra were utilized. Original spectra results account for the differences in fluorescence intensity and spectral line shape between control and post drug measurements. The advantage of normalization is that it cancels out inter-group variations in intensity and allows for a direct comparison of spectral line shape. In Tables 3(a-e) and 4(a-b) it was observed that in general normalized spectra yielded a higher accuracy rate than that of original spectra, indicating that spectral line shape or ratiometric measurements yield better accuracy in determining the effect of chemopreventive drugs.

When Table3a (category I. 4HPR) is compared with Table 3b (Category II, OCP), most of the results from Table 3b have a higher accuracy rate, indicating that OCP results are more consistent than 4HPR. In addition, Tables3c (category III, 4HPR+OCP), 3d (category IV, 4HPR after OCP) and 3e (category V, OCP after 4HPR) showed higher accuracy results than Table 3a and 3b at most excitation wavelengths, indicating a synergistic effect of the combination of both groups. The same is reflected in table 4(a-b). In particular, normalized emission spectra for lower excitation wavelengths (from 290 to

340 nm) from the combination groups yielded nearly perfect classification results(≈ 100%). These results suggest that each drug group, - 4HPR and OCP -, have unique chemopreventive properties and combination groups might result in a cumulative effect.

We observed that the accuracy rate for the fluorescence spectra for 290 (Figure 3a) and 300 nm excitations for all categories was excellent. The combination categories (III, IV and V) had rates close to or at 100%. These results indicate a consistent change of tryptophan associated signature with respect to chemopreventive drugs. Additionally emission spectra obtained at 320 (Figure 3b) and 360nm (Figure 3c) excitation in general performed well for all categories. From the tables 3, it is observed that the normalized spectra of most of the excitation wavelengths for all categories provided better results than the original spectra at the same excitation wavelengths. When comparing original spectra results for category I (4HPR) with category II (OCP), category II yielded better results than category I, though individual spectra of category I (4HPR) showed on average a larger spectral difference than category II (OCP). This indicates a higher variation in response in the 4HPR group. Normalization in general increased the accuracy but also the number of selected PLS components.

To investigate the effect of chemopreventive agents, the non-human primate is an excellent model for combining Bi-Linear PLS analysis with linear discrimination of the fluorescence spectroscopy data. There is evidence that 4HPR and OCP have different chemopreventive effects on the ovary. In addition, combination groups (4HPR+OCP, 4HPR after OCP, and OCP after 4HPR) seem to result in different optical response patterns, compared to the individual groups (4HPR and OCP) which suggest that the drugs reacts in different response mechanisms and that a combination treatment of 4HPR

and OCP may have a higher chemopreventive potential. The study was planned with a 3 week washout period to allow any residual drugs or drug effect to be dissipated. However, our results suggest that 4-HPR followed by the OCP or OCP followed by 4-HPR each has a unique effect on the ovary that was not washed out with the 3 weeks off of the drug. Further work is necessary to verify the chemopreventive effects of the combination of 4HPR and OCP group with various fluorophores as well as to investigate the action of these drugs when given in tandem. This primate model will allow us to develop similar non-human models for chemopreventive drugs for ovarian cancer, understand how these drugs affect the ovary, and develop rational chemoprevention strategies for this disease.

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	Treatment Group I	Treatment Group II	Treatment Group III
First Measurement	**1.** Control (38)	**2.** Control (39)	**3.** Control (38)
First treatment	***4HPR***	***OCP***	-
Second Measurement	**4.** 4HPR (35)	**5.** OCP (46)	**6.** Second Control (39)
Second treatment	***OCP***	***4HPR***	***OCP + 4HPR***
Third Measurement	**7.** OCP after 4HPR (31)	**8.** 4HPR after OCP (30)	**9.** Combination (4HPR+OCP) (31)

Table 1

Category	Control	Versus	Treatment groups (post-drug measurements)
I	Control (Groups 1, 2, 3)		4HPR (group 4)
II	Control (Groups 1, 2, 3)		OCP (group 5)
III	Control (Groups 1, 2, 3)		4HPR + OCP (group 9)
IV	Control (Groups 1, 2, 3)		4HPR after OCP (group 8)
V	Control (Groups 1, 2, 3)		OCP after 4HPR (group 7)

Table 2

Excitation Wavelengths	Original Spectra			PLS components selected
	Specificity (%)	Sensitivity (%)	Accuracy (%)	
290	91.3	94.3	92.0	6

300	89.6	97.1	91.3	6
310	71.3	88.6	82.0	2
320	86.1	88.6	84.7	7
330	80.9	90.4	83.3	6
340	80.9	80.0	80.7	5
350	84.3	82.9	84.0	8
360	80.9	88.6	82.7	6
370	79.1	85.7	80.7	7
380	91.3	97.1	90.7	9
390	89.6	82.9	88.0	7
Excitation Wavelengths	Normalized Spectra			PLS components selected
	Specificity (%)	Sensitivity (%)	Accuracy (%)	
290	94.8	85.7	92.7	7
300	91.3	77.1	88.0	5
310	72.2	71.4	72.0	3
320	87.8	94.3	89.3	8
330	88.7	85.7	88.0	7
340	88.7	74.3	85.3	7
350	87.0	88.6	87.3	7
360	93.0	97.1	94.0	9
370	88.7	80.0	86.7	7
380	92.2	97.1	93.3	8
390	98.3	91.4	96.7	9

Table 3a

Excitation Wavelengths	Original Spectra			PLS components selected
	Specificity (%)	Sensitivity (%)	Accuracy (%)	
290	95.7	97.8	96.3	6
300	92.2	100.0	94.4	3
310	80.9	71.7	78.3	7
320	92.2	87.0	90.7	6
330	93.9	91.3	93.2	6
340	85.2	87.0	85.7	5
350	80.0	80.4	80.1	5
360	80.9	73.9	78.9	4
370	93.9	76.1	88.8	10
380	93.9	93.5	93.8	6
390	88.7	84.8	87.6	11
Excitation Wavelengths	Normalized Spectra			PLS components selected
	Specificity (%)	Sensitivity (%)	Accuracy (%)	
290	100.0	91.3	97.5	6
300	98.3	97.8	98.1	4
310	82.6	76.1	80.7	7
320	96.5	82.6	92.5	6
330	100.0	89.1	96.9	6
340	100.0	89.1	96.9	6
350	100.0	97.8	99.4	11
360	98.3	97.8	98.1	8
370	90.4	78.3	83.9	10
380	100.0	97.8	99.4	10
390	92.2	87.0	90.7	10

Table 3b

Excitation Wavelengths	Original Spectra			PLS components selected
	Specificity (%)	Sensitivity (%)	Accuracy (%)	
290	99.1	100.0	99.3	7
300	95.7	100.0	96.6	6
310	84.3	64.5	80.8	2
320	100.0	87.1	97.3	8
330	99.1	90.3	97.3	6
340	99.1	90.3	97.3	6
350	98.3	90.3	96.6	7
360	96.5	96.8	96.6	7
370	87.0	90.3	87.7	9
380	98.3	96.8	97.9	7
390	79.1	74.2	78.1	4
Excitation Wavelengths	Normalized Spectra			PLS components selected
	Specificity (%)	Sensitivity (%)	Accuracy (%)	
290	100.0	100.0	100.0	7
300	99.1	100.0	99.3	6
310	88.7	83.9	87.7	8
320	99.1	100.0	99.3	9
330	100.0	100.0	100.0	10
340	98.3	100.0	98.6	9
350	98.3	93.5	97.3	7
360	100.0	100.0	100.0	9
370	83.5	90.3	84.9	8
380	93.0	87.1	91.8	5
390	93.9	96.8	94.5	10

Table 3c

Excitation Wavelengths	Original Spectra			PLS components selected
	Specificity (%)	Sensitivity (%)	Accuracy (%)	
290	100.0	100.0	100.0	6
300	100.0	100.0	100.0	6
310	86.1	80.0	85.3	3
320	100.0	90.0	97.9	8
330	100.0	86.7	97.2	5
340	100.0	98.6	98.6	5
350	100.0	93.3	98.6	8
360	100.0	96.7	99.3	7
370	83.5	86.7	84.1	7
380	99.1	93.3	97.9	6
390	74.8	73.3	74.5	4
Excitation Wavelengths	Normalized Spectra			PLS components selected
	Specificity (%)	Sensitivity (%)	Accuracy (%)	
290	100.0	100.0	100.0	5
300	100.0	100.0	100.0	8
310	80.9	90.0	82.8	3
320	100.0	100.0	100.0	6
330	100.0	100.0	100.0	6
340	100.0	100.0	100.0	6
350	99.1	100.0	99.3	6
360	100.0	100.0	100.0	8
370	94.8	86.7	93.1	10
380	99.1	100.0	99.3	6
390	97.4	93.3	96.6	10

Table 3d

Excitation Wavelengths	Original Spectra			PLS components selected
	Specificity (%)	Sensitivity (%)	Accuracy (%)	
290	99.1	100.0	99.3	5
300	98.3	100.0	98.6	6
310	87.0	61.3	82.1	3
320	100.0	80.6	95.9	8
330	100.0	90.3	97.9	6
340	100.0	87.1	97.3	6
350	100.0	90.3	97.9	8
360	100.0	93.5	98.6	6
370	75.7	64.5	73.3	5
380	99.1	90.3	97.3	6
390	80.0	77.4	79.5	5
Excitation Wavelengths	Normalized Spectra			PLS components selected
	Specificity (%)	Sensitivity (%)	Accuracy (%)	
290	100.0	100.0	100.0	6
300	99.1	100.0	99.3	3
310	74.78	70.96	73.79	3
320	100.0	96.8	98.6	6
330	100.0	100.0	100.0	7
340	100.0	100.0	100.0	9
350	99.1	100.0	99.3	8
360	100.0	100.0	100.0	8
370	96.5	90.3	95.2	9
380	100.0	96.8	99.3	10
390	90.4	80.6	88.4	7

Table 3e

Excitation wavelength	Control versus Individual groups			
	Specificity (%)	Sensitivity (%)	Accuracy (%)	Number of components
290	96.5	82.7	90.8	6
300	97.4	86.4	92.9	9
310	87.0	82.7	85.2	10
320	91.3	76.5	85.2	7
330	86.1	79.0	83.2	5
340	87.7	80.2	84.6	5
350	89.6	76.5	84.2	7
360	88.7	75.3	83.2	5
370	86.1	74.1	81.1	8
380	97.4	85.2	92.3	8
390	91.3	81.5	87.2	11
Excitation wavelength	Control versus Combination groups			
	Specificity (%)	Sensitivity (%)	Accuracy (%)	Number of components
290	100.0	100.0	100.0	7
300	100.0	98.9	99.5	7
310	82.6	84.8	83.6	8
320	98.3	98.9	98.6	8
330	98.3	91.3	95.2	5
340	99.1	97.8	98.6	7
350	99.1	96.7	98.1	8
360	97.4	96.7	97.1	9
370	71.3	71.7	71.5	4
380	99.1	95.7	97.6	8
390	89.5	83.9	87.0	8

Table 4a

Excitation wavelength	Control versus Individual groups			
	Specificity (%)	Sensitivity (%)	Accuracy (%)	Number of components
290	93.0	95.1	93.9	6
300	90.4	98.8	93.9	6
310	73.9	79.0	76.0	7
320	76.5	76.5	76.5	5
330	80.9	81.5	81.1	5
340	78.3	84.0	80.6	6
350	83.5	84.0	83.7	8
360	80.0	82.7	81.1	6
370	63.5	77.8	69.4	4
380	89.6	86.4	88.3	7
390	87.8	82.7	85.7	10
Excitation wavelength	Control versus Combination groups			
	Specificity (%)	Sensitivity (%)	Accuracy (%)	Number of components
290	99.1	100.0	99.5	7
300	96.5	100.0	98.1	8
310	88.7	82.6	85.9	8
320	100.0	81.5	91.8	6
330	97.4	79.3	89.4	6
340	98.3	87.0	93.2	5
350	94.8	83.7	89.9	5
360	96.5	92.4	94.7	6
370	67.0	75.0	70.5	5
380	97.4	81.5	90.3	5
390	96.5	95.7	96.1	11

Table 4b

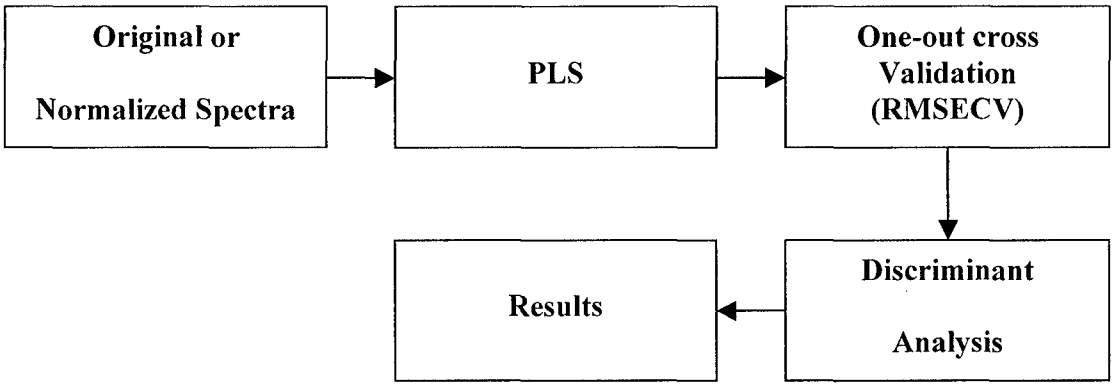

Original or
Normalized Spectra
PLS
One-out cross
Validation
(RMSECV)
Discriminant
Analysis
Results

Figure 1

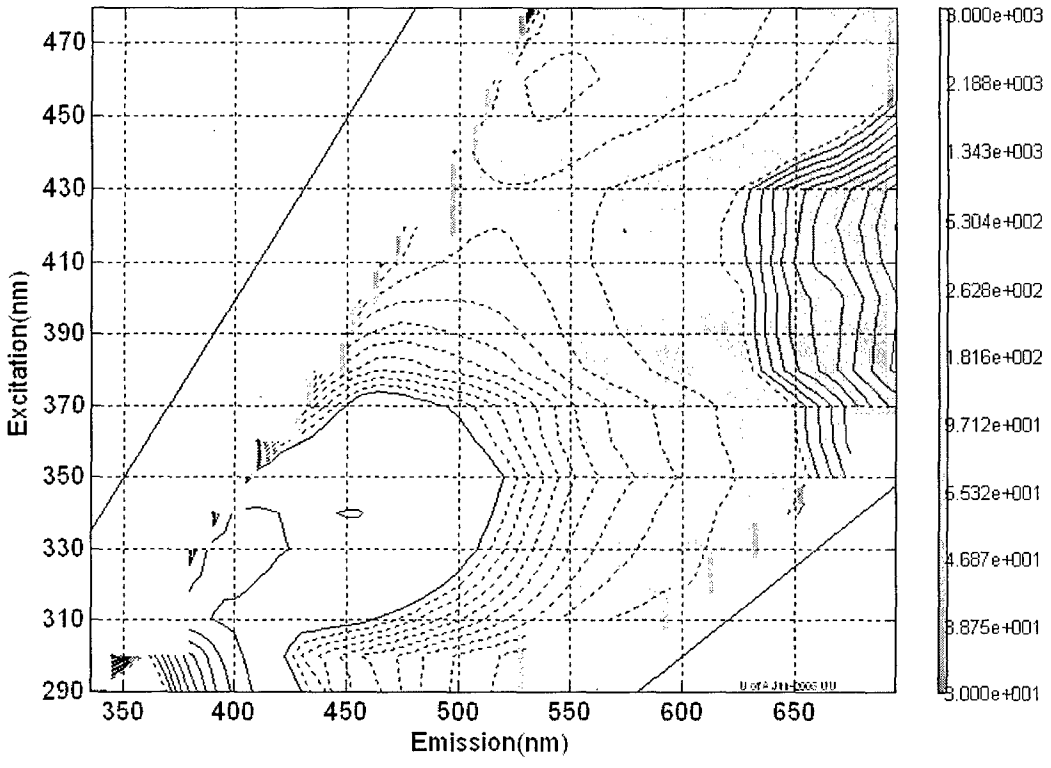
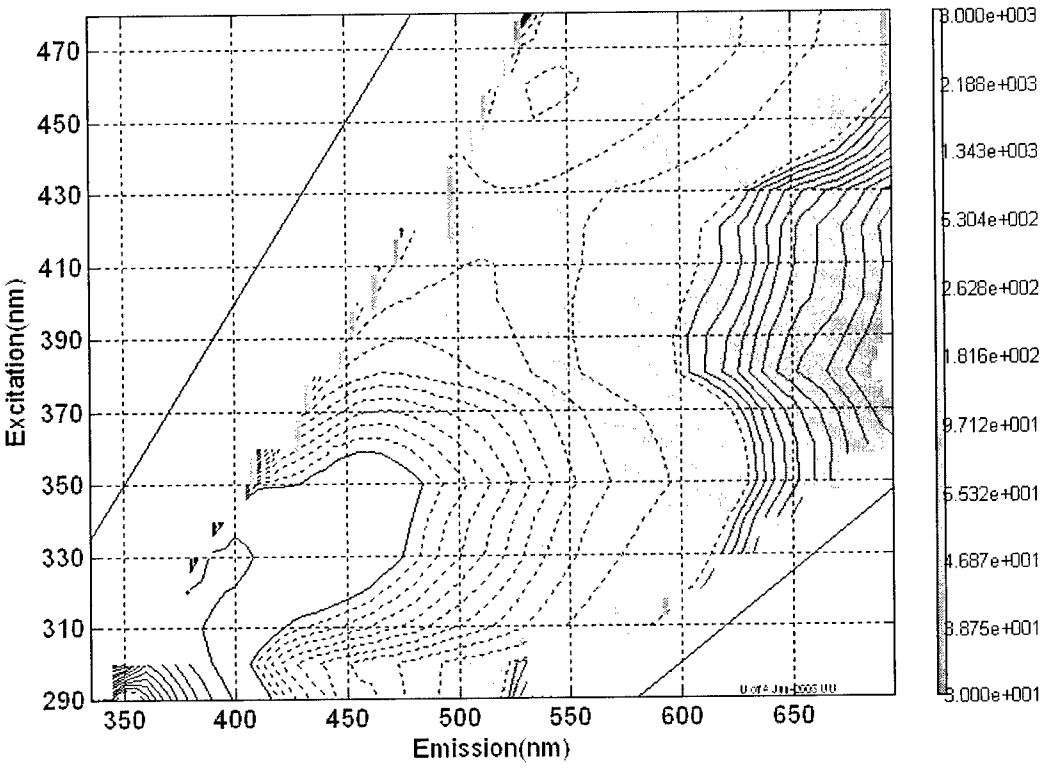

Excitation(nm)
470
450
430
410
390
370
350
330
310
290
350
400
450
500
550
600
650
Emission(nm)
3.000e+003
2.188e+003
1.343e+003
5.304e+002
2.628e+002
1.816e+002
9.712e+001
5.532e+001
4.687e+001
3.875e+001
3.000e+001

Figure 2a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Emission(nm)
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1.343e+003
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Figure 2b

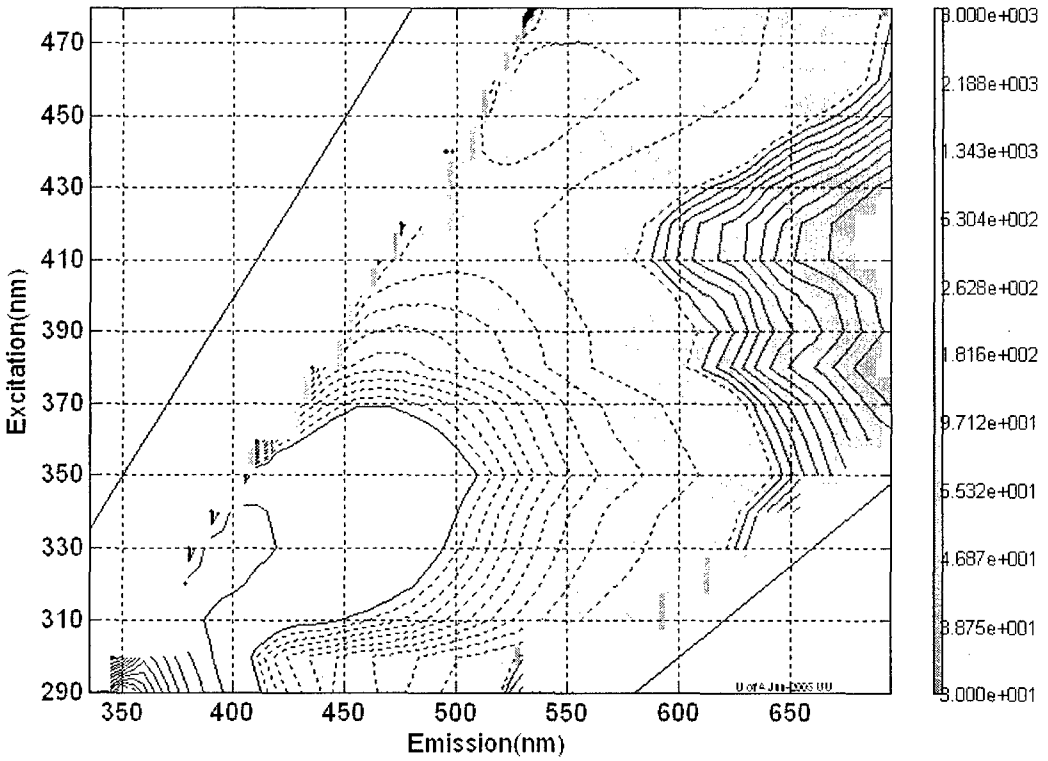
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Figure 2c

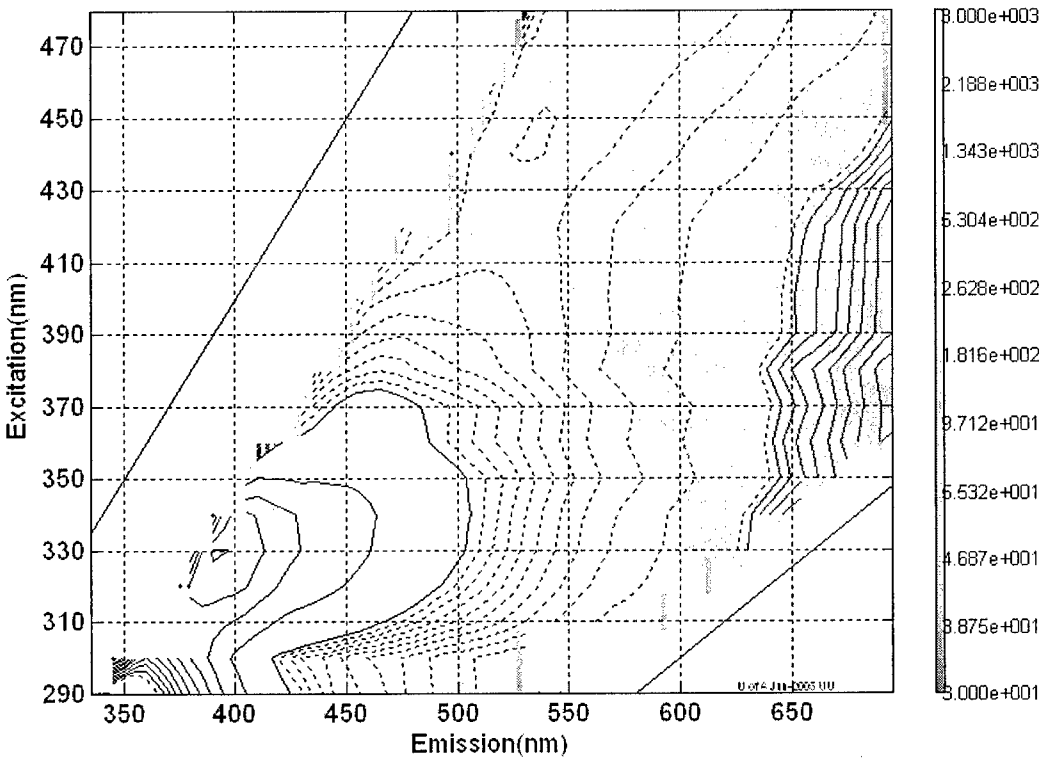
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Figure 2d

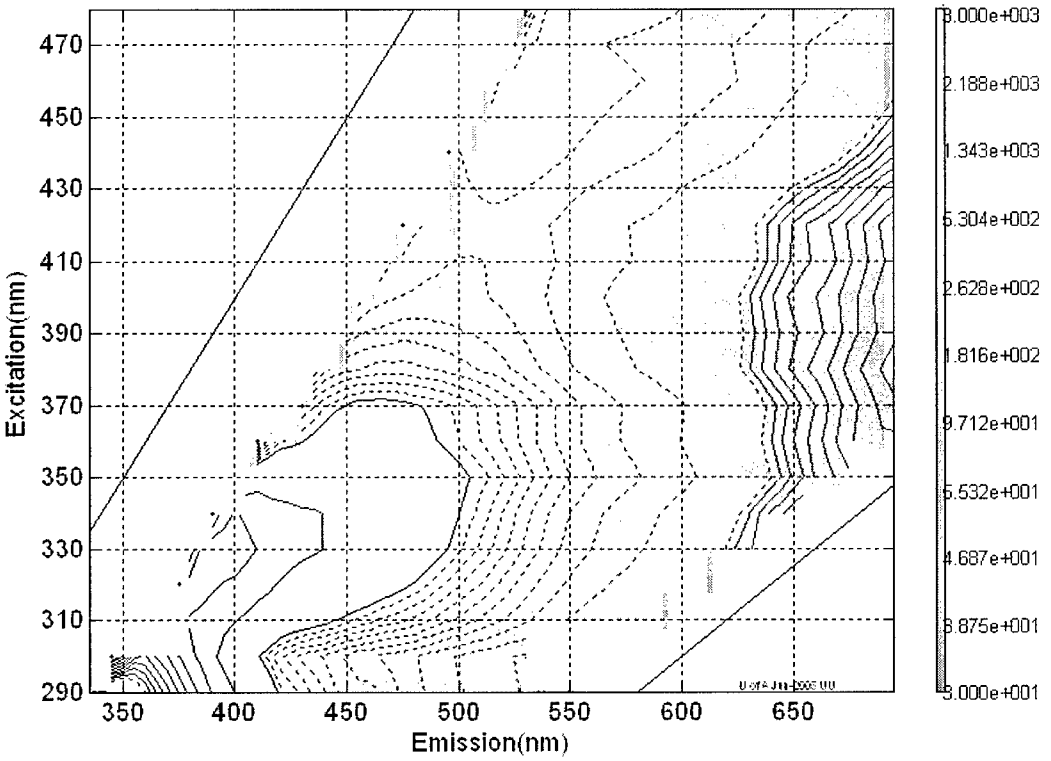
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Figure 2e

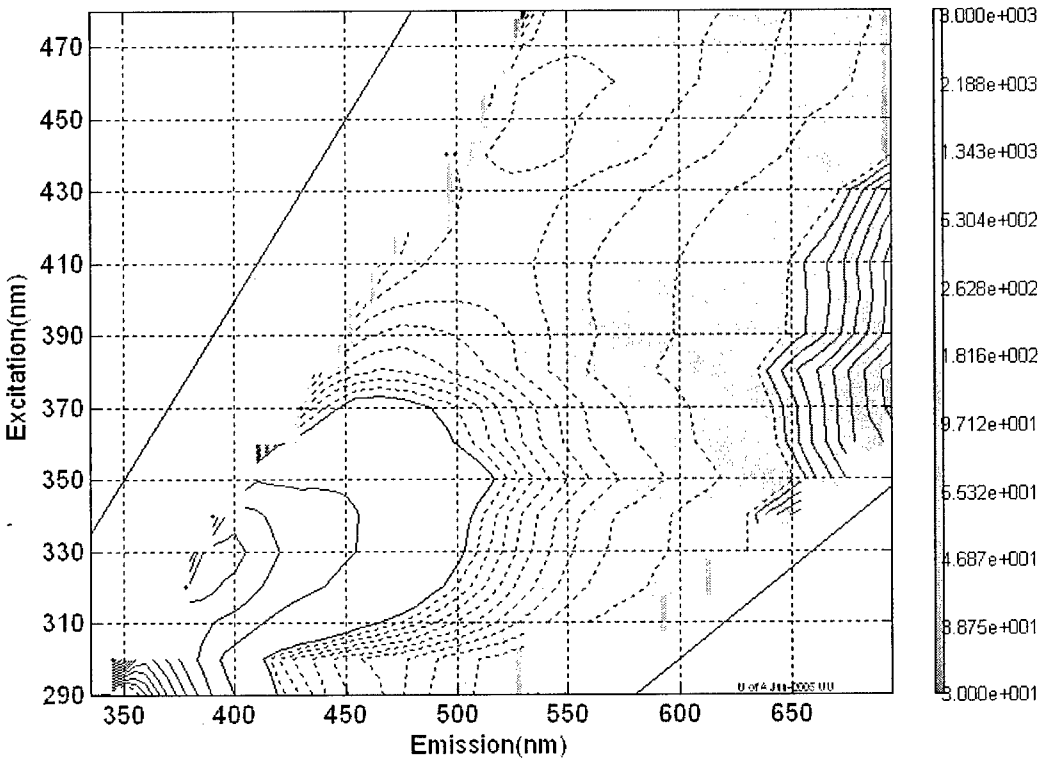
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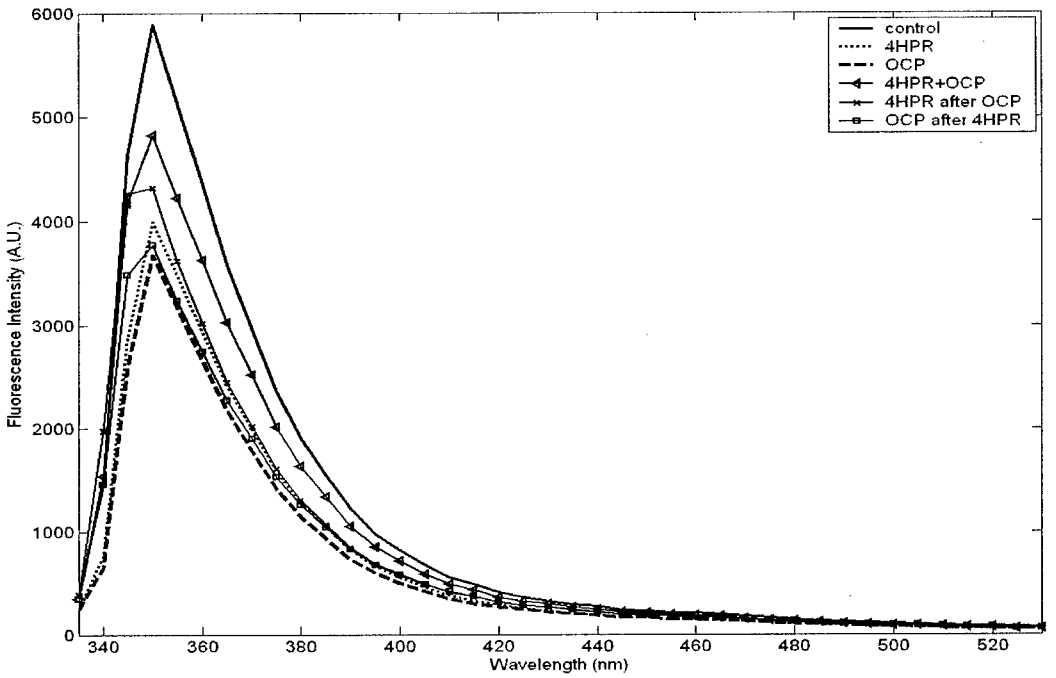
Figure 2f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OCP
4HPR+OCP
4HPR after OCP
OCP after 4HPR
Fluorescence Intensity (A.U.)
Wavelength (nm)
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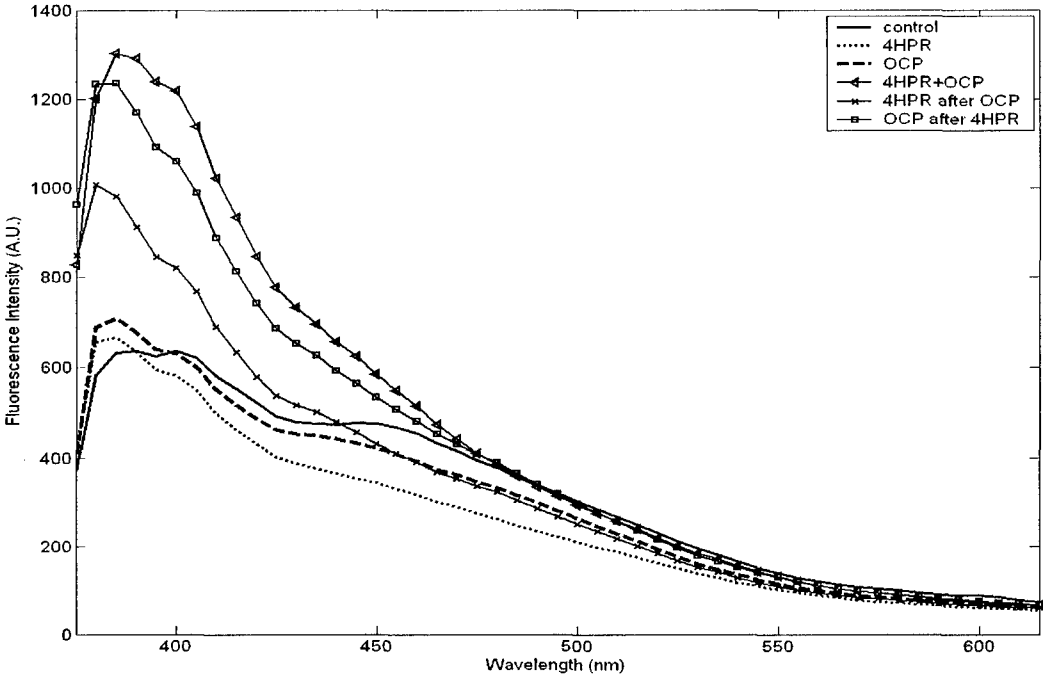
3000
4000
5000
6000
340
360
380
400
420
440
460
480
500
520

Figure 3a


control
4HPR
OCP
4HPR+OCP
4HPR after OCP
OCP after 4HPR
Fluorescence Intensity (A.U.)
Wavelength (nm)
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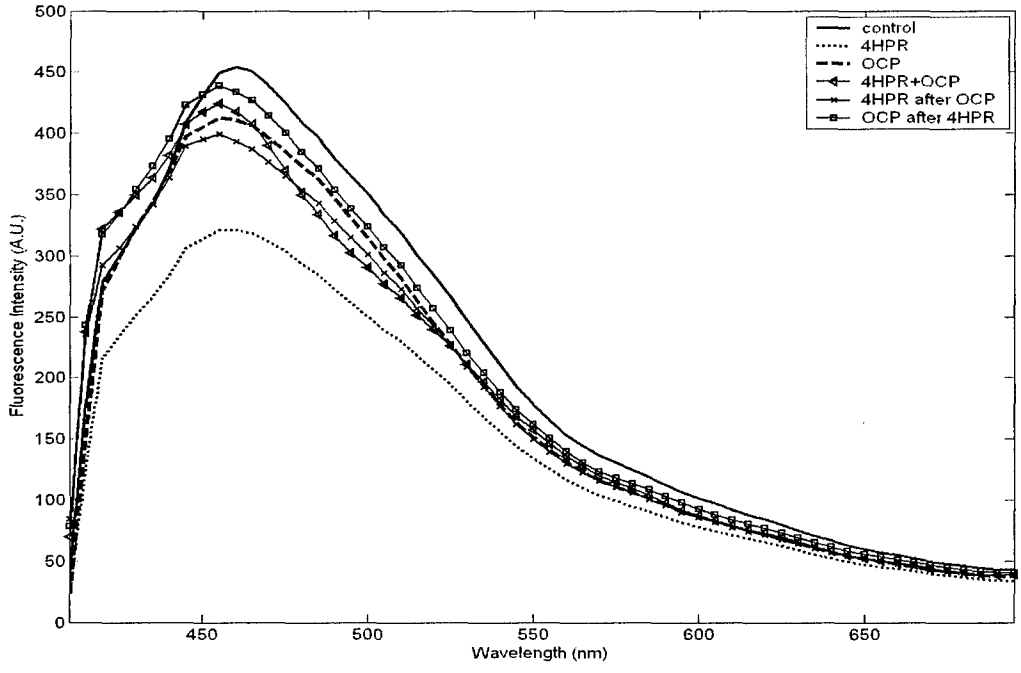
400
600
800
1000
1200
1400
400
450
500
550
600

Figure 3b


control
4HPR
OCP
4HPR+OCP
4HPR after OCP
OCP after 4HPR
Fluorescence Intensity (A.U.)
Wavelength (nm)
0
50
100
150
200
250
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400
450
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450
500
550
600
650

Figure 3c

4-HPR Modulates Gene Expression in Ovarian Cells

Molly Brewer, Nathaniel D. Kirkpatrick, J. Taylor Wharton, Jian Wang, Kenneth Hatch, Nelly Auersperg, Urs Utzinger, David Gershenson, Robert Bast, and Changping Zou

Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Arizona Cancer Center, Tucson, AZ 85724 (Molly Brewer, Kenneth Hatch, Jian Wang, Urs Utzinger and Changping Zou); Biomedical Engineering, University of Arizona (Molly Brewer, Nathaniel Kirkpatrick, Urs Utzinger); University of British Columbia, Vancouver, British Columbia (Nelly Auersperg), Department of Gynecologic Oncology (J. Taylor Wharton and David Gershenson), and Experimental Therapeutics (Robert Bast), The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030.

Running title: **Effect of 4-HPR on ovarian cells**

Key words: retinoids,4-HPR, apoptosis, ovarian cancer cells

Correspondence and Reprint Requests to:
Changping Zou, MD, PhD
Department of Obstetrics and Gynecology
Arizona Cancer Center
University of Arizona
1515 N. Campbell Avenue
Tucson, AZ 85724-5024
Tel: 520-626-8883
Fax: 520-626-9287
E-mail: czou@azcc.arizona.edu

Ovarian cancer has a high rate of recurrence and subsequent mortality following chemotherapy despite intense efforts to improve treatment outcomes. Recent trials have suggested that retinoids, especially 4-(*N*-hydroxyphenyl) retinamide (4-HPR), play an important role as a chemopreventive agent and are currently being used in clinical trials for ovarian cancer chemoprevention as well as treatment. This study examines the mechanism of its activity in premalignant and cancer cells. We investigated the modulation of gene expression by 4-HPR in immortalized ovarian surface epithelial (IOSE) cells and ovarian cancer (OVCA433) cells with DNA microarray. Real time RT-PCR and western blotting were used to confirm the microarray results and metabolic changes were examined with optical fluorescence spectroscopy. 4-HPR resulted in an up-regulation of expression of pro-apoptotic genes and mitochondrial uncoupling protein in OVCA433 cells and modulation of the RXR receptors in IOSE cells, and down-regulation of mutant BRCA genes in both IOSE and OVCA433 cells. 4HPR had a larger effect on the redox in the 433 cells compared to IOSE. These findings suggest that 4-HPR acts through different mechanisms in premalignant ovarian surface cells and cancer cells, with a preventive effect in premalignant cells and a treatment effect in cancer cells.

Introduction

Epithelial ovarian cancer is the leading cause of death from the gynecologic cancers.[1] In 2003, an estimated 25,400 women were diagnosed with ovarian cancer, and 14,300 women died from the disease.[1] It is most commonly diagnosed in Stage III or IV where the mortality rate is 70% or greater. [2] Currently, there is no generally accepted screening test in which sensitive and reliable biomarkers can be used to identify women destined to develop ovarian cancer. Although initial treatment for ovarian cancer has an excellent response rate, the recurrence rate is high following chemotherapy and drug resistance is a common problem. Better strategies for prevention and treatment of ovarian cancer are thus strongly warranted.

Retinoids have been intensively investigated as chemopreventive agents and have been shown to inhibit ovarian carcinogenesis based on both laboratory data and clinical trials. [3-7] The potential of the synthetic retinoid 4-HPR to prevent ovarian cancer was recognized in a large Italian breast cancer chemoprevention clinical trial. [5-7] Patients on the 4-HPR arm demonstrated a decreased incidence of ovarian cancer [7], with 6 patients developing ovarian cancer in the control group for the duration of drug ingestion but none in the 4-HPR group ($p=0.0327$) [6]. After cessation of the clinical trial, 6 patients in the 4-HPR group developed ovarian cancer compared to 4 in the control arm ($p=0.7563$). [6] This difference was not statistically significant suggesting that the effect of the retinoid was not durable. [6] The mechanism of action of 4-HPR's cancer chemoprevention is unclear. It may act partly through modulation of gene expression via retinoid receptors although modulation of retinoid receptors is still controversial. [8-14] Retinoid receptors are members of the steroid hormone receptor superfamily. Two types of receptors have been identified: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Each type includes three subtypes with distinct amino- and carboxyl-terminal domains. The RARs bind to all-trans-

retinoic acid (ATRA) and 9-cis-retinoic acid (9cRA), a natural retinoic acid isomer, whereas the RXRs bind only to 9cRA. [15-18] RARs can form heterodimers with RXRs and bind to retinoic acid response elements (RARE), specific DNA sequences that are characterized by direct repeats of (A/G)GGTCA separated by two or five nucleotides that act as ligand-dependent transcriptional regulators for retinoic acid-responsive genes. Some investigators hypothesize that both *all-trans* retinoic acid (ATRA) and 4-HPR bind to retinoic acid response element (RARE) and regulate gene expression (Zou and Lotan unpublished data). [19, 20]

DNA microarray is widely used in identifying gene expression in normal and cancer cells, and in evaluating molecular changes before and after treatment with drugs. [21-24] Use of array technology allows simultaneous evaluation of expression of many (up to thousands) genes. The challenge of such a powerful technique is to develop rigorous, quantitative methods for interpretation of such a wealth of data to identify the expression profile providing maximal biologic information.

Techniques based on quantitative optical fluorescence spectroscopy have shown promise to improve detection of epithelial lesions in the colon, cervix, bladder, head and neck, esophagus and other epithelial surfaces. [25-28] Certain molecules within a cell can be excited using light in the visible and UV range. This principle can be used to optically interrogate endogenous fluorophores with quasi monochromatic excitation light. Natural intra cellular fluorophores include electron carriers nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) and the aromatic amino acids tryptophan, tyrosine and phenylalanine, as well as structural proteins, each of which have a characteristic wavelength for excitation with an associated characteristic emission. [29] In particular, FAD and NADH can provide an estimate of mitochondrial metabolic activity through an estimate of cellular redox. [30] Fluorescence

spectroscopy of endogenous fluorophores has been used as a marker for both early detection and chemoprevention. [31-33]

Identification of biomarkers is important to detect abnormal cells so that invasive cancer can be prevented through chemoprevention. Furthermore, intermediate end point biomarkers are valuable in timely evaluation of the drug efficacy during chemoprevention trials. However, potentially useful biomarkers for evaluating ovarian cancer and 4-HPR's effect on the ovary are currently limited. To identify biomarkers in response to 4-HPR treatment and to investigate the mechanism of its action in ovarian cancer treatment and/or prevention, we used the *in vitro* model of normal cells (IOSE cells) and ovarian cancer OVCA433 cells. Ovarian surface epithelial (OSE) cells originated from ovarian epithelial carcinomas in which women with a strong family history of ovarian carcinomas or with a mutation in one of the two known cancer suppressor genes – BRCA1 and BRCA2. [34, 35] Since OSE cells are thought to be the site of origin of epithelial ovarian cancer, these cells are important to study their molecular and cellular properties compared to ovarian cancer cells to enhance our understanding of malignancy in ovarian cancer.

We compared changes in gene expression from treatment with 4-HPR in normal and malignant ovarian cells by microarray and examined 4HPR action in the context of growth inhibition, apoptosis induction, and mitochondrial permeability transition changes. The gene expression changes were verified further by real-time RT-PCR and Western blot while the metabolic status was evaluated with fluorescence spectroscopy.

MATERIALS AND METHODS

Cell Lines and Retinoids

Immortalized ovarian surface epithelial cells (IOSE) and ovarian cancer OVCA433 cells were grown as previously described. [8] The cells were incubated for 3 days with 4-HPR with different concentration of 4-HPR (1, 5 and 10 μM). Control cultures contained DMSO. N-(4-hydroxyphenyl) retinamide (4-HPR) was purchased from the Sigma Chemical Co. (St. Louis, MO), dissolved in dimethylsulfoxide (DMSO) at stock solutions of 10^{-2}M, and stored in an atmosphere of N_2 at -80° C.

RNA Preparation and Microarray

Total RNA was extracted as previously described. [8] We used DNA chip technology to identify gene expression in the Genomics Core Laboratory at the University of Texas Health Sciences Center. The cDNA chips (Agilent Technologies, Palo Alto, CA) with 8,000 human cDNA's, which had dual-labeled cDNA hybridization for use in high density cDNA microarrays. IOSE and OVCA433 cells were treated with 1 μM 4-HPR for 3 days. The RNA samples were evaluated for degradation and DNA contamination on an Agilent 2100 Bioanalyser (Agilent) using a RNA 6000 NanoKit. RNA from the control and the treated cells were submitted for comparison to each microarray chip. These RNA's were used with a Micromax TSA Labeling Kit (Perkin Elmer Life Sciences, Boston, MA) and Cyanine-3 or Cyanine-5 dyes. The recommended wash and labeling procedures for the Perkin Elmer TSA kit were followed except for increasing the post Cyanine 5 Tyramide labeling washes to 10 min each. The washed chips were read on a ScanArray Lite (Perkin Elmer) and the digital image output analyzed using their software (QuantArray). The resulting values were then compiled using either Excel or File Maker Pro macros written for the Microarray Core Laboratory.

Effect of 4-HPR on Cell Proliferation in Monolayer Cultures

IOSE and OVCA433 cells were placed in 96-well plates at 10^5 cells per well and grown for 24 hrs. The cells were incubated for 5 days with 4-HPR in 1, 5, and 10 μM concentrations. Growth inhibition was determined using the crystal violet method as previously described [8]. All experiments were performed in triplicate and the mean ± standard deviations calculated.

Analysis of Apoptosis Induced by 4-HPR

Terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-deoxyuridine-triphosphate (dUTP) nick-end labeling (TUNEL) assay was used (8). Flow cytometry used a FACScan flow cytometer (Epics Profile, Coulter Corp., Hialeah, FL) with a 15 mW Argon laser used for excitation at 488 nm. Fluorescence was measured at 570 nm. Computer analysis of the data provided information on the percentage of apoptotic cells. All experiments were performed in triplicate and the mean ± standard deviations calculated.

Caspase 3 Activity Assay and Protein Analysis

The cells were plated in 96-well tissue culture plates at densities ranging from 0.5-1 x 10^5 cells per well and treated with 4-HPR in 1, 5, and 10 μM concentrations for 12, 24, 48, and 72 hours. Control cultures and treated cultures contained the same amount of DMSO. The method for analysis of Caspase 3 activity as previously described [8].

Mitochondrial Permeability Transition (MPT) Assay

IOSE and OVCA433 cells were treated with 4-HPR in 1, 5, and 10 μM concentrations for 3 days to determine the time of maximal mitochondrial permeability transition. Cells were washed and resuspended in 40 nM MitoFluor medium, then incubated at 37°C for 30-45 min.

Cells were visualized under the fluorescence microscope at 490 nm excitation, 576 nm emission. A field of 20-30 cells was chosen using a photo amplifier to measure light intensity. Once the time of maximal mitochondrial permeability was determined, the remainder of the experiments were carried out using the pre-determined times of incubation.

Western Analysis of p53, p21, and p16 Gene Expression Modulated by 4-HPR

Genes showing alterations in expression by 4-HPR (> 2 fold increase or >2-fold decrease) were validated by real-time Q-RT-PCR or Western blotting. Nuclear and cytoplasmic protein extracts were prepared as previously described [8].

Real time Q RT-PCR Analysis for mRNA Expression of RARs and BRCA Genes

Real time Q RT-PCR performed in the University of Arizona Core facility by utilizing the 7700 sequence detector (Applied Biosystems, Foster City, CA) with a similar protocol as previously described. [8].

Optical Spectroscopic Analysis of Redox Ratio FAD/(FAD+NADH)

Fluorescence emission was measured on IOSE cells and OVCA433 cells. Cells were treated with 1 μM, 5 μM, or 10 μM 4-HPR 24 hr before fluorescence measurements, described previously. [36]

Results

Expression of Genes Altered by 4-HPR in IOSE and OVCA433 Cells Detected by Microarray

Microarray analysis was performed using total RNA purified from treated and untreated cells. The expression of genes modulated by 4-HPR was evaluated. Genes with a change of

expression >2 fold were recorded in Table 1a and b. In IOSE cells, there was up-regulation of apoptotic related genes and differentiation genes, as well as genes on chromosome 3 and 9. Cancer cells showed up-regulation of fewer genes associated with apoptosis and showed similar effects on up-regulation of the anti-oncogene segment on chromosome 9. Mitochondrial, NAD, NADH, and NADPH genes were modulated by 4-HPR in both IOSE and OVCA433 cells (Table 2).

Growth Inhibition and Apoptosis Induction by 4-HPR in Ovarian Cell Lines

IOSE and OVCA433 cells treated with different concentrations of 4-HPR, the growth inhibitory effect were compared in monolayer culture. Increasing the concentration of 4-HPR resulted in dose-dependent growth inhibition (Fig 1).

Apoptosis Induction by 4HPR in Ovarian Cells

Apoptosis induction in IOSE and OVCA433 cells were analyzed by TdT-labeling and flow cytometry after 3 days of treatment. Results showed 4-HPR apoptosis induction was dose-dependent (Fig 2). Cell cycle analysis demonstrated that 4-HPR increased the percentage of cells in the G_1 phase in OVCA433 cells, also in a dose-dependent manner (Fig 2).

Effect of 4-HPR on Caspase 3 Activity and Caspase 3 and 9 Protein Expression

Caspase 3 activity is a central mediator of apoptosis. Caspase 3 activity was measured in IOSE and OVCA433 cells at different time points with different concentration of 4-HPR. Caspase 3 enzyme activity was slightly increased at day 3 in the different concentration groups in OVCA433 cells (Fig 3a), which correlated with maximal apoptosis and growth inhibition in

these cells. However, Caspase 3 and Caspase 9 protein were not changed by 4-HPR in either IOSE or OVCA433 cells (Fig 3a and b).

Effect of 4-HPR on Mitochondrial Permeability Transition (MPT)

Mitochondrial permeability transition (MPT) changes are associated with mitochondrial mediated apoptosis. To investigate the mechanism of 4-HPR induced apoptosis in ovarian cancer cells, experiments were carried out to investigate the effect of 4-HPR in mitochondrial potential in IOSE and OVCA433 cells. 4-HPR decreased mitochondrial inner-membrane potential, which increased MPT in IOSE and OVCA433 cells (Fig 4). An inverse relationship in mitochondrial potential correlated in a dose-dependent manner with the increase in apoptosis and growth inhibition by 4-HPR in IOSE and OVCA433 cells (Figs 1 and 4), suggesting that these activities were mediated by changes in the mitochondrial membrane.

Expression of Apoptosis-associated Genes Modulated by 4-HPR

The effect of 4-HPR on the expression of the apoptosis-associated genes p53, p21, p16 and Rb were examined along with BRCA genes with Western blot and real-time PCR method. The expression of these genes was detected in both IOSE and OVCA433 cells (Fig 5). 4-HPR increased expression after 3 days of treatment in a dose-dependent manner in OVCA433 cells (Fig 5), which correlated with the microarray results, showing an increase in human p53 binding protein mRNA in these cells (Table 1a).

4-HPR Modulating Rtinoid Receptors and BRCA Genes Detected by Q RT-PCR

Microarray data showed that retinoid receptors were modulated by 4-HPR. Some receptors were induced by 4-HPR and others were suppressed by 4-HPR. We verified the effect of 4-HPR

on receptor expression and induction by Q RT-PCR. RARs were not significantly changed by 4-HPR in either cell line (data not shown); however, RXRs were modulated by 4-HPR in IOSE cells. 4-HPR increased RXRα and RXRβ expression and decreased RXRγ expression in IOSE cells (Fig 6a). The expression of RARs and RXRs were not altered by 4-HPR in cancer cells (Fig 6a).

BRCA1 and BRCA2 gene expression were decreased by 4-HPR in both IOSE and OVCA433 cells (Fig 6b). Real time RT-PCR result was consistent with microarray analysis (Table 1b and Fig 6b).

Redox Ratio Changed by 4-HPR Detected by Optical Spectroscopic Analysis

As shown in Fig 7a, the IOSE cell line exhibited a highly variable redox related fluorescence ratio compared to the OVCA433 cell line in which the estimated redox increased in a linear fashion. The OVCA433 cells demonstrated a strong sensitivity to 4-HPR treatment and Fig 7a illustrates that dose dependence as a linear increase with a slope of 0.0059 / μM 4-HPR ($p<0.001$). An increased redox ratio suggests less oxidative metabolism indicating the cells may be entering quiescence. When considering the relative ratios to untreated cells, as shown in the Fig 7b, the OVCA433 cells had a higher value at each drug dosage. At higher concentrations of 4-HPR the redox related fluorescence ratio increased for the IOSE cells but never reached the level of the OVCA433 cells. This is consistent with the result that the IOSE cell line was variable in response to 4-HPR treatment.

Discussion

The goal of chemoprevention is to prevent the progression of pre-cancerous cells to cancer. In practice, to achieve this goal, surrogate endpoint biomarkers are needed, because the biologic

endpoint (cancer development) may take many years and may be difficult to detect precisely. Large numbers of patients would have to be entered into such a trial to reach statistically significant conclusions. Using biomarkers that reliably predict progression and differentiation, a study can be completed with fewer patients in a reasonable length of time. [37, 38] Unfortunately, only a limited number of potentially useful biomarkers for chemoprevention studies in ovarian cancer have been described. [39-48]

An Italian trial that evaluated 4-HPR for prevention of secondary breast cancers demonstrated a decreased incidence of ovarian cancer in women receiving 4-HPR, suggesting that retinoids prevented the development of ovarian cancer. [5-7] After cessation of 4-HPR treatment, new ovarian cancers occurred in the treatment group, suggesting that this prevention was not durable [7]. Experimental studies have demonstrated that retinoids can affect human ovarian cancer cell growth by inhibiting proliferation and inducing apoptosis [4, 11-13], which are thought to be important mechanisms in cancer prevention, as well as in cancer treatment. However, the detailed mechanism of retinoid activity, including 4-HPR, in cancer chemoprevention has remained unclear. We have used DNA microarray to examine genes whose expression is modulated by 4-HPR in immortalized normal ovarian epithelial and ovarian cancer cells. The results are verified by real-time RT-PCR and western blot to further strengthen our conclusions.

P53, a tumor suppressor protein and transcription factor, is deleted or altered in many human cancers. P53 binds to DNA of cell cycle related genes to induce G1 arrest and allows cells to repair DNA damage or undergo apoptosis if DNA damage is too large for repair. P53 binds to DNA in response to DNA damage, a process that is redox sensitive and is inhibited by oxidizing conditions. Mutation of the p53 protein decreases DNA binding and inhibits activity in induction of apoptosis of genetically altered cells. [49] In our study, microarray data

demonstrated that 4-HPR increases human p53 binding protein mRNA expression and Rb binding protein in both normal and cancer cells (Table 1a), and p53/Rb expression were found in both IOSE and ovarian cancer cells. However, Western blot analysis showed p53 expression increased in OVCA433 cells only. In OVCA433 cells, p53 as well as downstream p21 and p16 proteins increase in a dose-dependent manner, suggesting the cells carry wild-type p53 (Fig 5). The concentration of 1 μM 4-HPR was chosen in microarray study and western analysis because most of clinical trials used this concentration.[5-7] A concentration of 1 μM is approximately equivalent to the plasma concentration when a dose of 200mg/day is administered. However, our results suggested that this dose may not be effective for ovarian cancer prevention. Although microarray results showed increasing gene expression, there were few changes at the protein level (table 1 and fig 5).

In OSE cells however, immortalized IOSE cells with catalytic subunit of telomerase (hTERT) and a SV40 Large T antigen inactivity of the p53/Rb pathway, the expression of p53 were detected but diminished when the temperature increased to 39°C for 5 and 7 days [50], supporting our findings. In downstream genes, p21 expression showed increased expression with the higher temperature but p16 expression was not changed by temperature. [50] The hTERT and a SV40 Large T antigen affected not only expression of p53, but also p16 in OSE cells. [50] 4-HPR treatment increased p21 expression in IOSE cells but the expression of p53 and p16 were not changed by 4-HPR, further confirming that the large T antigen affects p53 expression and thus is not modulated by 4-HPR (Fig. 5). This is a limitation of this cell line. However, primary cell cultures give highly variable results, limiting their usefulness.

p53/Rb mediates the action of 4-HPR on ovarian cancer cells which carry a functional or wild type p53. Up-regulation of p53, p21, and other downstream genes may be one of the mechanisms of retinoid-response in ovarian cancer cells, as is seen in other cell lines. [51, 52] A

previous study has reported [52] that ovarian cancer cell lines that are sensitive to retinoic acid have a higher expression of p53, p27, p21, and p16 compared to the retinoid resistant lines [52], which are concordant with our data. In OVCA433, which is sensitive to 4-HPR, there is an increase in p53, p21, and p16 expression in a dose-dependent manner, which correlates with growth inhibition and apoptosis. In early cancers, wild type p53 may still be present because p53 mutations may be a late event in carcinogenesis, specifically in ovarian cancer, suggesting one of the major effects of 4-HPR may require an intact p53 gene. This would also suggest that 4-HPR may be more active as a preventive agent rather than a treatment agent if a p53 mutation has occurred.

There is thought to be a role for nuclear retinoid receptors in mediating the retinoid regulation of growth, apoptosis, and gene expression. [15-18] RAR and RXR expression have different patterns in different tissues and organs. Retinoid treatment increased certain RAR mRNA levels in several normal and cancer cell lines [9-14] including several ovarian cancer cell lines. [4, 11, 12, 19, 20, 53] However, 4-HPR modulation of expression of retinoid receptors in ovarian cancer is still controversial. In this study, 4-HPR increased RXRα and RXRβ expression and decreased RXRγ expression in IOSE cells. Formelli's group reported that RARβ basal level expression and induction by 4-HPR play an important role in mediating 4-HPR response in ovarian cancer cells. [53] The over expressing RARα clone and RARβ clone increased the tumor-suppressive effect in ovarian tumors. [53] They also found that the most sensitive cell lines had RARβ expression and the highest levels of RARα and RARγ expression.[4] Moreover, ATRA inhibited ovarian cancer cell growth through RARα and RXRα [54, 55], with RXRα playing a critical role in mediating the growth inhibition in ovarian cancer cells. [54] In this study, the RXRs were regulated by 4-HPR in IOSE, but not in ovarian cancer cells, suggesting ovarian carcinogenesis may block some of the receptor expression and induction, and further study on

blocking these receptors to evaluate whether the effect of 4-HPR is altered will be forthcoming.

Retinoids, particularly 4-HPR, have been shown to increase aerobic glycolysis by increasing mitochondrial permeability to the co-enzymes NADH and FAD, as well as activity of the electron transport chain characterized by an increase in reactive oxygen species and cytochrome oxidase. [56, 57] There has been an increased interest in mitochondrial function in both normal and cancer cells; in particular, the mitochondria may be the site of induction of apoptosis by many preventive agents. 4-HPR induces a change in the mitochondrial permeability of the membrane permeability transition [57-59], which we hypothesize is one of the mechanisms of its suppressive activity in growth inhibition. Permeability of the mitochondrial inner membrane is increased by thiol agents and oxidative stress-inducing agents and is thought to be dependent on the opening of a non-selective pore [58]; intracellular redox potential increased along with increases in 4-HPR induced growth inhibition and apoptosis. [59] A shift towards a more oxidized condition increases membrane permeability, while the opposite occurs with reducing agents. Change in mitochondrial permeability allows cytochrome c to be released into the cytosol and is thought to initiate the Caspase system, ultimately with activation of Caspase 3 activity. [58, 60] Caspase 3 activity was investigated in this study because it is a pivotal step in both Caspase 9 and mitochondrial-induced apoptosis. We could not detect any significant change in Caspase genes after treatment with 4-HPR in our microarray and western analysis. Other Caspase genes besides Caspase 3 and 9 may be involved in 4-HPR induced apoptosis in ovarian cells and this requires further investigation.

The redox ratio of a cell defines the level of free radicals divided by the level of anti-oxidants. It is an indirect measure of the cells metabolic activity and functional ability of the electron transport chain. An increase in the ratio suggests a reduced metabolic activity under normoxic conditions. It is hypothesized that chemopreventive treatments reduce growth rate and

induce apoptosis which should result in reduced metabolic activity and an increased redox ratio. [32] The availability of free radical versus anti-oxidants within a cell can be measured by determining the ratio of FAD versus $FADH_2$ or NAD versus NADH. NADH is fluorescent but its oxidized complement NAD is only minimally fluorescent. On the other hand, $FADH_2$ is minimally fluorescent while FAD is fluorescent. Because both fluorophores are oxidized in the electron transport chain, measuring changes in the fluorescence intensity related to FAD serves as an estimate of changes in NAD. Given that the fluorescence from NADH and FAD can be measured non-invasively, a measure approximating the redox ratio can be obtained in vitro on cell cultures without the need for fixation and staining and chemical preparation. This may be an important non-invasive biomarker for the activity of chemopreventive agents *in vivo*.

Our results suggest the optically approximated redox status of the cells provides evidence of differences between the responses to 4-HPR treatment in the two ovarian cell lines. The IOSE cells exhibited a higher redox ratio that did not significantly increase with 4-HPR treatment while the redox ratio estimated from the OVCA433 cells increased in a dose dependant manner.

The BRCA family of genes regulates apoptosis and often has germ-line mutations in familial breast and ovarian cancer. [34, 35] 4-HPR down-regulated BRCA1 in the IOSE cells and decreased BRCA2 gene expression in OVCA433 cells detected by both microarray and real-time RT-PCR (Tables 1b and 3b). Both BRCA1 and BRCA2 function as tumor suppressor genes in the breast and ovary. [34, 35, 61] IOSE cells used in the study are thought to carry a BRCA mutation and originated from a woman with a strong family history of ovarian cancer [34, 61], The ovarian cancer cells OVCA433 had altered expression of BRCA genes, moreover, the expression of BRCA2 transcript in-frame exon 12 deletion (BRCA2Δ12) mRNA was also reported in these cells by Ho, et al. [61] 4-HPR could directly affect mutant BRCA gene to suppress the ovarian carcinogenesis, which is a very important finding in this study and has significant implications

for BRCA-related cancer prevention and merits further study.

Our study suggests that 4-HPR may be active in ovarian cancer cells through growth inhibition and apoptosis induction which is mediated by multiple mechanisms. We use both ovarian cancer cells and immortalized normal ovarian epithelium cells in the study. The IOSE cells represent a cell that has been immortalized as a premalignant cell. Although a true premalignant model in the ovary is still unclear, immortalized cells have been used from lung, cervical, and other cell lines to mimic premalignant cells. [8, 50] In immortalized normal ovarian cells, 4-HPR regulated RXR receptor pathways which increased RXRα and RXRβ expression and decreased RXRγ expression as well as down-regulating the anti-apoptotic genes ras and cyclin-dependent kinase and up-regulating the pro-apoptotic pathways p53 and BCL, although Western blot did not confirm an increase in p53 protein. However, in OVCA433 cells, 4-HPR mediated mitochondrial permeability and induced the pro-apoptotic p53 and downstream pathway. Early changes that have not undergone p53 mutations, for example, may have a response to 4-HPR with induction of the p53 pathway, while cells that have undergone p53 mutations may not be as responsive to drugs such as 4-HPR. Using biomarkers, such as RXR receptors and p53 up-regulation and downstream protein production as well as fluorescence spectroscopy, to evaluate patient response may be helpful to monitoring 4-HPR or other chemopreventive agents' activity as a preventive agent for ovarian cancer. Hence, this drug merits further study in the ovary, both as a preventive agent and as an agent which might aid in preventing future recurrences.

Natural and synthetic retinoids have been used in many different types of cancers for prevention and treatment. However, the mechanism is still not well studied and the suitable concentration of 4-HPR and/or retinoid has not been determined. [62-64] Negative trials were reported for bladder cancer and cervical cancer. [61, 64] A key result from our previous *in vitro*

studies suggested the concentration is important when applying retinoids to different cell types, correlating with different concentrations for various cell populations *in vivo*, i.e. normal and high risk patients as well as cancer patients need to use specifics concentrations. [8, 63, 65-67] Most of the clinical trials for prevention use the dose of 200 mg/day based on a breast cancer chemoprevention trial. [68] Compared with the bladder, cervix, and ovary, the breast is fat tissue that stores retinoids; consequently, the local concentration of 4-HPR in the breast is conceivably higher than that in other organs and this local concentration varies from organ to organ. Therefore, it is imperative that the concentration of 4-HPR in different type of cancer needs to be studied carefully before initiating a clinical trial.

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Figure Legends

Fig 1. *Effect of 4-HPR on growth and apoptosis in OVCA433 and IOSE cells.* Cells were grown in the absence (control) or presence of 4-HPR in concentrations of 1, 5, 10 μM. Growth inhibition assay were performed with crystal violet on day 5. The percentage of growth inhibition was calculated, as described in Materials and Methods. The data was presented as the mean ± SE of triplicate determinations.

Fig 2. *Effect of 4-HPR on apoptosis induction in OVCA433 and IOSE cells.* Cells were treated with the indicated 4-HPR concentrations for 3 days. The cells were then stained with fluorescein-labeled dUTP to label DNA fragments by the TUNEL method, as described in Materials and Methods. The percentage of apoptosis cell population and DNA contents, including cell cycle, were calculated.

Fig 3. *Effect of 4-HPR on Caspase 3 Activity in OVCA433 and IOSE cells.* Cells were grown in 96-well plate with absence (control) or presence of 4-HPR in concentrations of 1, 5, and 10 μM for 12, 24, 48 and 72 hrs and incubated in Caspase 3 buffer, as described previously in Materials and Methods. The plates were read at 400 nm excitation, 505 nm emission using a fluorescence plate reader immediately after adding Caspase 3 fluorescent substrate conjugate. The Western blot on Caspase 3 and 9 expression shown in the upper right corner were treated with same concentration of 4-HPR for 3 days.

Fig 4. *Effect of 4-HPR on Mitochondrial Permeability Transition in IOSE and OVCA433 cells.* Cells were treated with 4-HPR in concentrations of 1, 5, and 10 μM for 3 days and resuspended in 40 nM MitoFluor™ medium. Cells were visualized under the fluorescence microscope at 490 nm excitation, 516 nm emission. A field of 20-30 cells was chosen using a spectrophotometer to measure light intensity.

Fig 5. *Effect of 4-HPR on p53, p21, and other protein levels in IOSE and OVCA433 cells.* Nuclear proteins were extracted from cells treated with 1 μM 4-HPR for 3 days. Thirty microgram/lane of nuclear proteins were subjected to SDS-PAGE. The p53, p21, p16, and Rb proteins were identified by blotting with monoclonal antibodies. Immunoreactive bands were visualized using the enhanced chemiluminescence method described in Materials and Methods. The blots were stripped and reblotted to mouse anti-β-actin antibody for assessment of loading in each lane.

Fig 6. a. Estimated fluorescence redox ratios are presented from the OVCA433 and IOSE cells with increasing concentrations of 4-HPR. Standard error bars are shown and the value above each bar represents the number of times the treatment group was measured. **b.** Comparisons relative redox fluorescence ratio between the control and treatment measurements for both cell lines. The mean ratios for the treatment groups are normalized to the control group's mean ratio.

Table 1. *Microarray analysis of all genes induced by retinoid (greater than 2-fold changes).* Ovarian cells were grown in 10% FBS and DMEM/F12 medium treated with 1 μM of 4-HPR. Total RNA was purified and microarray was analyzed. The genes up- and down- regulated by 4-HPR were reported.

Table 2. *Microarray analysis of all genes related to NADH and NAD modulated by 4-HPR.*

Table 3. *Expression of RXR receptors and BRCA in ovarian cell lines determined by real-time RT-PCR analysis.* IOSE and OVCA433 cells were grown in 10% FBS and DMEM/F12 medium treated with 1 μM of 4-HPR for 3 days. The cells were then harvested and the total RNA was extracted and analyzed by real time RT-PCR. The number represented the fold changes. If fold change > 1.0, the gene is up-regulated relative to the control (equal to 1). If fold change is <1.0, the gene is down-regulated relative to the control.

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In vitro model of normal, immortalized ovarian surface epithelial and ovarian cancer cells for chemoprevention of ovarian cancer

Molly Brewer[a,d,*], J. Taylor Wharton[a], Jian Wang[d], Amanda McWatters[a], Nelly Auersperg[c], David Gershenson[a], Robert Bast[b], Changping Zou[d]

[a]*Department of Gynecologic Oncology, University of Texas, The M. D. Anderson Cancer Center, Houston, TX 77030, USA*
[b]*Experimental Therapeutics, University of Texas, The M. D. Anderson Cancer Center, Houston, TX 77030, USA*
[c]*University of British Columbia, Vancouver, British Columbia, Canada*
[d]*Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, University of Arizona, Tucson, AZ 85724, USA*

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Abstract

Background. Epithelial ovarian cancer has the highest mortality rate among the gynecologic cancers. The synthetic retinoid, N-(4-hydroxyphenyl) retinamide (4-HPR), has been used in the chemoprevention of ovarian cancer. However, the effectiveness of its application for different populations has been questioned because of the genetic differences among normal, high risk, and women with cancer.

Objective. To explore the similarities and the differences in 4-HPR effects on different ovarian epithelial cells which mimic different populations of women, normal ovarian surface epithelium to represent the normal population of women, immortalized ovarian surface epithelium to represent premalignant changes, and cells derived from ovarian cancer cells to represent malignant changes were used as in vitro models.

Methods. Normal ovarian surface epithelial cells, immortalized ovarian surface epithelial cells, and ovarian cancer cells were incubated for different intervals with increasing concentrations of 4-HPR. Growth inhibition, the fraction of apoptotic cells, the expression of apoptosis-related genes, including p53, p16, p21, and caspase-3, and mitochondrial permeability transition were measured before and after 4-HPR treatment.

Results. Treatment with 4-HPR produced growth inhibition and apoptosis in a dose-dependent manner for all 3 cell types. 4-HPR produced the strongest activation of the p53 pathway in normal ovarian epithelial (NOE) cells, while it caused the largest increase in MPT in the cancer cells, suggesting a different mechanism for growth inhibition and/or apoptosis in these cell lines. 4-HPR, at a concentration of 10 μM, had a maximal effect on caspase-3 activity at 72 h in normal cells and at 48 h in immortalized and cancer cells, although the effects were modest.

Conclusions. Normal ovarian surface epithelial cells, immortalized ovarian surface epithelial cells, and ovarian cancer cells showed a differential response to 4-HPR. Although the same endpoints of growth inhibition and apoptosis induction were present in response to 4-HPR, these endpoints may be regulated through different pathways.

Implications. Clinical trials with higher concentrations of 4-HPR should prove beneficial.

Keywords: Retinoids; Growth inhibition; Apoptosis; Cancer prevention

Introduction

Epithelial ovarian cancer has the highest mortality rate among the gynecologic cancers. Only 30–40% of patients survive 5 years despite aggressive treatment, in part, due to the advanced stage at diagnosis, with 70% of patients

* Corresponding author. Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Arizona Cancer Center, 1515 N. Campbell Avenue, Room 1968, Tucson, AZ 85724-5024, USA. Fax: +1 520 626 9287.
E-mail address: mbrewer@azcc.arizona.edu (M. Brewer).

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having widespread metastases [1]. Given our inability to consistently cure ovarian cancer, strategies for prevention merit at least as much attention as does treatment of the disease. Cancer chemoprevention is the administration of chemical agents to prevent or delay the development of cancer in healthy people. Biomarkers that are likely to be affected by the preventive agent and whose modulation supports the postulated chemopreventive activity [2,3] are important components of prevention studies.

N-(4-hydroxyphenyl) retinamide (4-HPR), a synthetic analog of Vitamin A, has been shown to have anti-neoplastic activity in both experimental models and clinical trials [4–12], particularly in the skin [5]. It has also been found to coincidentally decrease the risk of ovarian cancer in a breast cancer chemoprevention trial [13–15]. In both the skin [5] and the ovary [15], the response lasted for the duration of drug ingestion, suggesting that the use of retinoids may need to be longer than previously thought. This study, as well as others [16–20], shows activity by 4-HPR against tumor cell lines with increasing rates of apoptosis, suggesting that this drug has the potential for preventing and treating ovarian cancer. This is the first study to explore the effect of 4-HPR on normal ovarian epithelial cells and cells immortalized with viral T-antigen which are compared to ovarian cancer cell lines. Normal cells were used to mimic normal woman who carried no increased risk for ovarian cancer, and immortalized cells to mimic high-risk women who may harbor cells that have undergone various mutations. The in vitro model was chosen to evaluate the effect of 4-HPR on different cell types and to study the dose response in these cells because the efficacy of 4-HPR in clinical prevention trials has been contradictory for the high-risk population [3–15,21]. Early investigators using 4-HPR for prevention limited the dose to 200 mg due to safety concerns: 200 mg/day of 4-HPR was used in the breast cancer prevention trial for prevention of secondary breast cancers [14]; although there was no effect on the incidence of breast cancer, 4-HPR was found to decrease the risk of ovarian cancer at this concentration [15]. However, Dr. Follen's group found that 200 mg 4-HPR, which corresponds to 2 μM concentration in vitro [21], was not effective in inducing the regression of cervical pre-neoplasia in vivo. Moreover, their in vitro model showed that the higher dose of 10 μM concentration was needed to induce growth inhibition or apoptosis in both dysplastic and cervical cancer cells [22]. These studies have helped establish a relevant biologically active dose for clinical trials for premalignant lesions in the cervix, corresponding to >2 μM tissue concentration [21,22]. This study investigates the molecular and cellular events that occur in conjunction with exposure to different concentrations of 4-HPR.

Our research determines how 4-HPR inhibits growth and promotes apoptosis in normal ovarian surface epithelial cells, in immortalized ovarian surface epithelial cells, and in ovarian cancer cell lines at different concentrations. These results suggest that different doses of 4-HPR may be necessary for different populations of women to prevent and treat ovarian cancer.

Materials and methods

Cell lines, retinoids, and normal ovarian epithelial (NOE) cells

Surface epithelial cells were harvested at the time of oophorectomy for benign gynecological conditions. Primary cultures were established from the surgical specimens of normal ovaries [23], from patients without any increased risk for ovarian cancer based on their personal or family history of cancer, nor did any of these women have a known BRCA mutation. Prior to disruption of the blood supply, and without handling the ovaries, the ovary was gently scraped with a scalpel and the scalpel rinsed with sterile culture medium. Cells were maintained in a 1:1 mixture of MCDB 105 and medium 199 supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 2 mM L-glutamine, and 100 μg/ml streptomycin. Six different NOE primary cell lines were used in this study.

Immortalized ovarian surface epithelial (IOSE) cells were obtained from Nelly Auersperg (University of British Columbia, Vancouver, British Columbia). IOSE and ovarian cancer cells, OVCA420, SKOV3, and OCC-1 cells were grown in a 1:1 (volume/volume) mixture of Dulbecco's modified Eagle's medium (DMEM), and Ham's F12, with 10% fetal bovine serum, at 37°C in a humidified atmosphere of 95% air and 5% CO_2. N-(4-hydroxyphenyl) retinamide (4-HPR) purchased from Sigma Chemical Co. (St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at stock solutions of 0.1 mM and stored in an atmosphere of N_2 at −80°C.

Effects of 4-HPR on cell proliferation in monolayer cultures

Cells were plated in 96-well plates at a concentration of 10^5 cells per well and grown for 24 h. The NOE, IOSE, and OVCA420 cells were incubated with 1, 2, and 5 μM concentrations of 4-HPR and the ovarian cancer cells (OVCA420, SkOV3, and OCC-1) were incubated for 5 days with 2, 5, and 10 μM concentrations of 4-HPR. Control cultures contained DMSO, and the medium was replaced every 2 days. Growth inhibition was determined using the crystal violet method as previously reported [17]. After a 5-day treatment, cells were fixed with 5% glutaraldehyde in phosphate-buffered saline, rinsed with distilled water, and air dried. Cells were incubated with 1:1 (volume/volume) 200 mM (3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer (pH 9.5), and 0.2% CV at 25°C for 30 min, then washed and air dried. The intracellular dye was solubilized with 10% glacial acetic acid and the absorbance at 590 nm was determined using a

microtiter plate reader. Growth inhibition was calculated according to the equation: inhibition = $(1 - Nt/Nc) \times 100$, where Nt and Nc are the number of cells in treated and control cultures, respectively. All experiments were performed in triplicate and the means ± standard deviations calculated.

Analysis of apoptosis induced by 4-HPR

Terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-deoxyuridine-triphosphate (dUTP) nick-end labeling (TUNEL) assay was used [17]. Following incubation for 24, 48, and 72 h with different concentrations of 4-HPR, cells were fixed in 1% formaldehyde in PBS (pH 7.4) for 15 min at 4°C. The cells were washed twice with PBS. Cells were then resuspended in 70% ice-cold ethanol and stored in a −2°C freezer. For the assay, cells were first suspended in 1 ml wash buffer containing cacodylic acid, Tris–HCl-buffered solution and sodium azide (Phoenix flow cytometry kit, Phoenix Flow Systems, San Diego, CA). Approximately 10^6 cells were resuspended in 50 μl staining buffer containing Tris–HCl, TdT, and fluorescein-12-dUTP (Phoenix flow cytometry kit). Cells were incubated at 37°C for 60 min, rinsed twice with PBS, stained with 500 μl of propidium iodide/RNase A solution in the dark for 30 min at room temperature, then analyzed by flow cytometry using a FACScan flow cytometer, the Epics Profile (Coulter Corp, Hialeah, FL) with a 15-mW argon laser for excitation at 488 nm. Fluorescence was measured at 480 nm. The Phoenix flow cytometry kit included suspensions of cells that served as negative and positive controls for apoptosis. Computer analysis of the data provided information on the percentage of apoptotic cells, as well as the proportion of cells in the hypodiploid, G_1, S, and G_2 phases of the cell cycle.

Caspase-3 activity assay

The cells were plated in 96-well tissue culture plates at densities ranging from 0.5 to 1 × 10^5 cells per well and treated with 2, 5, or 10 μM 4-HPR for 12, 24, 48, or 72 h. Control cultures received the same amount of DMSO. Following culture, the 96-well plate was spun at 500 × g for 5 min, and the supernatant removed. The extraction buffer (EB) and assay buffer (AB) caspase-3 activity assay fluorometric kit (Oncogene Research Products) was prepared by adding 10 μl of 1 M DTT/ml EB and 10 μl of 1 M DTT/ml AB. Positive and negative controls were provided with the fluorometric kit, and 50 μl EB/well were added to each assay and mixed gently. The plate was incubated for 30 min at 4°C; 50 μl AB/well was added to each assay well, and the plate was incubated for 30 min at 37°C. The plate was read immediately after adding 10 μl/well of caspase-3 fluorescent substrate using a fluorescent plate reader at 400 nm excitation, 505 nm emission. Maximal caspase-3 activity was determined at different interval, and the remainder of the experiment was carried out when maximal activity was observed.

Mitochondrial permeability transition (MPT)

NOE, IOSE and OVCA420 cells were treated with 2, 5 and 10 μM 4-HPR for 12, 24, 48, and 72 h to determine the time of maximal mitochondrial permeability transition. Cells were then washed and resuspended in 40 nM MitoFluor® medium, incubated for 30–45 min at 3°C, and visualized under the fluorescent microscope at 490 nm excitation, 516 nm emission. Using a photo-amplifier connected to the epifluorescent microscope, a field of 20–30 cells was chosen to measure light intensity.

Western blot analysis of apoptosis-related genes and other gene expressions modulated by 4-HPR

Nuclear and cytoplasmic extracts were prepared from control and 2, 5, and 10 μM 4-HPR-treated NOE, IOSE and OVCA420 cells. Nuclear or cytoplasmic protein (80 μg/lane) were electrophoresed on 8% polyacrylamide gels in the presence of 0.1% SD, and transferred to a nitrocellulose membrane. The membrane was incubated with mouse IgG monoclonal antibodies against p53 (which recognizes both wild type and mutant), p21 and p16 (Santa Cruz Biotech, Santa Cruz, CA), then washed and incubated with a peroxidase-conjugated anti-mouse antibody (Amersham Biosciences, Piscataway, NJ). Immunoreactive bands were developed using an enhanced chemiluminescence reagent (Amersham Biosciences). The blots were stripped and then re-incubated with mouse anti-β-actin antibody (Sigma Chemical Co.) to normalize for loading differences. The quantization of the expression level relative to that of β-actin was calculated by using arbitrary units obtained from the densitometry.

Results

Growth inhibitory effects of 4-HPR in NOE, IOSE, and ovarian cancer cells

NOE, IOSE, and the three ovarian cancer cell lines were grown in monolayer cultures and treated with different concentrations of 4-HPR (1–10 μM) for 3 days. SkOV3, OVCA420, and OCC-1 were studied to determine which ovarian cancer cell line would serve as the best model for comparison between the normal, premalignant and malignant models. OVCA420 was chosen because it had the most sensitivity to 4-HPR and, thus, could be evaluated most comparably to the NOE and IOSE cell lines. We first compared low concentrations from 1 to 5 μM of 4-HPR in NOE, IOSE, and OVCA420 (Fig. 1a). Concentrations from 2 to 10 μM 4-HPR were used on the ovarian cancer cells (Fig. 1b); 10 μM 4-HPR had greater growth inhibitory

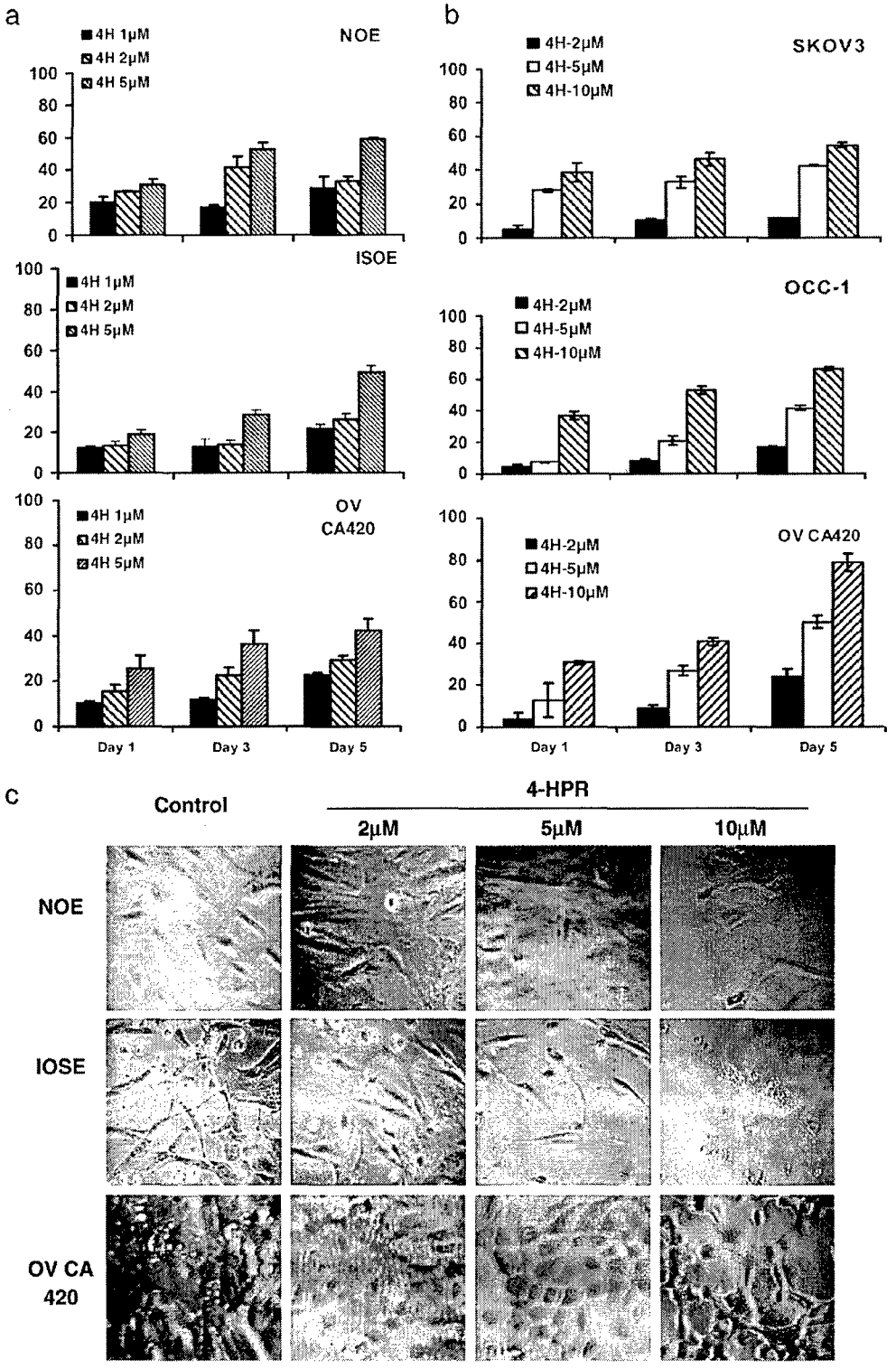

Fig. 1. Effect of 4-HPR on the growth inhibition of NOE, IOSE, and ovarian cancer cells. Cells were grown in the absence (control) or presence of 1, 2, 5, or 10 μM 4-HPR for 5 days, and stained with crystal violet on day 5 for analysis of growth inhibition. The percentage of growth inhibition was calculated as described in Materials and methods. (a) NOE* (average from 6 different NOE cells), IOSE, and OVCA420 cells were treated with 1, 2, or 5 μM 4-HPR. (b) Ovarian cancer cells, SkOV3, OCC-1, and OVCA420 cells, were treated with 2, 5, or 10 μM 4-HPR. (c) Morphologic change of 2, 5, or 10 μM 4-HPR in normal and ovarian cancer cells.

effects than the lower doses on the cancer cell lines. OVCA420 cells were the most sensitive cell line compared with other two cell lines (Fig. 1b). The graphical representations for the percentage of growth inhibition are shown in Figs. 1a and b. The morphologic changes of 1–10 μM 4-HPR in normal and ovarian cancer cells are shown in Fig. 1c. The NOE and IOSE cells were very sensitive to 10 μM 4-HPR; almost all cells were killed by day 3. Morphological changes consistent with differentiation (cells become more elongated) are observed along with cell death (Fig. 1c).

Apoptosis induction by 4-HPR

To assess other possible mechanisms of 4-HPR in ovarian cells, we analyzed the effects of 4-HPR on the induction of apoptosis in primary culture and cell lines by TdT labeling and flow cytometry. NOE and IOSE cells were treated with 5 μM 4-HPR. DNA content and apoptosis induction were analyzed (Fig. 2). The results showed that the apoptotic cell population did not change at this concentration, but a population of cells with hypodiploid (HD) DNA content increased 2- to 8-fold with increasing time of incubation with 5 μM 4-HPR in these cells (Fig. 2). The increase in hypodiploid cell population was shown as a population shift to the left (Fig. 2).

NOE, IOSE, and OVCA420 cells were treated with 2, 5, and 10 μM 4-HPR. Apoptosis induction was analyzed at different concentrations and different time points (Figs. 3a and b). Results demonstrated that 4-HPR-induced apoptosis was dose dependent in the NOE and OVCA420 cells (Fig. 3a); apoptosis was less marked in the IOSE cells than in the NOE and OVCA420 cells (Fig. 3a). 4-HPR-induced apoptosis was also time dependent in ovarian cells. A time course of apoptosis induction was carried out in the NOE, IOSE, and OVCA420 cells when treated with 4-HPR at 10 μM (Fig. 3b).

Effect of 4-HPR on caspase-3 activity

The 4-HPR effect on caspase-3 activation at different concentrations and different lengths of incubation was measured in NOE, IOSE, and OVCA420 cells (Fig. 4). Cells were treated with 2, 5, and 10 μM 4-HPR, and caspase-3 activity was measured at 12, 24, 48, and 72 h after treatment. The patterns of caspase-3 activity were different in the all three cell types, and 2 μM 4-HPR had little effect

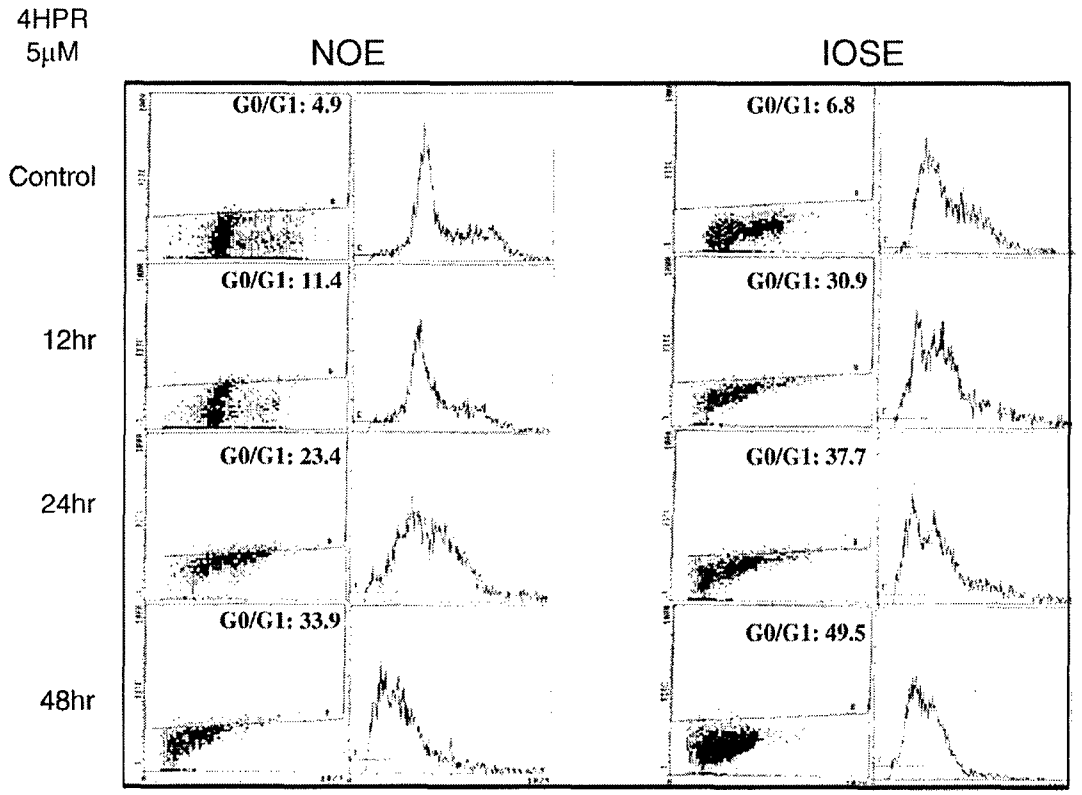

Fig. 2. Effect of 5 μM 4-HPR in NOE and IOSE cells. Cells were treated with 5 μM 4-HPR for 12, 24, and 48 h. The cells were harvested and then stained with propidium iodide for DNA content analysis and with fluorescein-labeled dUTP to label DNA fragments by the TUNEL method, as described in Materials and methods. The fluorescence of cells labeled by the TUNEL method is presented in the left columns, which fluorescence of viable and apoptotic cells represented by the dark dots below and above a demarcation line determined by a standard cell line provided with the labeling kit. The data on DNA content distribution are presented in the left column. The percentage of cell populations in the G0/G1 phase were labeled in the right corner at each time interval.

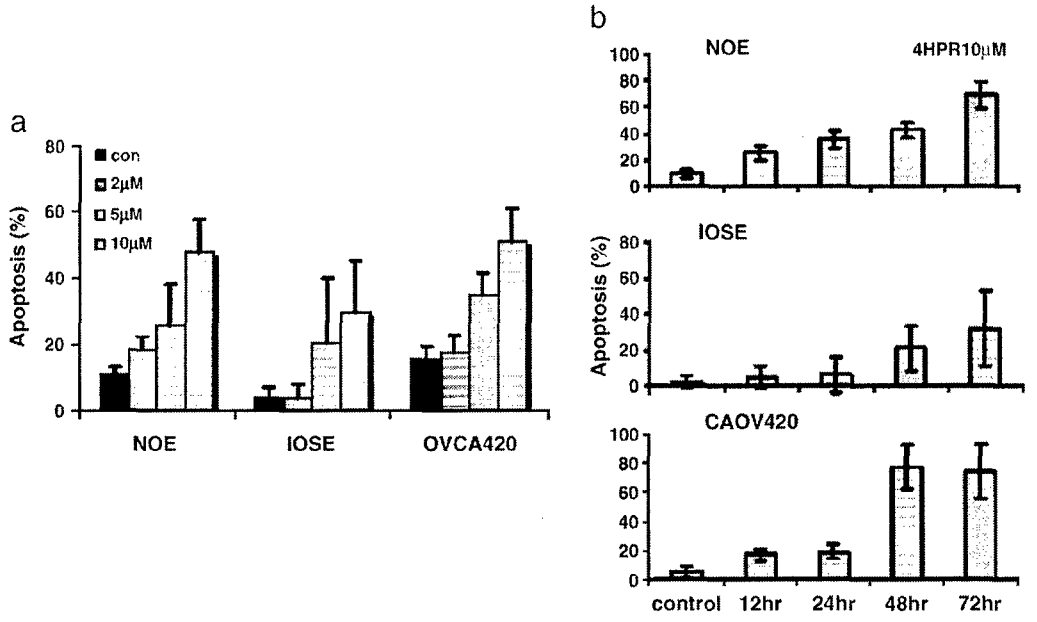

Fig. 3. Effect of 4-HPR on apoptosis induction in NOE, IOSE, and OVCA420 cells. (a) Dose response of 4-HPR in apoptosis induction. Effect of different concentration of 4-HPR on ovarian cells. Cells were treated with 2, 5, and 10 μM 4-HPR for 3 days. The apoptosis were determined by the TUNEL assay. The percentage of apoptotic cells is indicated in the right corner of each treatment analysis. (b) Time-course of 10 μM 4-HPR on apoptosis induction. Cells were treated with 10 μM 4-HPR for 12, 24, 48, and 72 h. The percentage of apoptotic cells was determined by the TUNEL assay. Data are presented as the mean ± SE of triplicate assays.

on caspase-3 activity in any of the cells. After 24 h of treatment, an increase in caspase-3 activity was detected in IOSE and OVCA420 cells treated with 10 μM 4-HPR. Caspase-3 activity increased when NOE cells were incubated for 3 days with 10 μM 4-HPR (Fig. 4).

Effect of 4-HPR on mitochondrial permeability transition

Mitochondrial permeability transition (MPT) changes are associated with apoptosis. To investigate the mechanism of 4-HPR-induced apoptosis in ovarian cancer cells, experiments were carried out to determine the effect of 4-HPR on MPT in NOE, IOSE and OVCA420 cells. OVCA420 cells had a greater change in MPT than did the NOE and IOSE cells after treatment with 4-HPR (Figs. 5a and b). 4-HPR decreased mitochondrial inner membrane potential, which increased MPT in these cells (Figs. 5a and b). An inverse relationship in mitochondrial potential correlated in a dose-dependent manner with both the increase in apoptosis and growth inhibition by 4-HPR in all three cell types (Figs. 1, 2, 5b).

Modulation of p53 and other gene expressions by 4-HPR in NOE, IOSE, and OVCA420 cells

The effect of 4-HPR on the expression of the apoptosis-associated genes p53, p21, and p16 was examined in NOE, IOSE, and OVCA420 cells; p53 expression was detected in the NOE, IOSE, and OVCA420 cells. 4-HPR increased p53 expression in the NOE cells in a dose-dependent manner, but not in IOSE and OVCA420 cells (Figs. 6a and b). The expression of the p21 gene was increased in NOE and IOSE cells but was not detectable in OVCA420 cells. The expression of p16 was modulated by 4-HPR in all three cell types (Figs. 6a and b).

Discussion

Retinoids have been studied as cancer chemopreventive agents [4–7] based on epidemiologic data, showing that diets high in Vitamin A are associated with lower odds of epithelial cancers [24]. An Italian trial that evaluated 4-HPR for the prevention of secondary breast cancers [14] demonstrated a decreased incidence of ovarian cancer in women receiving 4-HPR, suggesting that retinoids prevented the development of ovarian cancer [13,15]. Experimental studies have demonstrated that retinoids can affect human ovarian cancer cell growth by inhibiting proliferation and inducing apoptosis, which are thought to be important mechanisms in cancer prevention [12].

Programmed cell death is a physiological mechanism by which organisms eliminate cells during embryonic development and counterbalance cell division for homeostatic regulation of tissue mass in the adult. The term apoptosis was coined by Kerr et al. to describe this process, which is characterized by cell shrinkage, chromatin condensation, nuclear segmentation, and internucleosomal degradation of DNA [25]. Apoptosis can be induced by a variety of external and intracellular signals including those that induce

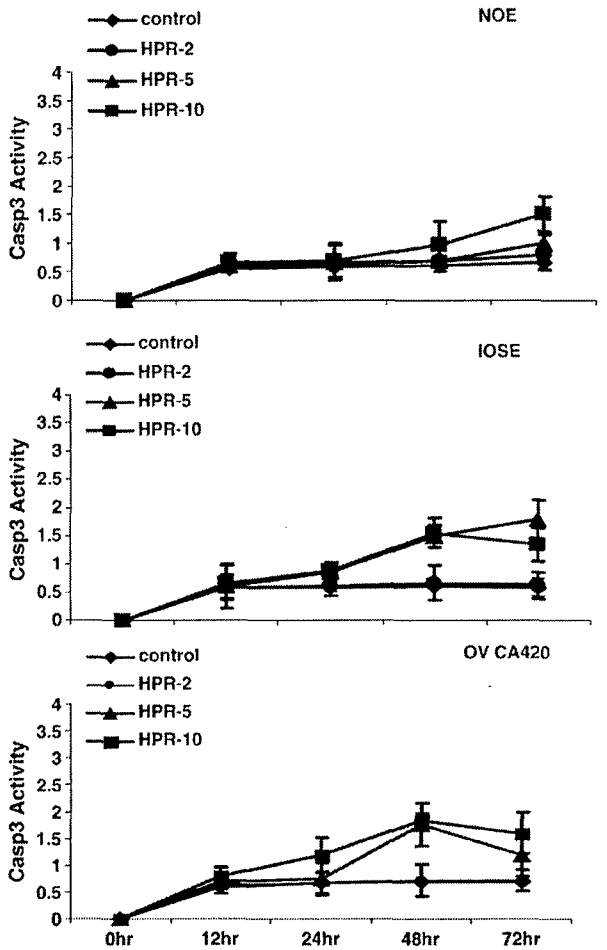

Fig. 4. Effect of 4-HPR on caspase-3 activity in NOE, IOSE, and OVCA420 cells. Cells were grown in 96-well plates in the absence (control) or presence of 4-HPR (2, 5 and 10 μM) for 12, 24, 48 and 72 h, and incubated in caspase-3 activity assay buffer, as described in Materials and methods. The intensity of staining was read using a fluorescent plate reader at 400 nm excitation, 505 nm emission, immediately after adding caspase-3 fluorescent substrate conjugate.

terminal differentiation or DNA-damage. This induction may constitute a protective anti-neoplastic mechanism to eliminate DNA-damaged cells and repair mutations [26]. Carcinogenesis is often associated with a decreased tendency to undergo apoptosis in response to certain physiological stimuli and cytotoxic agents. Therefore, agents that can induce apoptosis or restore the ability to undergo apoptosis in premalignant and malignant cells are expected to be effective in cancer prevention and treatment [26,27]. Several reports have demonstrated that certain retinoids, in addition to exerting cytostatic effects on tumor cells in vitro [28], also induce apoptosis in various cell types during normal development and in cultured normal and tumor cells [29–33].

This study is the first to use normal ovarian surface epithelial cells, immortalized epithelial cells, and cancer cells to investigate the effect of 4-HPR on growth and apoptosis. Different cell types showed different responses to 4-HPR in a dose-dependent manner. The normal cells harvested from normal women's ovaries were used to mimic normal ovarian surface cells. The immortalized cells were used to mimic epithelial cells from high-risk women. Although T-antigen immortalized cells may not bear a resemblance to preneoplastic cells, this is the best model currently available, and immortalized cells have been extensively studied in cervical [22] and lung epithelial cells [17,18] as premalignant models. Since the doses of 4-HPR were contradictory in human trials [13–15,21], we tested different concentrations of 4-HPR, ranging from 1 to 10 μM in these cell models, to determine an optimal concentration depending on cell type. The immortalized ovarian cells, which may correspond to what is present in high risk women, were not sensitive to 4-HPR compared to normal and cancer cells, and less apoptosis was detected by TUNEL assay, suggesting that cells containing mutations may be less sensitive to chemopreventive agents. This phenomenon has also been observed in immortalized lung epithelial cells in which these cells exhibit less sensitivity than lung adenocarcinoma cells in response to 4-HPR [17,18]. 4-HPR was thought to act mainly through induction of differentiation which takes 2–4 days in cell culture; however, Kelloff et al [34] as well as other groups have shown that this drug acts thorough induction of apoptosis and growth inhibition which occurs within 12–24 h, earlier and stronger than differentiation [7–9]. Dr. Follen's group reported that in both in vivo and in vitro, a higher 4-HPR dose may be needed to treat a premalignant lesion [21,22]. There are clinical trials currently being conducted with 4-HPR treatment for ovarian cancer in doses as high as 1800 mg/day without reported toxicity. Thus, the higher dose corresponding to the 5–10 μM in vitro concentration may be achievable without major toxicity and therefore merits intensive in vitro study.

Retinoids, particularly 4-HPR, have been shown to increase aerobic glycolysis by increasing mitochondrial permeability to the co-enzymes nicotinamide adenine dinucleotide (NAD(P)H) and flavin adenine dinucleotide (FAD), as well as activity of the electron transport chain characterized by an increase in reactive oxygen species and cytochrome oxidase [8,9]. There has been increased interest in mitochondrial function in both normal and cancer cells. In particular, the mitochondria are thought to be the site of induction of apoptosis for many of the chemopreventive agents. Retinoids were initially thought to act through nuclear receptors. However, 4-HPR has been shown to be receptor independent in many cells lines [35–39] and induces a change in the mitochondrial permeability of the membrane [29]. The permeability of the inner mitochondrial membrane, also called membrane permeability transition, can be increased by thiol agents and oxidative stress-inducing agents that are thought to be dependent on the opening of a non-selective pore [40–44]. A shift towards a more oxidized condition increases membrane permeability, while the opposite occurs with reducing agents [41].

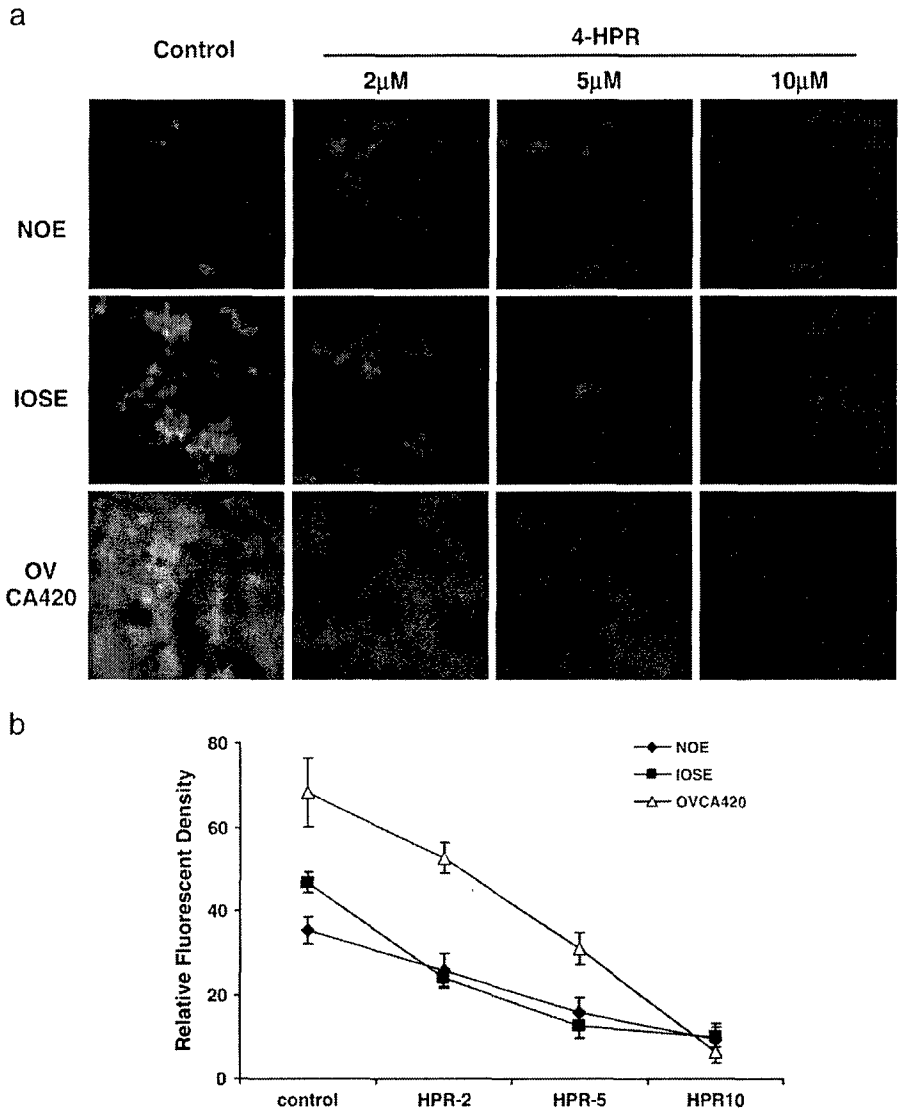

Fig. 5. (a) Effect of 4-HPR on mitochondrial permeability transition (MPT) in NOE, IOSE, and OVCA420 cells. Cells were treated with 2, 5, and 10 μM 4-HPR for 3 days and then resuspended in 40 nM MitoFluor medium. Cells were visualized under the fluorescent microscope. (b) Quantitative changes in relative fluorescent density which corresponds to change in mitochondrial permeability transition.

Mitochondrial dysfunction, in particular the induction of the mitochondrial membrane permeability transition (MPT), has been implicated in the cascade of events involved in the induction of apoptosis. Inhibition of the mitochondrial electron-transport chain reduces the mitochondrial transmembrane potential ($\Delta\Psi_m$), which induces the formation of the mitochondrial permeability transition pore and the subsequent MPT. Disruption or collapse of the $\Delta\Psi_m$ and the induction of the MPT results in the loss of matrix Ca^{2+} and glutathione, increased oxidation of thiols, and further depolarization of the inner mitochondrial membrane, which increase the gating potential for the MPT pore. The MPT is thought to function as a self-amplifying "switch" that, once activated, irreversibly commits the cell to apoptosis. We have clearly shown that treatment with increasing doses of 4-HPR has a major effect on the MPT, inducing formation of this non-selective pore and allowing dissipation of the dye that is sequestered inside the mitochondria inner membranes. These findings, which are the first to be reported in ovarian cells, suggest that these agents act through changes in the membrane potential by uncoupling the electron transport chain and inducing formation of the MPT which allows dissipation of not only the mitochondrial dye but also the small ions and molecules inherent in the functioning of the mitochondria.

Cancers are thought to create a more hypoxic environment which may affect mitochondrial function [45,46]. Growth in the presence of retinoids may alter the mitochondrial function by inducing growth arrest and apoptosis and thus create a more oxidized environment, or alternatively, retinoids may increase the oxidized condition and the mitochondrial permeability which may induce apoptosis.

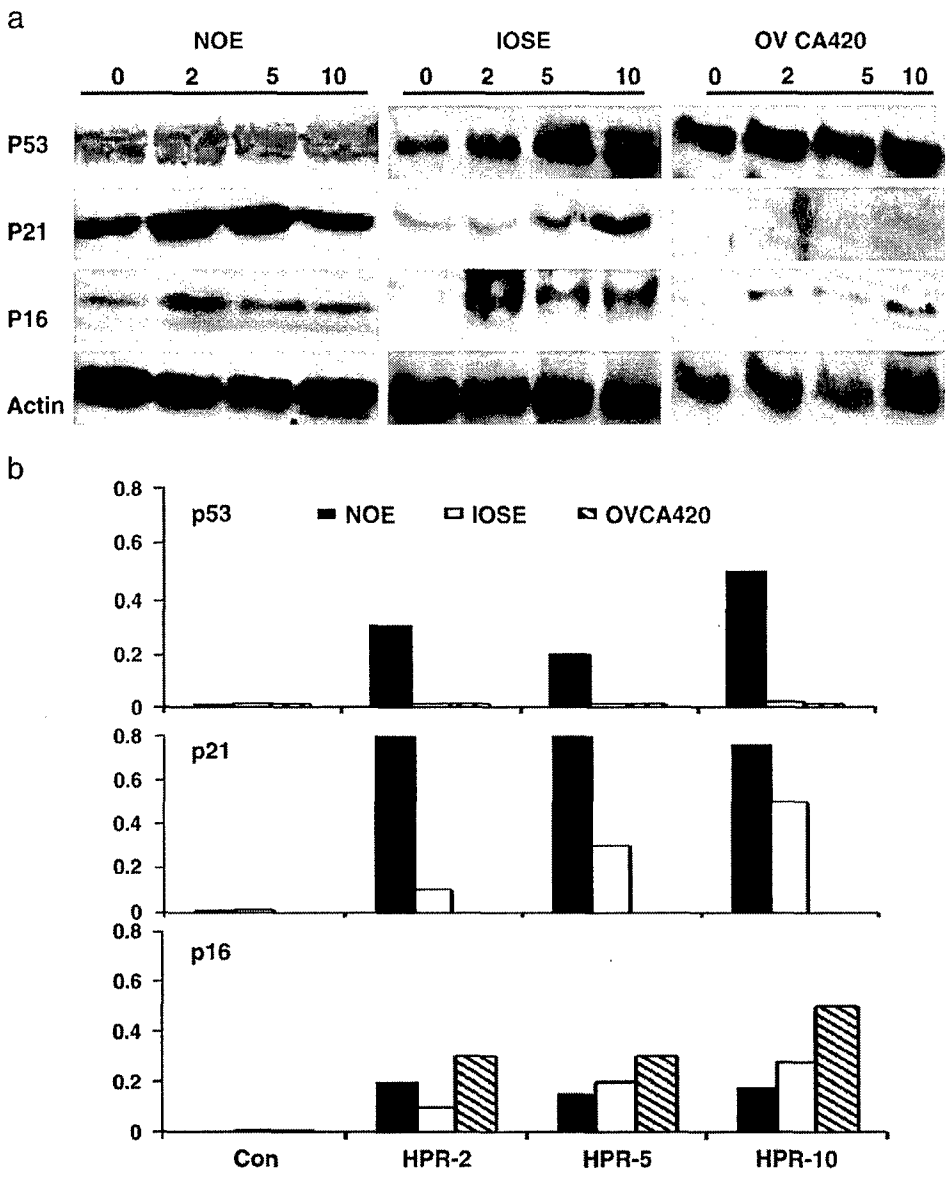

Fig. 6. (a) Effect of 4-HPR on p53, p21, and p16 expression in NOE, IOSE and OVCA420 cells. Nuclear proteins were extracted from cells treated with 5 μM 4-HPR for 3 days. Eighty microgram/lane of nuclear proteins were subjected to SDS-PAGE. The p53, p21, and p16 proteins were identified by blotting with monoclonal antibodies. Immunoreactive bands were visualized using enhanced chemiluminescence, as described in Materials and methods. The blots were stripped and reblotted to mouse anti-β-actin antibody for assessment of loading in each lane. (b) This graph shows the quantitative changes in p53, p21, and p16 in NOE, IOSE, and OVCA420 at 2, 5, and 10 μM 4-HPR.

In our study, all three cell types showed an increase in mitochondrial permeability, most notable in the ovarian cancer cell line, suggesting that 4-HPR induces apoptosis and inhibits growth, in part, by increasing mitochondrial permeability. Our work with fluorescence spectroscopy suggests a change in the oxidative state in cells treated with 4-HPR due to an increased generation of FAD and decreased generation of NADH [47]. The redox potential increased as both growth inhibition and apoptosis increased [47].

P53, a tumor suppressor protein and transcription factor, increases G_1, induces cell growth arrest, and allows cells to repair DNA damage or undergo apoptosis [47–49]. The binding of p53 to DNA induces cell cycle-related genes, the mechanism by which growth inhibition occurs. P53 binding is redox sensitive and is inhibited by oxidizing conditions, which decreases the growth inhibition or apoptotic effect of p53 while reducing conditions enhance the binding of p53 to DNA [48], increasing the response to some retinoids [49]. In addition, p53 has been hypothesized to induce apoptosis by stimulating the production of reactive oxygen species (ROS), causing mitochondrial damage, which is suggested by the change in the mitochondrial permeability that occurred [50,51]. In our study, 4-HPR induced apoptosis

and increased p53 expression in NOE cells. These results suggest that 4-HPR partially stimulates the p53 pathway with downstream markers p21 and p16 being activated, particularly in normal cells.

The NOE cells showed the most activation of the p53 pathway, while the cancer cells showed the most effect on the MPT, suggesting a different mechanism for the end result of growth inhibition and/or apoptosis. Caspase-3 is one of the effector caspases and may be p53 dependent or independent. The effect on caspase-3 activation was modest and occurred maximally at 72 h in the normal and immortalized cells and maximally at 48 h in the ovarian cancer cells with the higher concentration (10 μM 4-HPR), suggesting that the effect of 4-HPR may be through multiple pathways, including p53, and by a direct effect on the mitochondria. Different concentrations of retinoid may act in a different manner, with the lower doses inducing more growth arrest and the higher doses inducing more apoptosis. In addition, using these models of normal, premalignant and cancer cells may reflect some differences in vivo and may help determine which populations might benefit the most from these concentration.

These finding have implications for the approaches for chemopreventive recommendations. Women with normal, low-risk ovaries may be amenable to lower doses of retinoid and the higher-risk women with potentially more mutations (analogous to the immortalized cells) may respond to a higher dose of 4-HPR. One of the limitations of any study is the lack of a true premalignant ovarian surface epithelial cell line. However, it is a model that represents a cell that has undergone a mutation which could conceivably be a precursor to a premalignant lesion. Our results suggest that different concentrations of 4-HPR may be needed in different populations of women for prevention of ovarian cancer.

Acknowledgments

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Minireview

Prevention of Ovarian Cancer: Intraepithelial Neoplasia

Molly A. Brewer,[1] Karen Johnson, Michele Follen, David Gershenson, and Robert Bast Jr.

Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Arizona Cancer Center, University of Arizona, Tucson, Arizona 85724 [M. A. B.]; Division of Cancer Prevention, National Cancer Institute, Bethesda, Maryland 20892 [K. J.]; Departments of Gynecologic Oncology [M. A. B., M. F., D. G.] and Translational Research [R. B.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030; and Department of Obstetrics, Gynecology and Reproductive Science, University of Texas Medical School, Houston, Texas 77030 [M. F.]

Abstract

To reduce the incidence and mortality associated with invasive cancers, the Intraepithelial Neoplasia (IEN) Task Force recommends that carcinogenesis be viewed as a disease that requires treatment. This publication outlines the current knowledge of IEN of the ovary and reviews chemoprevention possibilities for ovarian cancer. Ovarian cancer has the highest mortality of all of the gynecological cancers and is the fourth leading cause of death from cancer in women. The IEN Task Force has defined precancer as a noninvasive lesion that has genetic abnormalities, loss of cellular control functions, and some phenotypic characteristics of invasive cancer with a substantial likelihood of developing invasive cancer. The IEN Task Force recommends targeting moderate to severe dysplasia for new IEN treatment agents in clinical trials. Ovarian cancer does not have a clear preinvasive lesion yet merits considerable study for new prevention strategies because of the high mortality associated with ovarian cancer. There is a great unmet clinical need for treatments that can prevent ovarian cancer by providing nonsurgical options that treat the entire epithelial layer. New prevention strategies hold significant promise to reduce the mortality from ovarian cancer.

Introduction

Carcinogenesis must be viewed as a disease and disequilibrium that requires treatment to dramatically reduce the incidence and mortality associated with invasive cancers. The IEN[2] Task Force has defined precancer as a noninvasive lesion that has genetic abnormalities, loss of cellular control functions, some phenotypic characteristics of invasive cancer, and predicts a substantial likelihood of developing invasive cancer. In demonstrating effectiveness and patient benefit of new IEN treatment agents in clinical trials, the IEN Task Force recommends targeting moderate to severe dysplasia, which is close in stage of progression to invasive cancer and thus substantially elevates the risk of developing cancer.

Epithelial ovarian cancer has the highest mortality rate of any of the gynecological cancers; the 5-year survival is no more than 30% despite aggressive treatment. Seventy percent of these cancers are diagnosed with widespread intra-abdominal disease or distant metastases, which partially accounts for the poor prognosis associated with ovarian cancer. Although up to 90% of stage IA tumors and 70% of stage II tumors can be cured by current management, the cure rate drops below 30% for Stage III and IV tumors. Even ovarian cancer limited to the pelvis has a 5-year survival of only 50% (1). This dismal overall prognosis for women with ovarian cancer results from an inability to detect ovarian cancers at an early, curable stage, from the lack of effective therapy for advanced disease, and from our incomplete understanding of both the early changes in the ovary that predate the development of cancer and the initiators of these changes. Although radical surgery, radiation therapy where appropriate, and new methods of chemotherapy have improved survival times, cure rates have stayed essentially the same over the last 20 years. Thus, early intervention with chemopreventive agents merits serious consideration as a desirable alternative to suboptimal treatment of invasive disease.

Cancer chemoprevention is a rapidly growing area of research because of the possibility to prevent disease and to restore cancer-suppressing cellular functions. Chemopreventives are micronutrients or medications that prevent or delay cancer in at-risk populations. Fundamental elements for chemoprevention studies include (*a*) a suitable cohort of patients with sufficient incidence to establish an acceptable risk:benefit ratio; (*b*) appropriate agents that are safe and whose use is supported by both epidemiological and mechanistic data; and (*c*) measurable biomarkers that are likely to be affected by the agent and whose modulation is predictive of the postulated chemopreventive activity (2). Biomarkers are important because they can be used in lieu of following patients prospectively until a cancer occurs, if they indicate a protective response to a chemopreventive agent. Several criteria must be met for biomarkers to be useful: (*a*) they are relevant to the development of neoplasia either phenotypically (proliferation, angiogenesis, or nuclear morphometry) or mechanistically (molecular markers); (*b*) they

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[1] To whom requests for reprints should be addressed, at Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, The University of Arizona, Arizona Cancer Center, 1515 North Campbell Avenue, Salmon Building, Room 1968, Tucson, AZ 85724-5024. Phone: (520) 626-9283; Fax: (520) 626-9287; E-mail: mbrewer@azcc.arizona.edu.

[2] The abbreviations used are: IEN, intraepithelial neoplasia; OSE, ovarian surface epithelial; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; 4-HPR, (4-hydroxyphenyl) retinamide; TKI, tyrosine kinase inhibitor; COX, cyclooxygenase; PI3K, phosphatidylinositol 3′-kinase; OCP, oral contraceptive; RA, retinoic acid; RAR, RA receptor; KGF, keratinocyte growth factor; NOE, normal ovarian epithelial; HGF, hepatocyte growth factor.

are modulated by chemopreventive agent; and (*c*) they should predict a decrease in carcinogenesis (3).

Epidemiology

High Epidemiological Risk. The etiology of ovarian cancer remains unknown; low prevalence rates, low participation rates, small sample sizes, and potential bias in the selection of control groups have limited the interpretation of results from epidemiological studies. However, multiple epidemiological studies agree that an increased risk of epithelial ovarian cancer has been linked to advancing age, family history of breast or ovarian cancer, and frequency of ovulation (4–15). Reproductive factors have been extensively studied, but interpreting these results has been complicated by the intercorrelation of reproductive characteristics (4–15). Despite these limitations, several factors related to ovulation have been consistently associated with increased or decreased risk of developing ovarian cancer. Risk is increased with uninterrupted ovulation (nulliparity), larger number of lifetime ovulatory cycles (early age at menarche and late age at menopause), and possibly hyperovulation (fertility drugs), whereas risk is reduced by factors that suppress ovulation [pregnancy, breast feeding, and OCP use (4–15)].

High-Risk Population

High Genetic Risk. Premenopausal women with a family history of breast and/or ovarian cancer constitute an important high-risk group and are excellent candidates for prevention strategies. Although only 10% of ovarian cancers are attributable to germ-line mutations, this high-risk population is an ideal patient population to target for preliminary chemoprevention studies because of the higher prevalence of ovarian cancer as compared with that seen in the general population. Women from a cohort of high-risk families carrying the BRCA1 mutation were observed to have an approximately 40–60% risk of developing ovarian cancer and an 85% chance of developing breast cancer (15). Multiple methods of calculating risk are available. The Parmigiani method (16) uses Bayes theorem and calculates likelihood ratios. The probability of a mutation in the general population is 0.04%–0.20%, with part of the variation attributable to the ethnic mix of the population. A family history of breast and/or ovarian cancer determines the risk calculation in a particular patient. High-risk family histories with an elevated risk of developing ovarian cancer include >2 breast cancers and 1 or more cases of ovarian cancer at any age, >3 cases of breast cancer before age 50 years, sister pairs with cancers less than age 50 years, cases of breast cancer occurring at or before age 40 years, Ashkenazi Jewish descent (which carries a 2% or greater risk of mutation; Ref. 17), or 1 or more cases of breast/ovarian cancer.

The ideal design for a chemoprevention trial includes a high-risk population with an identifiable and easily accessible preinvasive lesion (*e.g.,* IEN), a safe and effective chemopreventive agent, and surrogate end point biomarkers that have been validated as markers of regression of such lesions. For ovarian cancer chemoprevention trials, the targeted population should include high-risk women with a strong family history of breast/ovarian cancer, with or without a BRCA mutation, or with Ashkenazi Jewish descent. Although there is as yet no identifiable preinvasive lesion of ovarian cancer (18), there is strong evidence for one based on the increased numbers of inclusion cysts and areas of proliferation noted in the ovaries of high-risk women seen in some studies (19); thus, this has the potential for use as a biomarker.

Pathology

IEN in the Ovary. We hypothesize that there is an IEN precursor to ovarian cancer; however, the natural history of ovarian cancer and the location of the ovary have made it difficult to characterize precursor lesions. The ovary is not routinely biopsied because of the inaccessibility of the ovary in its i.p. location and concern about the effect on fertility that might result from biopsy. Scully (20) has qualitatively described early histological changes in the ovary, whereas Deligdesch *et al.* (21, 22) have described them quantitatively with the use of nuclear texture analysis. These studies support the concept that ovarian cancer behaves in a similar manner to other epithelial cancers with an identifiable precursor. Other authors (23, 24) have described a pathological process in the ovary consistent with the IEN seen in other organ systems that occurred adjacent to existing cancers. Although progression from IEN to cancer has not yet been validated, there is accumulating evidence including a chain of underlying molecular events that supports the ovarian IEN concept.

The cell of origin of epithelial ovarian cancer remains controversial, although most investigators think it is the OSE cell. Many ovarian cancers are thought to arise from OSE cell-lined inclusion cysts (see Fig. 1); these small, subsurface cysts are hypothesized to arise from involution of ovarian surface epithelium at ovulation (20), but some inclusion cysts are thought to antecede ovulation because they are often present in fetal and juvenile ovaries (25). Small collections of malignant cells contiguous with normal ovarian epithelium suggestive of an IEN but not involving underlying tissues can be found in: (*a*) ovaries removed from women who eventually develop primary peritoneal carcinomatosis (19); (*b*) high-risk women who undergo prophylactic oophorectomy (19), particularly those with the BRCA1 mutations (24); and (*c*) in areas adjacent to stage I cancers that show a transition from malignant to normal epithelium (23, 24). However, a characteristic histological precursor lesion for ovarian cancer is not apparent in all prophylactic oophorectomy specimens (26–29). Whether these contradictory findings are due to differences in patient populations or differences in pathological techniques is not clear. However, these findings underscore the discrepancies present in our understanding of IEN of the ovary and suggest that the ovarian surface epithelium is probably the precursor for most epithelial ovarian cancers. OSE cells differ from peritoneal mesothelial cells because they overlie the ovarian stroma and are in close contact with the hormones secreted by the ovary. The chronic repair after ovulation and/or the influence of ovarian hormones are thought to increase the propensity of the OSE cells to undergo tumorigenesis and may account for the higher incidence of ovarian cancer compared with primary peritoneal cancer.

The molecular mechanisms leading to the initiation and progression of ovarian cancer remain elusive, partly because of the ovary's location and the consequent difficulty in identifying

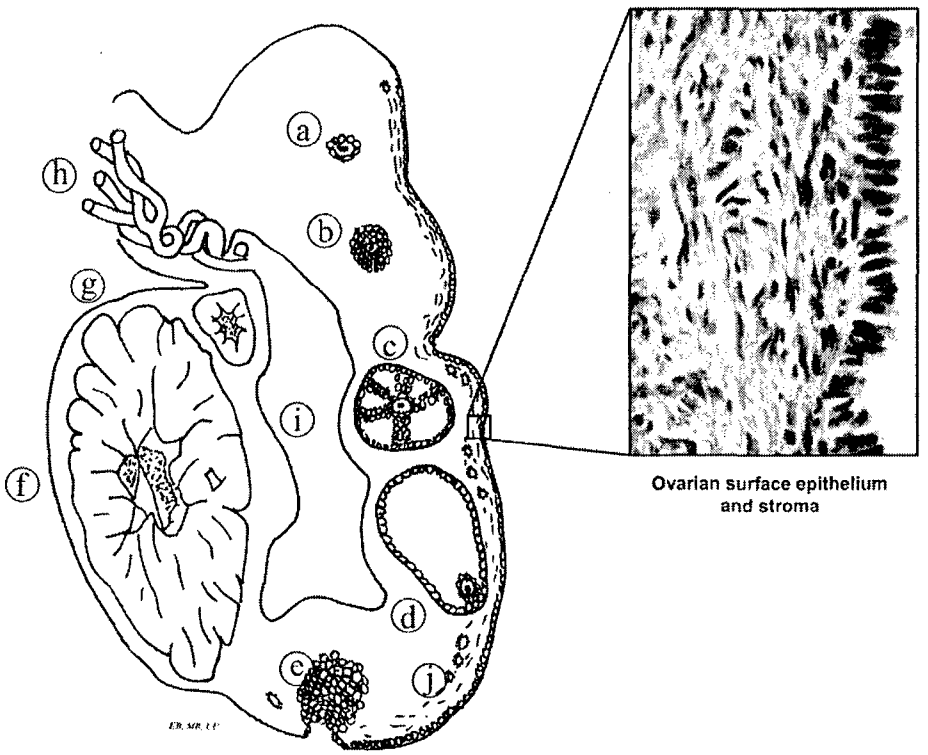

Fig. 1 Normal ovarian structures. *a*, primary follicle; *b*, secondary follicle; *c*, tertiary follicle; *d*, ovulatory follicle; *e*, hemorrhagic cyst; *f*, corpus luteum; *g*, corpus albicans; *h*, ovarian artery and vein; *i*, medulla; *j*, inclusion cyst.

early or precursor lesions. In contrast, analysis of colon, cervix, and head and neck cancers have resulted in a rapidly emerging understanding of the genetic events underlying the initiation and progression of these diseases and of the biological events that result from these genetic changes.

As with all cancers, ovarian cancer is a consequence of either germ-line or acquired somatic changes in genetic function. The acquired changes in gene expression or function can result from mutations or epigenetic alterations such as changes in methylation. Therefore, one important challenge is to link the genotypic changes that occur in ovarian cancer cells to the phenotypic and biological changes observed in human tumors and cell lines.

Although the association between ovulation and ovarian cancer is well accepted, little is known about the underlying biological mechanisms of this association. Ovulation is thought to be important for the development of inclusion cysts from which ovarian cancers may arise. In addition, ovulation, and particularly the high estrogen associated with ovulation, may provide a stimulus for proliferation of ovarian surface epithelium. Ovaries removed prophylactically from women with a strong family history of ovarian cancer demonstrate increased frequency of occult carcinomas, epithelial hyperplasia and atypia, and increased stromal activity (30). Epithelial hyperplasia and increased number of crypts (which are deep indentations of the ovary covered with surface epithelial cells) with an associated increase in proliferation may contribute to tumorigenesis by increasing the risk that a genetic alteration will occur.

OSE cells are generally quiescent but proliferate after ovulation to repair the defect created by the release of an oocyte from a mature follicle. Increased proliferation may contribute to the accumulation of genetic defects in the OSE cells. Furthermore, growth factors produced during wound healing may promote the survival of OSE cells with accumulated mutations. Alternatively, ovulation may be important for the development of inclusion cysts from which ovarian cancers may arise. Entrapment of ovarian epithelium in the stroma of the ovary may disrupt the normal relationship between the ovarian surface epithelium and the underlying stroma. Disruption of normal epithelial stromal interactions can increase mutation rates, directly contributing to ovarian cancer development. Furthermore, growth factors normally produced by the ovarian epithelium that would diffuse into the large potential space of the peritoneal cavity may be present at higher levels in the microenvironment of entrapped ovarian epithelium. Finally, ovarian epithelium in the stroma may be exposed to higher concentrations of paracrine growth factors produced by the stroma or to higher hormone levels than are present on the surface of the ovary. In support of the concept of increased ovulation conferring increased risk are the pathobiological data that show a strong correlation between the lifetime number of ovulations and the frequency of p53 mutations (19, 30). One biological hypothesis is that ovulation may result in genomic instability that occurs as a result of the repeated turnover of cells that renders cells sensitive to the high levels of gonadotropins or gonadotropin-releasing factors present postmenopausally.

Animal Models

A reliable animal model is invaluable in providing optimal flexibility for examining mechanistic, dose-response relationships and comparative efficacy. Ovarian chemoprevention studies of specific molecular targets could be accomplished with greater efficiency if an accepted rodent model of ovarian cancer were available. Under current circumstances, the opportunity to test the many hypotheses being generated by a growing list of potential agents with activity against targets implicated by the new molecular technologies is limited. Research to evaluate several candidate rodent models is being supported by the National Cancer Institute and its Mouse Consortium, and there has been investigation of both syngeneic models (31) by injecting malignant cells or by transfecting epithelial cells with oncogenes (32) to simulate development of a tumorigenic phenotype with variable success. In the meantime, the most widely accepted animal model for ovarian cancer prevention is the domestic white Leghorn chicken (33). Although investigators at The University of Texas M. D. Anderson Cancer Center (Houston, TX) are performing primate studies because of their close similarity to humans, the absence of tumor formation is a drawback, and little is known about primate ovarian carcinogenesis. The chicken, however, has an extremely high rate of Müllerian cancer, of which 30–50% are oviductal in origin. The spontaneous rate of ovarian cancers was approximately 19% in hens ranging from age 2–7 years (33). The potential utility of immunohistochemical markers in the chicken has been investigated to further develop the chicken (*Gallus domesticus*) as a model for spontaneous ovarian carcinoma. Antibodies used to characterize human tumors that were cross-reactive in chicken carcinomas included cytokeratin AE1/AE3, pan-cytokeratin, EGFR, HER-2, Lewis Y, carcinoembryonic antigen, TAG 72, proliferating cell nuclear antigen, p27, and TGF-α (34). Antibodies that were not cross-reactive included CA125, Ki67, Muc-1, and Muc-2. Oviductal cancers were not differentiated from ovarian cancers, which could limit the applicability of these markers. However, the white leghorn chicken model has considerable merit and is being investigated as a model for chemoprevention with the University of Illinois collaborating with Duke University.

Advantages of the chicken model include a high incidence of epithelial cancers. Disadvantages include a high incidence of oviductal cancers and lower correlation with humans than in primates. A study of progestin and 4-HPR in the chicken model is currently in progress. It will be interesting to see if marker modulation in this system corresponds to observations in humans and in cynomolgus macaques.

TKIs. Although the role of the EGFR family in ovarian carcinogenesis is not fully documented, it has been appreciated for some time that growth regulation and differentiation, in response to these receptors in normal ovarian surface epithelium, follow a complex system of interactions that are tissue specific. EGFR, along with the other three members of this receptor family (HER-2, HER-3, and HER-4), is detected immunohistochemically in normal OSE cells at low or moderate intensity (35, 36). Under normal conditions, epidermal growth factor, TGF-α, and amphiregulin provide growth-stimulatory signals to EGFR, and growth inhibition is mediated by TGF-β autocrine feedback (36).

Expression of the various EGFRs in ovarian cancer has been studied extensively by immunohistochemistry. Abnormal expression implying abnormal signaling through EGFR pathways is a common finding in ovarian malignancy. In contrast to the levels observed in normal ovarian surface epithelium, EGFR may be overexpressed in 50–70% of ovarian cancers (37–40). HER-2 is intensely expressed in approximately 10–20% of ovarian cancers and moderately expressed in another 20–40% more cases (41). Because of heterodimer formation among EGFR family members, multiplex expression of certain receptors may be of particular biological relevance. For instance, coexpression of EGFR and HER-2 has been reported in 30–50% of some case series (38, 39). Although intense expression of EGFR and HER-2 is not seen in normal cells and appears to confer a negative prognosis in malignancy, conflicting results regarding prognostic implications are found in the literature. This confusion should resolve as the understanding of EGFR dysregulation in ovarian cancer is updated. For instance, EGFR overexpression is reported to be associated with serous histology (40), and amphiregulin expression is reported to be associated with mucinous histology (42). These observations suggest that histological heterogeneity of ovarian cancer may contribute to the complexity of interpreting the results of growth factor pathway analysis.

A model of the expression patterns underlying EGFR dysregulation has been developed on the basis of EGFR-mediated signals in human ovarian cancer cell lines (43). In brief, the major observation is that coexpression of EGFR and HER-2 facilitates transactivation by epidermal growth factor and produces a strong mitogenic signal. Coexpression of *Her-2* gene product and EGFR was present in 68% of ovarian cancers (44). Two other observations include: (*a*) heregulin activates HER-4, either on its own or with HER-3; and (*b*) HER-3 and HER-4 do not cross-react with EGFR and HER-2 after stimulation with heregulin. Activation of the EGFR and HER-2 heterodimer may be the predominant growth-stimulatory signal in ovarian epithelium, as suggested by the commonly observed overexpression of these two receptors in ovarian cancer (45). If unattenuated, it is plausible that this signal could be a critical driving force in ovarian carcinogenesis. This hypothesis is the basis for testing the potential usefulness of TKIs for preventing the abnormal proliferation of ovarian epithelium that is postulated to precede malignant transformation (see "New Agents").

COX-2 Expression. Abnormal COX-2 expression is found in ovarian cancer. Matsumoto *et al.* (46) have reported a series of 28 ovarian carcinomas, of which 79% were positive for COX-2 expression overall, with 61% strongly positive and 18% weakly positive. In contrast, COX-2 expression was not found in the surface epithelium or inclusion cysts of the uninvolved ovaries in this series (46). In these samples, COX-2 expression was correlated with vascular endothelial growth factor expression. Ovarian cancer cell lines that express COX-2 include SKOV3, CAOV3, and OVCAR3, all of which undergo growth suppression by a COX-2 inhibitor *in vitro* (47).

PI3K. PI3K represents a node in a pathway downstream to the proliferation signals originating from the EGFR family. The potential importance of PI3K in the malignant process is

suggested by its position in signal transduction between EGFR and AKT2, which has been identified as another site of oncogene abnormality in ovarian cancer (48). PI3K has become a focus of attention because amplification of PI3K activity is observed in approximately 40% of ovarian tumors confined to the subset of tumors with serous histology (49, 50). PTEN mutations predominate in endometrioid ovarian cancers. In addition to increased proliferation, decreased apoptosis is also found in association with increased PI3K activity (51). Amplification of PI3K activity is observed in several ovarian cancer cell lines, including OVCAR3. Mechanistic studies have shown that enzyme activity of PI3K is reduced more than 80% by specific inhibitor LY294002 (52). LY294002 causes a dose-dependent reduction in growth of OVCAR3 cells in culture and reduces tumor burden in nude mice inoculated with OVCAR3 (52).

Agents

Well-known agents are being considered for use in reducing the risk of developing ovarian cancer. One of these is the OCP. The OCP reduces the risk of developing ovarian cancer with odds ratios ranging from 0.25 to 0.8 (12–14, 53–55). Its protective effect is independent of study design (case-control or cohort study) and study population (population based or hospital based). The reduction in risk appears to persist for up to 10 or more years after discontinuation of OCP. Based on epidemiological findings and assuming a lifetime protective effect and similar protection by all formulations of OCP, it is estimated that more than half of all ovarian cancers in the United States could be prevented by the use of OCP for at least 4 years (53, 54). A recent case control study (56) that compared high-dose OCP with mid-dose OCP showed greater protection from the high-dose OCP. Both progestin and estrogen concentrations are higher in the high-dose pill, but the greater protective effect is attributed to the progestin component. There have not been studies comparing the mid-dose pill with the low-dose pill, but it would be important to determine the effectiveness of the low-dose pill. In monkey studies using both progestins and OCP (57), the progestin arm had more apoptosis of the OSE cells than the OCP arm, suggesting that progestin may be the active component in the OCP, although variances were large, and median values were used because of the large variability. A reanalysis of the data from the cancer and steroid hormone study (58), which examined the strength of OCP components taken by 390 women diagnosed with ovarian cancer compared with controls, found the greatest reduction in ovarian cancer risk associated with the highest progestin potency. In addition to this information, a recent report has explored the relationship between progestin, TGF-β expression, and apoptosis in the ovarian epithelium of cynomolgus macaques. Exposure to progestin changed the expression of TGF-β, lowering TGFβ-1 with a corresponding increase in TGF-β2/3, correlating closely with induction of substantial apoptosis in the ovarian epithelium (59). Consequently, the progesterone receptor is a prime candidate as a preventive target. Definitive mechanistic and clinically based studies are of great importance in refining the opportunity to specify a role for progestins, agent/dose/schedule, in ovarian cancer risk management. This body of evidence makes the OCP (and potentially the progestin component) an excellent candidate chemopreventive agent for ovarian cancer, although more needs to be learned about the mechanism of its protective effect.

Use of the OCP for 5 years decreases the risk of ovarian cancer by 50% (53–55) but reduces the number of ovulatory cycles by approximately 15 percent. Consequently, there is not a linear correlation between the duration of OCP use and the impact on ovarian oncogenesis, suggesting that more complex mechanisms other than just ovulation suppression may be at work in the chemopreventive activity of OCP. Inhibition of gonadotropin release from the pituitary, one of the effects of OCP use, or other unknown effects of estrogens and progestins may also play a role in this chemopreventive activity.

Retinoids. As with OCP, the antineoplastic effect of 4-HPR is not completely understood. In the laboratory, the activity of retinoids in ovarian cancer has been studied in various ways. In four different ovarian cancer cell lines, 4-HPR was the most effective at suppressing growth compared with all-*trans*-RA, 9-*cis*-RA, and 13-*cis*-RA (60). Studied in more detail in cell line OVCAR3, 4-HPR was found to weakly activate only RARγ, with induction of apoptosis appearing to occur independent of retinoid receptors. This result is consistent with the classification of 4-HPR as a receptor-independent apoptotic retinoid. In addition to retinoid receptors, several other targets have been suggested to mediate their effect. They include the destruction of the mitochondrial membrane by reactive oxygen species and stabilization of the Rb2/p120 protein that mediates retinoid induced growth arrest (61).

Epidemiological and laboratory data suggest that retinoids may have a role as preventive or therapeutic agents for ovarian cancer (58, 62, 63). Fenretinide or 4-HPR has few side effects compared with other vitamin A derivatives and is currently being used in chemoprevention studies of other organ sites, including lung, head and neck, cervix, and bladder. Experimental studies have demonstrated that retinoids can affect human ovarian cancer cell growth by inhibiting proliferation and inducing apoptosis (58, 62); preliminary data from our laboratory show that 4-HPR induces apoptosis in immortalized OSE cells and in normal OSE cells (64). Some cells respond to 2 μM/ml 4-HPR, which might be achievable by oral administration, but others require as high as 10 μM/ml to have an effect. The 10 μM/ml dose would not be achievable by an oral dose because of side effects, particularly skin and ophthalmic effects (nyctaglia). Because epithelial ovarian cancer is thought to arise from a neoplastic process that results from a series of mutations in the OSE cells, the probability of developing a neoplasm would decrease if premalignant or genetically altered cells were eliminated by apoptosis.

In Italy, a randomized trial for the prevention of breast cancer has provided preliminary evidence that retinoids, specifically 4-HPR, may prevent or delay the development of ovarian cancer (63). After surgical treatment for breast cancer, 2972 patients were assigned to treatment with 4-HPR (1422) or placebo (1427) to prevent development of new primary breast cancers. After a median follow-up of 51.9 months, no overall difference in the development of new primary breast cancers was evident; however, there was a significant difference in the numbers of ovarian cancers that developed. During the treatment period, six new cases of ovarian cancer were diagnosed in

the placebo group *versus* none in the 4-HPR group (P = 0.0162). After cessation of treatment, there were four additional cancers in the control group and six in the 4-HPR group, suggesting that the effect was not durable. There was also a difference in the characteristics between groups: the control group was more likely to have a BRCA mutation than the 4-HPR group, which suggests that the difference may be due to the BRCA status rather than drug effect (65). However, when combined with the cell work, there is the suggestion that retinoids may have chemopreventive activity in the ovary but may need to be administered for long periods of time or to have a different dosing developed so that higher doeses could be achieved without toxicity.

Some low-toxicity retinoids, known as heteroarotinoids, share the receptor-independent apoptotic profile of 4-HPR and may be candidates for development as chemopreventive agents (61). In addition to this group of retinoids, AHPN/CD437, a conformationally restricted retinoid engineered to bind selectively to RARγ, also shares the apoptotic profile of 4-HPR. In ovarian tumor cell cultures, exposure to AHPN was associated with increased expression of *BAX* and decreased expression of *Bcl*-2 (66, 67). Agent development for ovarian risk reduction will benefit from mechanistic studies of apoptotic induction by 4-HPR and functionally similar compounds such as AHPN. Retinoids that suppress both growth and survival of abnormal cells hold more promise as chemopreventive agents. Prioritizing retinoids for ovarian cancer prevention will ultimately depend on the overall interrelationship among antiproliferative, differentiating, and apoptotic properties of these compounds.

Although there are still too little data to definitively guide chemopreventive studies, there is compelling evidence that as many as 50% of ovarian cancers may be prevented with OCP, and possibly even more may be prevented if a combination of the active ingredients (presumably progesterone) and a vitamin A derivative could be combined for use in high-risk women. Likewise, a similar reduction might be obtained if the active component of OCP could be administered to low-risk women, the group that develops 90% of the ovarian cancers.

Biomarkers

Biomarkers need to be identified that are predictive of drug or micronutrient activity and pharmacodynamics because the modulation and utility of particular biomarkers will depend on the drug-specific mechanism of action and conditions of use for any given agent. At the current time, little is known about effective biomarkers for drug activity in ovarian cancer chemoprevention. Markers of proliferation and apoptosis are key biomarkers in prevention. Apoptosis is hypothesized to be one of the major mechanisms by which cells with genetic alterations are eliminated (68). In a pivotal trial, primates were treated for 2 years with OCP, estrogen, progestin, or placebo (69). After this period, ovaries were removed, and their surface epithelia were assessed for apoptosis. OCP and progestin significantly increased apoptosis in OSE cells from 5% (baseline) to 25%, whereas estrogen had no effect. If OCP increased the rate of apoptosis in epithelial cells from human ovaries, as they have been found to do in primate ovaries, apoptotic elimination of aberrant cells might be one of the mechanisms underlying the chemopreventive effects of OCP. In the primate, the high rate of apoptosis (25%) also indicates that either a very large proportion of OSE cells had underlying genetic abnormalities or a number of NOE cells underwent apoptosis in response to components of the OCP. This 25% frequency of apoptosis in the primates appears particularly high when it is considered that it represents a "snapshot" in time and that many cells may already have undergone apoptosis and been cleared from the system. One possible explanation may be the use of a Triphasil pill that has the highest concentration of the progestin in the third week. These monkeys were sacrificed in the third week of their OCP use.

In cell culture, different retinoids (including 4-HPR and all-*trans*-RA) inhibit proliferation and induce apoptosis in a number of tumor cell types [including ovarian cancer cell lines (57, 70–74)]. In at least some cell lineages, 4-HPR is more effective than other retinoids at inducing apoptosis (57) and can induce apoptosis in cells resistant to conventional retinoids. However, the mechanism by which 4-HPR and other retinoids induce growth inhibition and apoptosis is unclear. Most, but not all, studies indicate a correlation between RARβ expression or the ability to induce its expression and the ability of 4-HPR to induce growth inhibition and apoptosis (70–72). However, in ovarian cancer cells, 4-HPR effect appears to be receptor independent (61). Induction of reactive oxygen species is another proposed mechanism (74) of 4-HPR, but there are no data available on OSE cells or ovarian cancer cells. These biomarkers of retinoid activity warrant further exploration.

Growth Factor Specific. TGF-β is another potential biomarker of chemoprevention. It can induce growth arrest and apoptosis of ovarian cancer cell lines, as well as ovarian cancer cells isolated directly from patients (73, 74). In several cell types, including ovarian stroma, steroid hormones can increase TGF-β production and activation (75–79). Whether TGF-β, in concert with retinoids or steroids, induces apoptosis in ovarian epithelial cells *in vitro* or, more importantly, *in vivo* remains to be assessed. A recent histochemical evaluation of the primate ovary from primates receiving either progestin alone or the Triphasil OCP found an increase in expression of TGF-β2, but not TGF-β1, suggesting that TGF-β2 may be modulated by OCP, serving as a marker of progestin activity (59). Thus, TGF-β and its receptors definitely merit exploration. Other potential biomarkers include proliferative markers, such as Ki67, which are currently being used to evaluate the antiproliferative effects of progesterone.

Stromal cells may play an important role in epithelial carcinogenesis. Both types of cells contribute to the extracellular matrix (80), and stromal cells may influence some of the premalignant changes that occur in the epithelial cells (81). Epithelial ovarian cells express both cytokeratin and vimentin, suggesting a dual phenotype consistent with a multipotential stem cell origin. These epithelial cells produce both epithelial and mesenchymal components of extracellular matrix in tissue culture, which is consistent with their dual phenotype (80). KGF, a mesenchymal growth factor that mediates epithelial-stromal interaction, has been recently studied as a factor in early carcinogenesis (82). Fresh NOE cells, but not stromal cells, were found to highly express KGF, which subsequently growth-stimulated NOE cells in an autocrine manner (83). HGF and its

receptor, Met, have also been studied. HGF and KGF, as well as Kit ligand, have been found to interact and promote NOE cell growth and growth factor expression, suggesting that these may play a role in the growth stimulation that accompanies the carcinogenic process (84). Wong *et al.* (85) found that NOE cells from women without a family history of ovarian cancer down-regulated the HGF receptor with increasing passage, whereas the NOE cells from women with a family history of ovarian cancer did not, suggesting that HGF may be a growth regulator, particularly in cells destined to develop cancers (85). Telomerase may be an additional marker warranting both study and targeting. These biomarkers need further study to understand their role in modulating growth and to determine which growth modulators might be a target for prevention.

Optical Spectroscopy

One of the more exciting prospects for an early marker of ovarian cancer is optical spectroscopic signatures. In the last decade, substantial research has lead to useful optical methods of diagnosing early cancers (86–92). Fluorescence spectroscopy is being used to detect cancers noninvasively in many organ systems using a probe that can interrogate the tissue. The system utilizes redox potential (ratio), which is calculated by (FAD/(FAD + NADH) and is, in part, a measure of the relative hypoxia of the tissue (93). FAD and NAD(P) are reduced in the citric acid cycle (anaerobic glycolysis) to FADH and NAD(P)H, which are used as coenzymes in the electron transport chain. In tumors, these coenzymes will accumulate in their reduced states (NADH and FADH) and produce a unique fluorescence signature (NADH high, FAD low) as a result of alterations in blood flow, decreases in tissue pH, and abnormalities in mitochondria and in transport of electron carrier molecules into the mitochondria (93, 94), where the electron transport chain functions. In a pilot study (95), patterns of fluorescence called excitation/emission matrices differed between normal ovaries and areas of invasive cancer and thus are promising for early detection of ovarian cancer. As anticipated, redox potentials were 50% lower in the cancer than in the normal ovary with peaks at 350 nm (excitation) and 460 nm (emission), representing both collagen and NADH. Even more exciting, however, was our recent primate study of fluorescence spectroscopy as a marker for drug activity in the ovary (96). Unlike cancer, where redox potentials are reduced, redox potentials were increased in response to both OCP and 4-HPR. Changes in fluorescence signatures were hypothesized to be due to a decrease in NAD(P)H and an increase in FAD in response to the drugs. 4-HPR had the least effect on the fluorescence signature of NAD(P)H and the greatest effect on the area corresponding to FAD, in contrast to OCP, suggesting that each agent has a unique effect on cellular metabolism. These agents also produced an increase in the redox-related potential of the target organ, suggesting that hypoxia was less extensive and that the system was more quiescent. Thus, optical spectroscopic signatures may serve as an early marker of drug activity.

Cell culture data (97) show that retinoids can induce apoptosis or inhibit growth in both normal and immortalized OSE cells. Cell studies measuring fluorescence emission compared with percentage of apoptosis and growth inhibition showed that both apoptosis and growth inhibition correlated with redox ratio ($P = 0.0274$), FAD fluorescence intensity correlated with apoptosis ($P < 0.001$), and NAD(P)H fluorescence intensity correlated with cell survival ($P = 0.04$; Ref. 68).

New Agents

Given the preponderance of evidence that OCP and/or retinoids have a role to play in ovarian cancer prevention, an initial consideration for the development of new agents would be optimizing the effect of these two categories of agents on ovarian cancer risk reduction. In general, recent progress in pharmacological intervention has been achieved by identifying a specific target that is critical to a pathological process. Disruption of a critical pathological signal by eliminating target function can be the key to disease prevention as well as disease control. Evidence reviewed above suggests that OCPs disrupt a critical pathway in early ovarian carcinogenesis in a substantial proportion of women at risk, but a specific target has not been clearly identified. A recent study supports the theory that the progesterone component of OCPs is responsible for reducing ovarian cancer risk.

In addition to the targets suggested by the observation of ovarian cancer risk reduction associated with the use of OCP or 4-HPR, the molecular analysis of malignant ovarian tumors has identified other candidate prevention targets. Selected oncogenes, growth factors, and signal transduction pathways are connected with ovarian carcinogenesis. The focus on targets related to the EGFR family, P13K, and COX2 is guided not only by preclinical studies but also by the presumption that relatively nontoxic oral agents in these categories are either available or about to become available.

TKIs. TKIs are of interest to ovarian cancer prevention because of their effect on the activation of EGFRs. In general, the dose-limiting toxicities for TKIs are skin rash and/or diarrhea. In the prevention setting, it may be possible to identify conditions of use that will avoid these toxicities. Alternatively, if preclinical concepts of prevention are demonstrated with compounds like these, it may be possible to identify less toxic versions of these agents that reduce cancer risk. Beyond EGFR-selective agents, there are TKIs in development with a different range of activity. For instance, CI-1033 is a potent inhibitor of kinase activity across the entire EGFR family. Agents selective for HER-2 are also in development.

Examples of TKIs that are of theoretical interest for testing in ovarian cancer prevention studies are ZD1839 (Iressa), OSI774, and PKI-166 (98, 99). As a pyrolopyrimidine, PKI-166 is structurally different, but like ZD1839 and OSI774, targets EGFR. All of these agents have been used in clinical trials and could be studied in model systems to generate preclinical data relevant to ovarian cancer prevention. EGFR, HER-2, and HER-4 all have a tyrosine kinase domain that can be activated when particular EGFR dimer-ligand complexes are formed. As members of a structural class of compounds known as quinazolines, ZD1839 and OSI774 are structurally similar and selectively deactivate the tyrosine kinase of EGFR. These agents might be particularly useful if it could be demonstrated that an EGFR-selective drug deactivates abnormal EGFR/HER-2 heterodimer expression in ovarian epithelium. In breast cancer cells

that overexpress HER-2, blocking transmodulation through EGFR with an EGFR-specific TKI is known to halt growth (100).

PI3K Inhibition. Because of the position downstream to the proliferation signals originating from the EGFR family, agents targeting PI3K might have chemopreventive activity on the basis of an antiproliferative effect. P13K inhibitors have not reached clinical trials, so future use of agents in this class for chemoprevention awaits their further pharmacological development.

COX-2 Inhibition. Another target of chemopreventive interest is COX-2 (101). COX-2 is an inducible enzyme of inflammation, mediating the conversion of arachidonic acid to prostaglandins. Attention has been called to the role that inflammation might play in ovarian carcinogenesis (102). It remains to be seen whether COX-2 inhibitors can be used to modulate COX-2 expression in ovarian epithelium and whether such modulation has a role early in carcinogenesis. As with the other agents mentioned in this review, it would be helpful to have a valid animal model of ovarian carcinogenesis to use in the development of preclinical data.

Clinical Trials

There are currently ongoing trials of both high low- and high-risk women for investigating chemopreventive agents in the ovary. Fox Chase Cancer Center has a trial using 4-HPR in high-risk women undergoing oophorectomy. The University of Texas M. D. Anderson Cancer Center and the University of Arizona have chemoprevention trials in both low- and high-risk women using OCPs and 4-HPR alone and in combination for women undergoing oophorectomy. This is early exploratory work, and findings from these trials will serve as templates for additional trials. Markers that are elucidated as a result of these trials will help determine which are the best biomarkers for drug activity. Following the results of these and other trials that may be starting in the next decade, larger randomized prospective trials will be important to determine the true preventive activity of these and other agents.

Discussion

Of the four criteria given in the opening definition of IEN, the most difficult to pinpoint in ovarian cancer is the abnormal phenotype. Although access to early ovarian neoplasia is limited by anatomical circumstance, there may, in fact, be biological reasons why an understanding of ovarian IEN is elusive. Ovarian cancer has been described as diseases (37), and, in fact, more than 40 histological entities contribute to the WHO classification of epithelial tumor types. Heterogeneity in ovarian cancer histology suggests a corresponding complexity in IEN. Also, it is recognized that malignant cells with a specific and identifying molecular fingerprint are not always histologically unidentifiable in seemingly normal epithelium adjacent to tumor (34). Given these observations, it may ultimately be necessary to rely on the genetic and consequent functional abnormalities to identify the precursor population of cells that gives rise to invasive ovarian cancer. In addition to the spectroscopic technique described above, genomic and proteomic methods are now being developed that may facilitate the definition of localized precursors of ovarian cancer (103). The possibility of analyzing proteins characteristic of cancer risk and shed from ovarian IEN into serum (104–107) appears to offer an attractive alternative to the direct assessment of the ovarian epithelium by microlaparotomy for examination by spectroscopy or biopsy and subsequent microdissection.

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