

Biomarker Modulation in a Nonhuman Rhesus Primate Model for Ovarian Cancer Chemoprevention¹

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Abstract

Objective: The objective of this study was to explore whether a nonhuman primate model could be developed to test drugs for the prevention of ovarian cancer.

Methods: Nineteen adult female Rhesus macaques were given fenretinide (4HPR), oral contraceptives (OCP), the combination (4HPR + OCP), or no medication for 3 months. Exploratory laparotomy was done pre- and postdrug to assess intermediary biomarkers of neoplastic phenotype, proliferation, response pathways, and growth-regulatory and metabolic markers. Fluorescence emission spectra were plotted for each group pre- and postdrug and means were overlaid on these plots and normalized. Fluorescence intensities were compared using the 2-tailed Student *t* test, ($P = 0.1-0.01$).

Results: All monkeys tolerated drugs and surgeries without difficulty. Histochemical markers showed no significant trend. However, fluorescence spectroscopy showed increased intensity at 450 nm excitation, 550 nm emission correlating with increased FAD presence. The 4HPR group ($P = 0.01$) showed higher intensity than the OCP group ($P = 0.05-0.07$) when compared with the controls. Decreased emission was seen at 350 nm excitation, 450 nm emission correlating with decreased NAD(P)H presence. The OCP group showed the largest change ($P < 0.01$), and the control group showed the smallest change.

Conclusions: The nonhuman primate is an excellent model to test drug effect on the ovarian surface epithelium and merits additional study. Fluorescence

spectroscopy was the most sensitive marker for drug activity and the apparent increase in NAD and FAD in the 4HPR group is consistent with the effect of 4HPR observed in cell culture. The differences between the OCP and the 4HPR groups suggest a different mechanism of activity of these drugs.

Introduction

Epithelial ovarian cancer has the highest mortality rate of any of the gynecological cancers, with a 5-year survival rate of 30% or less, despite aggressive treatment. Seventy percent of these cancers are diagnosed with widespread intra-abdominal disease or distant metastases, which accounts for the dismal prognosis for ovarian cancer. Even ovarian cancer limited to the pelvis has a 5-year survival rate of only 50% (1). Thus, cancer prevention merits at least as much attention as does treatment of disease that has already occurred. Cancer chemoprevention is a rapidly growing area of research because of the difficulties in treating advanced cancers. Chemoprevention refers to the administration of chemical agents that prevent or delay cancer in normal or undiseased people. Necessary elements for chemoprevention studies include appropriate agents that are safe and which have both epidemiological and mechanistic data that support their use; a suitable cohort with adequate incidence of disease and in which the risk:benefit ratio is acceptable; and measurable biomarkers that are likely to be affected by the agent and whose modulation supports the postulated chemopreventive activity (2). Biomarkers are important because they are relevant to the development of neoplasia, either phenotypically (proliferation, angiogenesis, or nuclear morphometry) or mechanistically (molecular markers), they are likely to be required for a response to the chemopreventive agent, or they are relevant to carcinogenesis (3).

Two drugs that have received attention as agents that can prevent ovarian cancer are OCPs³ and retinoids. There are multiple epidemiological studies that show that OCP use of at least 5 years' duration is associated with a 50% or greater reduction in the odds of developing ovarian cancer (4–8). The mechanism of this prevention is unclear; ovulation suppression may be one factor, but other mechanisms are hypothesized. A single prospective study showed that OCP and progesterone increased the rate of apoptosis of ovarian surface epithelial cells in primates (9). Premenopausal women with a family history of breast cancer, ovarian cancer, or both are excellent candidates for prevention strategies; however, in premenopausal women the potential teratogenicity of retinoids is a concern (10). Epidemiological and laboratory data suggest that retinoids may have a role as preventive or therapeutic agents for ovarian cancer (11–12). 4-HPR has the fewest side effects of the vita-

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³ The abbreviations used are: OCP, oral contraceptive; 4-HPR, fenretinide; EEM excitation and emission matrix.

min-A derivatives and is currently being used in chemoprevention studies in other organ sites including lung, head and neck, cervix, and bladder. Preliminary results from the 2972 women in the Italian randomized chemoprevention trial for prevention of secondary breast cancers suggest that retinoids have preventive activity against ovarian cancer (12). Ovarian cancer developed in six women in the placebo arm, but there were no cancers in the 4HPR arm. Although this response was not durable, it suggested retinoid activity in the ovary. Experimental studies have demonstrated that retinoids can affect human ovarian cancer cell growth by inhibiting proliferation and inducing apoptosis (13–15). Preliminary data from our laboratory 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,⁴ compared with the baseline rate of 1–3% in untreated cells. The probability of developing a neoplasm will decrease with increasing apoptosis.

Substantial research has been done to develop optical methods as early diagnostic tools for cancers in the last decade (16–19). Optical spectroscopy has the potential to decrease the necessity for pathological diagnosis; to aid in early, near-real-time diagnosis; and to identify abnormal areas that may not have a visible lesion for localization of biopsy. Fluorescence spectroscopy uses energy in the form of a light photon to activate certain molecules within a cell; the subsequent radiative relaxation of the molecule and the release of a reemission photon is the process called fluorescence. It is an optical method of illuminating tissue with monochromatic light and exciting the natural fluorophores within tissue, which then emits fluorescent light when a paired electron in an excited state returns to a lower or ground state. Natural fluorophores in tissue include NADH; FAD; structural proteins such as collagen, elastin, and their cross-links; and the aromatic amino acid tryptophan, each of which has a characteristic wavelength for excitation with an associated characteristic emission. Fluorophore concentrations change as tissue progresses from normal to a preneoplastic state to cancer (20). Theoretically, different changes in the fluorescence signature should occur in response to an agent that induces quiescence or apoptosis.

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 that are useful for understanding basic mechanisms, but these animals differ from the human reproductively. Primates, however, are much closer to humans in reproductive function and anatomy, hormonal and menstrual patterns (9, 21), and histochemistry and will be a better model with which to develop strategies for prevention of ovarian cancer in the future. This study describes the use of the *Macaca mulatta* as a nonhuman primate model to test drugs for the prevention of ovarian cancer.

Materials and Methods

Initially, a pilot study was done with three animals to test drug tolerance, tolerance for two surgical procedures, and the feasibility of the model. After this pilot, 19 female adult *M. mulatta* were brought together for this preliminary exploratory 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, an Association for the Assessment and Accreditation of Laboratory Animal Care International-accredited facility, in accordance with the *Guide for the Care and Use of Laboratory Animals*. Numbers were chosen based on cost and availability of animals: five animals were initially placed in each drug group, and four were placed in the control group. This study was a pilot study designed to generate measurements so that future studies could be designed with adequate numbers.

The animals were given 4HPR (five monkeys), OCP (five monkeys), the combination of 4HPR+OCP (five monkeys), or no medication (four monkeys) daily for 3 months. Doses of 4HPR and OCP were calculated by allometric scaling, which calculates a dose based on both weight and basal metabolic rate (22). This is derived from the equation $Y + K(W_{kg}^{0.75})$, where Y is the resting animal's energy output in kcal/24 h (also termed the minimum energy cost), K is a constant based on core temperature, which is specific for each species, and W is mass in kilograms. Monkeys ranged in weight from 6–8 kg, but doses were calculated for a 7 kg monkey. The OCP used was Ortho-Novum 1/35, a medium-dose OCP known to suppress cyst formation, which has 1 mg of norethindrone and 35 μ g of ethinyl estradiol in each pill. The 4-HPR dose was calculated in the same manner from the human dose of 200 mg daily. The OCP dose was 0.2 mg norethindrone/0.07 mg of ethinyl estradiol, and the 4HPR dose was 35 mg.

Before starting medication and after 90 days of medication, monkeys underwent laparotomy 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. Fluorescence measurements were taken from each site before biopsy. Biopsy and healing of the ovary was hypothesized to interfere with the spectroscopic signature because of the increased collagen associated with healing as well as with histochemical assessment. Thus, to avoid this bias, one ovary was biopsied on the first surgery and the other ovary was biopsied on the second surgery.

Biomarkers. Biomarkers chosen for this trial include markers of neoplastic phenotype (p53), proliferative markers (Ki67), markers of intact response pathways (estrogen, progesterone, and nuclear retinoid receptors), or inducible growth-regulatory molecules (TGF- β and receptors HER-2, BAX, and BCLx). Apoptosis was evaluated with Apotag. The remainder of the markers were either mouse or rabbit antibodies that were evaluated by immunohistochemistry in the standard manner. The choice of these markers was based on previous studies (apoptotic, proliferative, neoplastic, and retinoid markers), known pathways (retinoid markers), and hypothesized mechanisms (TGF β and receptors and p21).

Complete characterization of the fluorescence properties of an unknown sample requires measurement of a fluorescence EEM, with the fluorescence intensity recorded as a function of both excitation and emission wavelengths (23). Our custom-made system records EEMs in <1.5 min and consists of a xenon arc lamp coupled to a scanning spectrometer that provides excitation light between 300–480 nm. A fiber optic probe directs excitation light to the tissue and collects emitted fluorescence light, which it delivers to an imaging spectrograph and CCD camera. Fig. 1 shows the probe placed on a monkey ovary. Fluorescence emission spectra ranging from 320–850 nm were collected sequentially at 19 excitation wavelengths ranging from 300–480 nm. Before assembling the data into fluorescence EEMs, system-dependent response and background signals were removed. Tissue exposure to broadband UV radiation from this device is below the total exposure limits

⁴ Unpublished data.

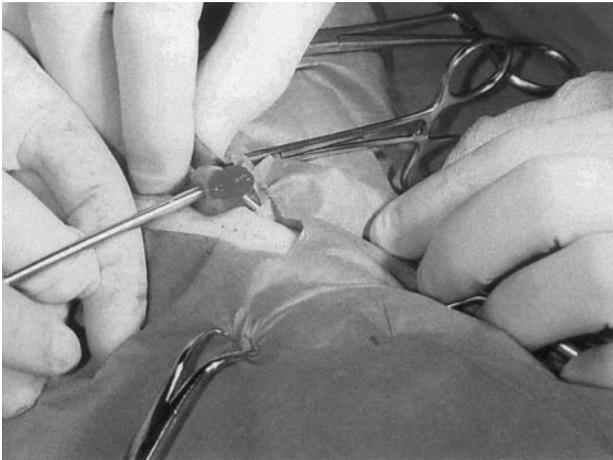


Fig. 1. Fast EEM probe placed on the monkey ovary at the time of laparotomy.

developed by the American Conference of Governmental Industrial Hygienists (ACGIH) for epithelial tissues.

Data Analysis. A visual analytic tool was developed to explore fluorescence differences grouped by monkey, drug, and time (pre- and postdrug), and emission spectra were plotted for each group pre- and postdrug at each excitation wavelength measured. Means were calculated and overlaid on these plots to view the relationship. The data were examined in two fashions: the raw intensities were compared, and emission spectra were normalized to a common wavelength to enhance relative spectral contributions. Using this information, we compared all fluorescence measurements between the first and second measurements using the two-tailed Student *t* test. *Ps* were between 0.1 and 0.01 for this exploratory study. Areas where statistically significant differences were observed between the first (pre-drug) and second (postdrug) measurements were identified in the EEM and plotted as contour lines.

Animal Care. Monkeys used in this study were culled from the specific pathogen free rhesus colony because they developed herpes-B-indeterminate status, they were poor breeders, or they had chronic diarrhea. The monkeys were prevented from eating for 12 h before surgery. Anesthesia was induced with an i.m. injection of tiletamine HCl/zolazepam HCl (Telazol; Fort Dodge Laboratories, Inc., Fort Dodge, IA). When the monkey was sedated, she was removed from the cage and intubated, and anesthesia was maintained on 2–2.5% isoflurane gas with oxygen. After each initial procedure, monkeys were maintained in separate housing and were fed the drug in a flavored treat. Menses were recorded daily, and progesterone levels were measured 2 weeks apart to evaluate ovulatory status.

Pathology. Biopsy specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4-mm sections, and stained with H&E for light microscopic examination. Sections were evaluated for morphological characteristics and pathological changes.

Results

Animal Tolerance. The pilot study showed that the three animals tolerated the drugs and two surgeries without difficulty. Rhesus monkey ovaries are easily accessible through a midline laparotomy incision and are 1–1.5 cm in length and 0.75–1 cm

in the other two dimensions (see Fig. 1). At the time of the second surgery, the scar from the previous biopsy was completely healed and there was a small visible area <1 mm where the defect had filled in. In the larger study, there was one death of a monkey in the 4HPR group that was not related to this study. She was a 9-year-old obese macaque with severe amyloidosis on necropsy.

Fluorescence Measurements. There were consistent differences in the absolute fluorescence intensities and relative contributions noted between the pre- and postdrug measurements in each drug group as well as in the controls. However, the differences noted in the control group that were attributable to a time effect were much smaller than those seen in the three drug groups. Fig. 2 shows areas of significant differences between the pre- and postdrug measurements for all 4 groups. To the right of each graph is the *P*, from 0.01–0.1. Two main areas of change can be identified. The center of the first area is located at 450 nm excitation and 550 nm emission wavelength, consistent with the FAD emission peak. This area of fluorescence intensity is increased in the three postdrug groups, which correlates with increased FAD presence and decreased FADH. The 4HPR group (*P* = 0.01) shows a larger area of difference in pre and postdrug intensity than the OCP group (*P* = 0.05–0.07), whereas the combination group is in between the 4HPR and OCP groups. There were no differences attributable to treatment seen in the control group in this area of the EEM. The second area is located in the 350 nm excitation, 450 nm emission wavelength, which is consistent with the NAD(P)H emission peak. All four groups show decreases in these intensities that correlate with a decreased NAD(P)H presence and thus an increase in NAD(P) (the oxidized form). However, the OCP group shows the largest change (*P* < 0.01), the control group shows the smallest change (very small area of significant change), followed by the 4HPR and OCP group; and, again, the drug combination lies in between the 4HPR and OCP groups.

Other Biomarkers. Markers were evaluated visually in a 2 × 2 table. Missing data in the 4HPR as well as the OCP group precluded any data analysis, inasmuch as there were only two monkeys with histochemical marker data in the 4HPR group and four in the OCP group because of difficulties in slide preparation. The markers did not show consistent changes in response to drug group, but there were trends noted in marker expression. EGFR was consistently present, in both pre- and postdrug measurements; Her-2, p21, and p53 were rarely present in either group, whereas BAX was usually present in both groups. Estrogen receptors were absent, whereas progesterone receptors were usually present and did not change in response to drug exposure. Two monkeys in the OCP group showed apoptosis and TGFβ declining after exposure to drug. However, there were two monkeys that did not show either marker present before or after drug exposure; However, if trends were actually present, they were not large enough, given our numbers, to have adequate power to detect a difference statistically.

Discussion

The pilot study of three monkeys was done to assess and plan the feasibility of developing a primate model for a chemoprevention study. After that, we did a larger study to develop additional expertise in administering the drugs for a longer period of time, in multiple surgical procedures on a larger number of animals, and in handling and processing the ovary. The primate was chosen because of the close association with humans, and the rhesus monkeys were chosen because of their availability, their cost, and their ease of handling. A “before and

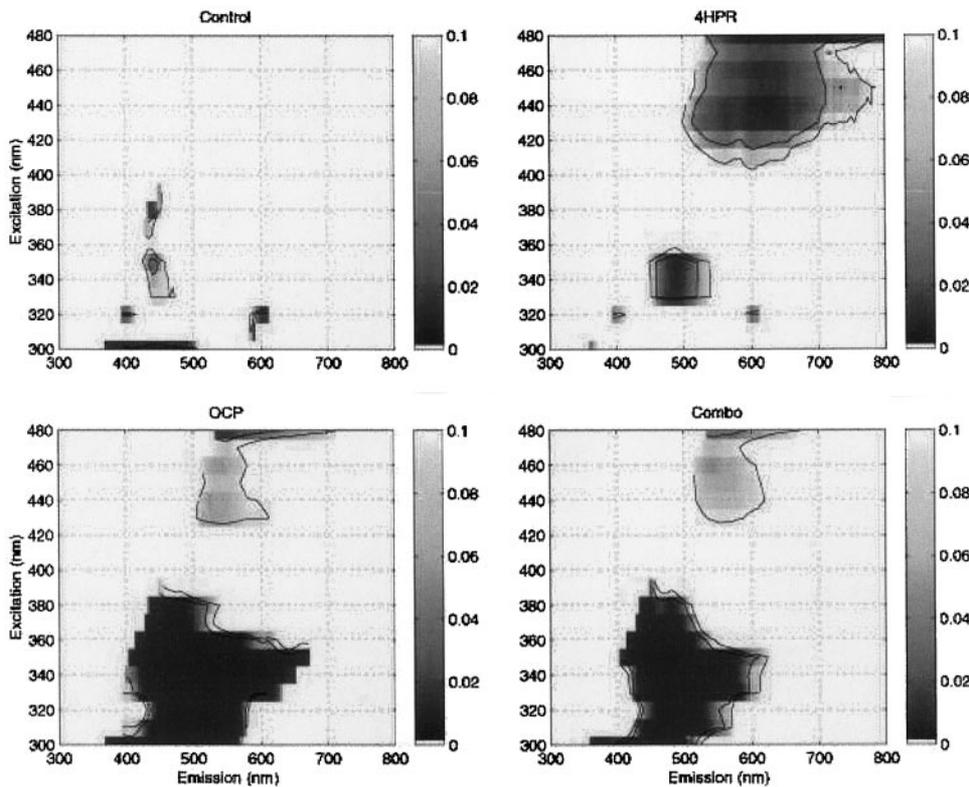


Fig. 2. Results from a two-tailed Student *t* test of pre- and postdrug measurements are presented for the control group (top left), the 4HPR group (top right), the OCP group (bottom left), and the combination group (bottom right). Each graph illustrates *P*s between 0 and 0.1 at all measured fluorescence intensities. Abscissa, the fluorescence emission wavelength; ordinate, the fluorescence excitation wavelength. *P*s are color coded, and a low *P* is represented by a dark color, indicating an area where the drug treatment has affected significantly the average fluorescence emission.

after” drug study has the advantage of controlling for intraspecies variation as well as variations attributable to time—things that historical controls or separate control groups cannot account for.

Animal models are developed to reconcile biological phenomena between species (24) and to allow for extrapolation of knowledge from one species to another. Humans are difficult to use as an experimental model because of ethical limitations, cost limitations, and lack of volunteers. Transgenic mice are commonly used because of their availability and cost, but, in addition to their different reproductive physiology (21) from all primates, their compromised immune systems cannot approximate a normal host for chemoprevention. The nonhuman primate more closely approximates the human in its reproductive physiology and thus remains an important model that needs additional development. The cost of procuring and caring for rhesus monkeys and their low reproductive rates, long developmental periods, and relative scarcity have limited their use as animal models for human diseases. However, the close genetic similarity and, thus, the high probability of producing results that can be extrapolated to humans more than overcomes the above limitations (25). Histochemical analysis (26) and hormone activity (21) of the nonhuman primate ovary show remarkable similarities to that of humans, and the microanatomy of all primates, both human and nonhuman, is almost identical, which is consistent with their close phylogenetic linkage. The fact that all animals in this study were not “reproductively healthy” should not affect our results, and in fact should make this study population more analogous to the diversity seen in women, rather than the selection seen in most breeding colonies.

Fluorescence spectroscopy is being used to noninvasively detect cancers in many organ systems. In this study,

we evaluated it as a marker for drug activity in the ovary. Redox potential can be calculated by $(FAD)/(FAD + NADH)$ and is a measure of the relative hypoxia of the tissue (20). 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 state (NADH or FADH) with a unique fluorescence signature (NADH, high, and FAD, low) as a result of alterations in blood flow, decreased pH of the tissue, and abnormal mitochondria as well as abnormal transport of electron carrier molecules into the mitochondria (27), where the electron transport chain takes place. Although these coenzymes have a unique signature, there is considerable overlap in tissue from other fluorophores, such as structural proteins, and reabsorption of emitted fluorescence by blood. Therefore, results in living tissue are affected by several factors and not these coenzymes alone. This study shows that the opposite occurs in response to the chemopreventive agents. NAD(P)H is reduced consistently by the drugs, and FAD is increased. 4HPR decreases NAD(P)H less than does OCP or the combination of the two drugs, and it increases FAD more than either OCP or the combination, suggesting that each agent has a unique effect on cellular metabolism. These agents show an increase in the redox potential of the target organ, suggesting that less hypoxia is present and that the system is more quiescent. Although numbers were small in this study, variances were also small, suggesting that there is a consistent effect of these drugs on the fluorescence signature of the ovary. By measuring fluorescence from endogenous fluorophores with this system, we were able to identify two areas in the EEM that are affected by these drugs. Thus, a system could be constructed easily to interrogate the entire ovary

and to potentially quantify the drug effect on the ovary as well as to identify areas of occult cancer, which are thought to be more common in BRCA1-positive women (28). The use of fluorescence as a biomarker has a distinct advantage over the repeat biopsy of the ovary and, with the additional development of algorithms, could be used for an "optical biopsy," in lieu of a tissue biopsy, to reassess biomarkers in the original field of investigation.

Although the histochemical marker data were not definitive, some data were gathered regarding the ability to test these histochemical methods in the monkey as well as some data on markers that are present or absent in the rhesus. One of the hypotheses of our group and others is that both progesterone and 4HPR induce apoptosis in the ovarian surface epithelial cells. This may be by a direct effect on the epithelial cells or by induction of TGF β by the stromal cells. Preliminary data from our laboratory suggests that 4HPR and TGF β act synergistically in induction of apoptosis in normal ovarian epithelial cells. We did not document this effect with histochemistry. TGF β 1 was the marker tested, and there is evidence from Rodriguez (9) that TGF β 1 may be decreased, whereas TGF β 2 and -3 are increased. BAX and BCLx are proteins produced by the *bcl2* gene family that are thought to be modulated by retinoids, with BAX being proapoptotic and BCLx being anti-apoptotic. The 4HPR group did not have enough data to evaluate this effect on either protein. The numbers in this pilot were not adequate to show a statistically significant drug effect in any of the categories, if such an effect exists.

The nonhuman primate is an excellent model for investigating the effects of chemopreventive agents for ovarian cancer. There is evidence that OCP and 4HPR have different effects on the ovary that merit additional study. Although there was no rationale for the sample size except cost and the availability of monkeys, the results of this study will allow us to plan for additional animal studies with better power. In addition, changes caused by the chemopreventive agents have the potential to be followed optically, and this may prove helpful in assessing responses to the drugs. The clear difference in activity in the ovary as a result of 4HPR activity suggests that this is a drug meriting additional study in both monkeys and women. The largest effect consistent with decreased NADH presence was seen in the monkeys who were given OCP, the largest effect consistent with increased FAD presence was seen in the monkeys given 4HPR, whereas a combination of effects was seen in the monkeys given both OCP and 4HPR. The additional development of this nonhuman primate model for assessing chemopreventive agents for ovarian cancer will allow us to make significant progress in understanding how these drugs affect the ovary and in developing rational chemoprevention strategies for this devastating disease.

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