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To complete the first specific aim of this project VP-16, taxol, okadaic acid, and calyculin A were used to screen for apoptosis in the human breast carcinoma cell line MDA-MB-231. Characterization of apoptosis was investigated by concentration-response and time course studies using MDA-MB-231 cells evaluated by morphology. Appropriate concentrations of calyculin A were selected for additional characterization of apoptosis in MDA-MB-231 cells since calyculin A was the most effective compound. DNA fragmentation was the next hallmark of apoptosis measured using calyculin A. Calyculin A does not promote internucleosomal DNA fragmentation, but does induce heavy molecular weight DNA fragmentation at concentrations that induce apoptotic morphology in MDA-MB-231 cells. This compound instigates apoptotic morphology and heavy molecular weight DNA fragmentation in MDA-MB-231 cells characteristic of apoptosis. Future experiments on calyculin A's ability to induce apoptosis, and the effects of Protein Kinase C on calyculin A induced apoptosis, will be carried out using MDA-MB-231 cells as outlined in the Statement of Work.

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Robert L. Rice
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9/25/85
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E. Table of Contents

A. Front Cover.....1

B. SF 298 Report Documentation Page.....2

C. Foreword..... 3

E. Table of Contents..... 4

F. Introduction..... 5
 1. Background..... 5
 2. Technical Objectives..... 6
 3. Methods..... 6

G. Body of Proposal..... 7
 1. Experimental Procedures..... 7
 2. Results..... 8

H. Conclusions..... 10

I. References.....11

J. Addendum.....12
 1. Statement of Work.....12
 2. Training Environment.....13
 3. Program Director and Participating Faculty...14
 4. Training..... 16

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F. INTRODUCTION

1. Background

Cancer is a balance between cell growth and death. Two forms of cell death have been described in tumor cells: necrosis and apoptosis. Apoptosis was originally discovered on the basis of morphological changes in cells¹. The morphological changes are cell shrinkage, dense chromatic condensation and margination, loss of intracellular contacts such as detachment from a monolayer of cells, preservation of organelle and membrane integrity, and formation of apoptotic bodies that are phagocytosed by neighboring cells¹. Transcription or translation inhibitors block apoptosis in many cell types showing that apoptosis is an active process. Since the original morphological criteria, biochemical changes with apoptosis have been described such as DNA fragmentation. Agarose gel electrophoresis of DNA from apoptotic cells demonstrated a 180-200 base pair "DNA ladder" indicating internucleosomal degradation of the genome¹. These were adopted as one of the hallmarks of apoptosis when internucleosomal DNA cleavage was correlated with DNA morphological DNA condensation and margination. The idea that production of 180 base pair ladders is necessary for apoptosis has been challenged².

Previous studies indicate that altered protein phosphorylation functions in the signaling of apoptosis. It has been suggested that the premature activation of a subset of protein kinases called cyclin dependent kinases (CDK) may generate apoptosis. CDK activation can be activated by the dephosphorylation of a threonine residue. Ceramide generated through the sphingomyelin pathway has also been implicated in the induction of apoptosis in several cell lines³⁻⁵. Ceramide can activate both a serine/threonine protein phosphatase called CAPP (ceramide-activated protein phosphatase) and a PK_C like kinase called CAPK (ceramide-activated protein kinase) respectively³⁻⁵. Therefore, a role exists for protein phosphorylation in the signaling pathway of apoptosis.

The purpose of this project is to test the hypothesis that apoptosis has a role in human breast carcinoma cell life, and that PK_C plays a significant part in this apoptotic process. The first specific aim of this project was to characterize apoptosis in MDA-MB-231 cells using the compounds VP-16, taxol, okadaic acid, and calyculin A. VP-16 is a topoisomerase II inhibitor while taxol is an anti-mitotic agent; both compounds have induced apoptosis in mammalian cell lines. Calyculin A and okadaic acid are specific inhibitors of serine/threonine protein phosphatases. Calyculin A has an IC₅₀ of 0.4 nM, 0.25 nM, and 0.25 nM for the serine/threonine protein phosphatases PP1, PP2A, and PP3 respectively while okadaic acid has an IC₅₀ of 40 nM, 0.25 nM, and 0.25 nM for PP1, PP2A, PP3 respectively. Studies indicated okadaic acid and calyculin A induce apoptosis in MDA-MB-231 cells. Using apoptotic morphology and DNA fragmentation as hallmarks to investigate apoptosis, these compounds were used to screen for apoptosis in MDA-MB-231 cells to complete the first specific aim of this project.

2. Technical Objectives

- A) Characterize apoptosis in the cultured human breast carcinoma cell line MDA-MB-231 cells.
- B) Determine the effects of pharmacological activation, inhibition, and down regulation of PK_C on the induction of apoptosis in MDA-MB-231 cells.
- C) Investigate the effects of different isoforms of PK_C in MDA-MB-231 cell line, by transfection of PK_C isoforms and antisense of PK_C isoforms, on apoptosis in MDA-MB-231 cells.

3. Methods

Characterization of apoptosis caused by the compounds VP-16, taxol, okadaic acid and calyculin A was first studied by concentration-response and time course studies using MDA-MB-231 cells. These concentration response and time course studies were evaluated by morphological examination of the cells. From this data appropriate concentrations of calyculin A were selected for additional characterization; okadaic acid, VP-16, and taxol were not tested further since calyculin A was the most effective compound used on MDA-MB-231 cells. Conventional agarose gel electrophoresis tested for 180-200 bp DNA "ladder" fragments for calyculin A treated of cells, and Field Inversion gel Electrophoresis (FIGE) tested for heavy molecular weight fragments for calyculin A treated of cells.

G. Body of Proposal

1. Experimental Procedures

Cell culture. The MDA-MB-231 cell line was obtained from American Type Culture Collection (ATCC) and was subsequently carried for no longer than 20 passages. The cells were grown in RPMI- 1640 (Gibco/BRL) containing 10% fetal bovine serum (HyClone), 100 U/ml penicillin G sodium, and 100 ug/ml streptomycin (Gibco/BRL) (complete medium) at 37° C under an atmosphere of 95% air and 5% CO₂. Cells were routinely passaged at 1:5, or 1: 10 in 150 cm² flasks (Corning). Cells were routinely tested and found to be free of Mycoplasma contamination. For experiments, cells were plated at a density of 1x 10⁶ cells/T-150 flask and were cultured for two days prior to drug treatments (unless otherwise indicated). Drugs were dissolved in 100% DMSO (VP-16, Taxol, and Calyculin A). Drugs were added to the medium such that the final concentrations of DMSO was between 0.1 - 0.2%. Unless otherwise indicated, controls received equivalent amounts of vehicle (final concentration) in PBS.

Morphological Examination of Cells. Attached and detached cells were harvested and centrifuged at 1000 x g for 5 min and resuspended in Puc's Saline A (Gibco/BRL) containing 20 ug/ml Hoechst 33342 (Molecular Probes) for 15 min at room temperature. Nuclei were visualized using a Nikon Microphot-PX photomicroscope with an epifluorescence attachment. Microscopy was performed on attached cells and detached cells *in situ* (100 - 150 cells examined).

Internucleosomal DNA cleavage. Cells were treated for 24, 48 and 72 h with 1, 10 and 100 VM VP-16. Attached cells were washed three times with PBS, harvested by trypsin treatment, and when indicated combined with detached cells from the same flask; then centrifuged at 1000 x g for 5 min. Cells analyzed for internucleosomal DNA cleavage as previously described⁶.

Field Inversion Gel Electrophoresis (FIGE). Attached cells were washed three times with PBS, harvested by trypsin treatment, and when indicated combined with detached cells from the same flask; then centrifuged at 1000 x g for 5 min.. After centrifugation cells were suspended to a final concentration of 2.5 x 10⁴ cells/ml in a buffer containing 20 mM NaCl, 50 mM EDTA, and 10 mM pH 7.2 and equilibrated to 50° C. An equal volume of 2% InCert (50° C) agarose (FMC) was added and plugs were cast (1x10⁶ cells per plug) for FIGE in disposable molds (BioRad). After solidification at 4° C, plugs were incubated at 50° C overnight in 4.8 mM (0.2%) sodium deoxycholate, 1% sodium lauryl sarcosine, 100 mM EDTA, pH 8.0 and 1 mg/ml Proteinase K (Boehringer Mannheim) and then washed four times

with 50 ml of 50 mM EDTA and 20 mM Tris, pH 8.0. For electrophoresis, plugs were uniformly cut such that 5×10^5 cells per lane were electrophoresed. Electrophoresis was performed using a FIGE Mapper Electrophoresis System (BioRad) in 1.5% Pulsed Field Certified Agarose (BioRad) with a 0.5X TBE solution (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH8.3) that was recirculated at 400 ml/min through a 6°C water bath. During electrophoresis the buffer maintained a constant temperature of $10 \pm 0.5^\circ \text{C}$ in the electrophoresis chamber. An initial 12 min pulse of forward voltage at 150 V was applied to promote migration of the DNA from the plug into the gel. This initial pulse was followed by electrophoresis for 30 h at 150 V using a 21% ramp from 0.9 sec to 30 sec in the forward direction and 0.3 to 10 sec in the reverse direction at 10°C . Using a 21% ramp, the midpoint of the switch time ramp was reached after 21% of the run time past. This non-linear shaped ramp was conducted in both the forward and reverse directions always producing a 3:1 forward:reverse ratio and afforded increased resolution of higher molecular weight DNA as compared to a linear (50%) ramp. After electrophoresis, gels were stained with 1.0 ug/ml ethidium bromide for 1h. The gels were viewed using a UV transilluminator and photographed. Molecular weight size standards were *Saccharomyces cerevisiae* chromosomes (225 - 2200 kbp, BioRad), k phage digested with Eco RI and IEndIH (8 - 48.5 kbp, Gibco/BRL), and k phage DNA concatamers (48.5 - 1309.5 kbp, MegaBase 11 standard, Gibco/BRL).

Statistical analysis. All statistical analyses were performed using InStat (GraphPad). Unless otherwise indicated, data were analyzed by ANOVA analysis followed by either Dunnett's comparison post tests. Dunnett post tests were used when experimental groups were compared to a control group. The threshold for determination of significance in all experiments was $p < 0.05$.

2. Results

Taxol, VP-16, okadaic acid, and calyculin A were screened for their ability to induce apoptosis in MDA-MB-231 cells by apoptotic morphology. Taxol and VP-16 induced significant apoptotic morphology in MDA-MB-231 cells when treated with 0.3-10.0 uM taxol or 30.0-100.0 uM VP-16 continuously for 48h. After 72h of continuous treatment, taxol and VP-16 produced significant apoptosis in MDA-MB-231 cells after 0.1-10.0 uM taxol or 10.0-100.0 uM of VP-16. At 96h of continuous treatment, taxol at 0.1-10.0 uM taxol instigated apoptosis in MDA-MB-231 cells while VP-16 only produced statistically significant apoptosis at 100.0 uM. 10.0-30.0 uM VP-16 produces apoptosis in MDA-MB-231 after 96h of continuous treatment. This result for VP-16 for 96h of continuous treatment is probably due to the number of data points for this treatment ($n=3$). Okadaic acid induced significant apoptosis at 60.0 nM after only 24h of continuous treatment. Calyculin A launched apoptosis in MDA-MB-231 cells when treated continuously

with 10.0 nM for 1-24h of treatment and at 3.0 nM for 4-24h of treatment, but not at 0.3-1.0 nM for 1-24h of continuous treatment. Of the four compounds, calyculin A bestowed the most dramatic results in the shortest amount of time at the lowest concentration. These results demonstrated that calyculin A was the best candidate to test further for apoptosis.

DNA fragmentation was the next hallmark of apoptosis measured on MDA-MB-231 cells using calyculin A. Conventional agarose gel electrophoresis tested for 180-200 bp DNA "ladder" fragments for calyculin A treatment of cells. This was accomplished using the concentrations and time of treatment found to be most optimal to promote apoptosis in MDA-MB-231 cells; 3.0 nM and 10.0 nM for 24h. Calyculin A does not promote internucleosomal DNA fragmentation at 24h of treatment for 3.0 nM and 10.0 nM of calyculin A. A longer incubation of 48h for 3.0 nM and 10.0 nM calyculin A does not produce internucleosomal DNA fragmentation; neither do shorter incubations at 8h or 12h. Previous studies demonstrate that some cell lines that undergo apoptosis do not produce internucleosomal DNA fragmentation, but will produce heavy molecular weight DNA fragmentation.

Field Inversion Gel Electrophoresis (FIGE) tested for heavy molecular weight fragments for calyculin A treatment of cells. MDA-MB-231 cells were treated continuously with calyculin A for 24h at 1.0 nM, 3.0 nM, 10.0 nM. At 1.0 nM the predominant DNA fragmentation is 30-50 Kb. At 3.0 nM the predominant DNA fragmentation is at 30-50 Kb, 400-600 Kb, and some at >1 Mb. The predominant DNA fragmentation of cells treated with 10.0 nM calyculin A for 24h is 400-600 Kb and >1 Mb. This demonstrates a unique and repeatable pattern of DNA fragmentation for increasing concentrations of calyculin A.

H. Conclusions

To complete the first specific aim of this project, VP-16, taxol, okadaic acid and calyculin A were used to screen for apoptosis in MDA-MB-231 cells. Characterization of apoptosis was first studied by concentration-response and time course studies using MDA-MB-231 cells evaluated by morphological examination. From this data appropriate concentrations of calyculin A were selected for additional characterization; okadaic acid, VP-16, and taxol were not tested further since calyculin A was the most effective compound used on MDA-MB-231 cells.

DNA fragmentation was the next hallmark of apoptosis measured with MDA-MB-231 cells using calyculin A. Calyculin A induced apoptotic morphology, but does not promote internucleosomal DNA fragmentation in MDA-MB-231 cells. This is not unusual since there are cell lines that undergo apoptosis, but do not possess internucleosomal DNA fragmentation such as the prostatic carcinoma cell line DU-145. Next, Field Inversion Gel Electrophoresis (FIGE) assayed for heavy molecular weight fragments for calyculin A treatment of MDA-MB-231 cells. A unique and repeatable pattern of DNA fragmentation for increasing concentrations of calyculin A unveiled itself. As the concentration of calyculin A is increased during a 24h continuous treatment, the molecular weight of the predominant DNA fragments increases. As calyculin A is a serine/threonine protein phosphatase inhibitor, this raises interesting questions about serine/threonine phosphorylation involvement in apoptosis, and the possible role of serine/threonine kinases such as Pk_c , Raf-1, and CDKs.

Calyculin A promotes apoptosis in MDA-MB-231 cells very effectively. This compound instigates apoptotic morphology and HMW DNA fragmentation in MDA-MB-231 cells characteristic of apoptosis. Calyculin A will be used in the future to probe the effects of PK_c on apoptosis in MDA-MB-231 cells as outlined in the Statement of Work.

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J. Appendum

1. Statement of Work

Protein Kinase C Processes and Their Relation to Apoptosis in Human Breast Carcinoma Cells

Task I. Characterize apoptosis in cultured human breast carcinoma MDA-MB-231 cells. Months 1-12

A. Initial evaluation of the compounds VP-16, taxol, okadaic acid, and calyculinA: apoptotic morphology and DNA fragmentation

- 1.** Concentration -response curves
- 2.** Time course studies

B. Further evaluation of compounds will be performed using the appropriate concentrations and time-points for the best compound from the above studies.

- 1.** Meridian ACAS 570c confocal microscopy and flow cytometry of attached and detached cells.
- 2.** Ultra structural morphology by TEM by Simon Watkins in the Dept. of Cell Biology/Physiology U. of Pittsburgh.
- 3.** Agarose gel electrophoresis for 180 base pair DNA ladders.
- 4.** Field inversion gel electrophoresis(FIGE) analysis for 50 and 300 kilobase fragments.
- 5.** Determine the effects of protein synthesis inhibitor cycloheximide upon apoptosis in these cells

Task II. Determine the effects of pharmacological activation, inhibition, or down regulation of PK_C on the induction of apoptosis in MDA-MB-231 cells. Months 9-15

A. Activate(PDBU or n-hexyl ILV), inhibit (UCN-01 or Calphostin C) , or down regulate PKC (PDBU or TPA) prior to induction of apoptosis and determine the effects by:

- 1.** Examination for apoptotic morphology
- 2.** Quantification of cell survival
- 3.** Quantification of loss of intercellular contacts(detachment from the monolayer)
- 4.** Measurement of DNA fragmentation as above by conventional gel electrophoresis
- 5.** FIGE

Task III. Investigate the effects of different isotypes of PK_C in MDA-MB-231 cell line by transfection of PK_C isoforms and antisense of PK_C isoforms on apoptosis in MDA-MB-231 cells. Months 15-36

A. Transfect MDA-MB-231 cells with the proper isoforms and antisense of PK_C; determining the effects on apoptosis by:

1. Examination for apoptotic morphology
2. Quantification of cell survival
3. Quantification of loss of intracellular contacts (detachment from the monolayer)
4. Measurement of DNA fragmentation as above by conventional gel electrophoresis
5. FIGE

2. Training Environment

The University of Pittsburgh, founded in 1787, is one of the oldest institutions of higher education in the United States. At present it comprises 16 schools having more than 2,800 faculty and 35,000 students. Of the total student population, 9,940 are currently enrolled in Ph.D. degree programs and 1752 are in professional schools of Medicine, Law or Dentistry. There are currently 150 students pursuing Ph.D. degrees at the University of Pittsburgh SOM, 103 Ph.D. graduate students in the GSPH and 17 in the Biopsychology Program of the Department of Psychology in FAS.

The Pittsburgh Cancer Institute (PCI) was established in 1984 to strengthen and expand cancer core and educational resources in the Western Pennsylvania region by developing new, more effective approaches to the prevention, diagnosis and treatment of cancer and by enhancing professional and lay educational programs. This is especially important because the Western PA region has the oldest population of any in the US; this extends to women. Thus, the projected incidence of tumors in the female population is extremely high. In less than 10 years the PCI has become the major focal point for research and education not only in Western Pennsylvania but also Northern West Virginia and Eastern Ohio; it is now ranked 6th in the United States among recipients for NCI funding with more than \$29 million annually; the PCI has dedicated basic and clinical research facilities totaling over 250,000 square feet for laboratory studies, 28,000 square feet for out-patient services and over 100 beds for cancer in-patients; over 200 women are treated annually for breast cancer at the PCI. The PCI has been responsible for recruiting more than 100 cancer researchers to the Institution, including both the Training Program Director and the Co-Director, and in 1990 the PCI was designated by the NCI as a National Comprehensive Cancer Center. Thus, there is a cohort of young and very enthusiastic investigators available for the educational and research mission of the PCI.

The PCI is a Vanguard Center for the Women's Health Initiative. In addition, Carnegie Mellon University, which is affiliated with the PCI and shares educational programs with the University of Pittsburgh, is physically contiguous with the University of Pittsburgh. Carnegie Mellon has 7,259 students of which 1,164 are enrolled in Ph.D. programs. Thus, within a very small geographical area there is large density of students with a wide variety of interests and talents. This results in a very dynamic and exciting academic environment, which is conducive for interdisciplinary programs. Indeed one of the hallmarks of the University of Pittsburgh's campus has been the successful development of joint educational programs with Carnegie Mellon University, such as the current NSF Fluorescence Center, the NSF/DoD Supercomputer Center, the Biotechnology Center and NIH supported M.D./Ph.D. program (T32-GM08208-05, Joseph M. Furman, PI, 10 positions). As one of the top fifteen recipients of NIH grants, the University of Pittsburgh Medical Center has placed particular emphasis on the importance of external research funds as a vehicle for stimulating high caliber research experience and education. Thus, there is a robust environment for interdisciplinary graduate education in and around the SOM, GSPH and FAS of the University of Pittsburgh.

Grants to support training of predoctoral students are usually given to a particular training program in an established scientific discipline or a subdiscipline, rather than for training in a specific disease entity or in a particular model system. Thus, training grants are relatively common in pharmacology, virology, immunology, epidemiology, psychology or biochemistry, regardless of the specific problems various investigators from these disciplines are addressing. What distinguishes these discipline-based predoctoral training programs from our **Training Program in Breast Cancer Biology and Therapy** is our multidisciplinary approach and the focus on a specific and important disease. The overall philosophy of this training proposal is to identify qualified graduate students in existing basic science departments, to educate them in the problems in breast cancer and to enhance their research capabilities in this field. Our Training Program intends to expand the existing pool of investigators studying breast cancer. Moreover, the program is designed to encourage currently funded investigators to focus on breast cancer as an area of study; this is an important programmatic by-product because it fosters ongoing interdisciplinary research efforts by an array of well-funded investigators.

3. Program Director and Participating Faculty

The **Predocutorial Training Program in Breast Cancer Biology and Therapy** was formally initiated in September 1994 with the awarding of the US Army Training Grant DAMD1-94-J-4039. The Program Director is John S. Lazo, Chair of the Department of Pharmacology and Co-Director of the PCI Experimental Therapeutics Program, and the Co-Program Director is Ohvera J. Finn, Associate Professor of Molecular Genetics and Biochemistry and Director of the PCI Immunology Program. Dr. Lazo has had more than 20 years of

research experience in cancer biology and experimental therapeutics. Much of his early work has been directed at mechanism of drug action and drug resistance. Most of this research has been tumor type-independent in focus. He has been a member of the Board of Directors of the American Association of Cancer Research and Chair of the 1992 Gordon Research Conference on Chemotherapy of Experimental and Clinical Cancer. He is collaborating with Dr. Ohvera Finn to couple antimucin antibodies to DNA cleaving agents and is examining the role of protein kinase C signalling systems in breast cancer cells. Dr. Lazo has been a Ph.D. thesis advisor or Committee Member for 16 Ph.D. candidates and has trained 21 postdoctoral fellows; he currently my thesis advisor. Two of his previous postdoctoral fellows are now investigating new anticancer agents as clinical pharmacologists at a major pharmaceutical firm (Bristol Myers Beecham) and 1 is designing new diagnostic agents at a biotechnology company. Since 1976 Dr. Lazo has been intimately involved in both graduate and medical education and since 1979 he has taught a graduate level course almost every year. His basic medical science preparatory book for second year medical students published by Wilhams and Wilkins (*Review of USMLE Step One*) is among the most popular books of its kind (almost 50,000 copies published) and is about to enter its fourth edition. Writing and reviewing this book has given the Program Director a broad background in both basic and clinical issues related to malignancies including those associated with the breast. He is also PI of an NIH Predoctoral Training Grant in Pharmacological Sciences. The CoDirector, Dr. Olivera Finn, has been investigating breast cancer biology and immunology since 1985. She has trained 7 Ph.D. students and 7 postdoctoral fellows. Three of her Ph.D. students and 2 postdoctoral fellows have continued their research in breast cancer immunology in their new positions as postdoctoral fellows or assistant professors. She has also been a thesis committee member for 22 Ph.D. candidates. There are currently 3 graduate students in her laboratory, 2 of whom are doing research in breast cancer. In addition there are 3 postdoctoral fellows in her laboratory investigating the biology and immunology of breast cancer. In February 1993 Dr. Finn was invited to testify before the President's Cancer Panel, the Special Commission on Breast Cancer, on the future direction of breast cancer and breast cancer vaccines.

The participating faculty members have been drawn from the over 175 members of the Graduate School at the University of Pittsburgh, who are eligible to train students enrolled in a Ph.D. degree granting program. We have selected these 32 faculty members by carefully evaluating them for excellence in the following categories: extramural research support; previous educational experience; research interest in cancer, particularly breast cancer; diversity of research interest and, suitability as a mentor. We have made a special effort to include a significant number of clinically trained investigators (30% of the total faculty have an M.D. degree) to ensure the appropriate exposure of students to clinically relevant issues associated with breast cancer. Participation in this training grant is not viewed as exclusionary and new members will be

considered by the Training Program Executive Committee throughout the training program funding period. The members of the faculty, their departmental affiliation and a brief description of their research interest as related to breast cancer to illustrate the diversity of the faculty members and their interactions.

4. Training

In fulfillment of my doctoral training, I am in the process of finishing my course work and examinations to become a PhD candidate. I have accomplished all course work, and am now taking my comprehensive exam in order to become a PhD candidate. Besides required course work, I am now taking a scientific ethics course, participate in the Predoctoral Training Program in Breast Cancer Biology and Therapy, and regularly attend seminars.