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14. ABSTRACT

Year 04 of the Undergraduate Training Program in Breast Cancer Research has been completed successfully. In addition to the eight students funded by this grant, two additional students were recruited and funded by the Dean of Pharmacy or by individual grants. Eight different mentors were involved. In the four years of the program, 24 of the 42 participants have been women, which are underrepresented in the biomedical research community. Students carried out research in their mentors' laboratories, attended a weekly journal club, attended a weekly seminar of guest speakers, presented a formal oral report on their work, and wrote a final report.

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Introduction

This report describes progress in implementing the fourth and final year of a three-year summer undergraduate training program (that was extended at no cost to the Army BCRP to four years) in breast cancer research at Purdue University in the School of Pharmacy & Pharmaceutical Sciences and the School of Veterinary Medicine.

Body of Report

Task 1: Publicity and Notification. We contacted a number of small colleges in the vicinity of Purdue and solicited applications to the Breast Cancer Program. In addition, professional students in the Purdue Schools of Pharmacy and Veterinary Medicine were notified both by a web site and by direct class visitations. We also posted the program applications on our undergraduate research web site. This posting attracted students from other universities and in fact ultimately resulted in an offer to a student from Florida State. We felt that the best possibility of recruiting minority students was to coordinate our efforts with those of the MARC-AIM program at Purdue.

Task 2: Schedule Seminar Speakers. Speakers were scheduled for a weekly seminar in Breast Cancer Research. For these seminars, faculty participating in the program gave talks directed to the undergraduate researchers about their research relevant to breast cancer research.

Task 3: Mail Publicity and Mount Web Site. (See also Task 1.) We agreed to allow minority applicants to take part in the MARC-AIM program. Unfortunately, no MARC-AIM applicants expressed interest in the program. As noted in Task 1, applications were made available through the Undergraduate Research Web Site.

Task 4: Accept and Review Applications. Applications were accepted through February 24, 2005. As was the case in previous years of the program, our office was operating a more general undergraduate research program with about the same application deadlines. This program was funded by the Office of the Dean of the School of Pharmacy. This year the Dean's Program funded three additional positions and a research grant partially supported one of these positions. In other words, the Army grant funded eight positions, and the School and individual research grants funded two. *Over the past three years, the Army grant has leveraged support from other sources for a total of ten additional undergraduate research opportunities* beyond the 32 supported on this grant.

Task 5: Make Offers to Students. Prof. Riese (the co-PI) and the PI, with consultation by other participating faculty, made offers to students in mid-March. We had no indication of interest by students from the MARC-AIM program. Despite out disappointing results with minority students, it should be pointed out that 24 of the 42 participants to date have been women, which are underrepresented in the research community. The high percentage of qualified women in the Purdue Pharmacy Program (about 65%) provides fertile ground for recruiting women researchers.

Task 6a: Assign Students to Laboratories of Program Staff. This assignment was made at the time of offer; that is, students were informed with whom they would work.

Task 6b: Coordinate Assignment of Students with MARC/AIM Program. Because we had no indications in interest from MARC-AIM students, this task was not applicable.

Task 7: Complete Program Setup Tasks. Program setup tasks were accomplished prior to the start of the program on June 1.

Task 8: Preside over First Week of Program. An official meeting of students was held on June 5, 2005. Students who had not already done so were briefed on expectations for safety, seminar attendance, and reports.

Task 9: Conduct Program. The program was conducted in the manner described in the proposal. Students took part in a weekly journal club, a weekly seminar by a guest speaker, and in weekly laboratory meetings.

Tasks 10 & 11: Final Reports. Students were scheduled about one month in advance for their final reports, and a letter was sent to each describing expectations for their final written report. Each student presented a 15-minute PowerPoint seminar describing his/her work in a public seminar. Each student also provided the PI and Prof. Riese with a short report summarizing their research and evaluating the program in answer to specific questions. Summaries of the research conducted by each student are provided at the end of this report.

Task 12: Review Students' and Members Final Reports. Student final reports were due, and were received, on or before September 1, 2005.

Task 13: Survey Students about Attitudes and Career Choices. As part of the summary report, students were asked about their attitudes toward research, and whether they were contemplating a career path in research.

Task 14: Make Yearly Report to Breast Cancer Program. This report fulfills this task.

Summary Reports of Key Research Accomplishments

Names of Students, Home Institutions, and Mentors

Katie Behr (Purdue University School of Pharmacy), Prof. Ross Weatherman
Megan Fitzgerald (Purdue University School of Pharmacy), Prof. Ross Weatherman
Mohammad Hassan (Purdue University School of Pharmacy), Prof. David Riese
Kyle Hodges (Purdue University School of Pharmacy), Prof. Jo Davisson
Steve Kaverman (Purdue University School of Pharmacy), Prof. David J. Riese
Jack Laney (Florida State University Chemistry), Prof. Richard F. Borch
Michael Placzek (Elms College Chemistry), Prof. Mark Cushman
Jonathan Ritter (Wilkes University College of Pharmacy), Prof. Richard Gibbs and Prof. Marietta Harrison
Katie Seipel (Purdue University School of Pharmacy), Prof. Richard Gibbs
Zeina Shtaih (Purdue University School of Pharmacy), Prof. Chang-deng Hu

Reports of Students' Accomplishments
(These reports were provided by the students themselves with minor edits.)

Katherine E. Behr. This summer I continued working in the Weatherman laboratory, which was the same lab I had the opportunity to research in last summer. However, this summer my project was a bit different. I worked primarily with cell culture and how certain drugs affected certain cell promoters. The cells I worked with were called SKBR3 cells which were ER negative and had a high level of HER2. I transfected the cells with different promoters using lipofectamine.

The promoters I used were AP-1, ERE, CRE, and NFKB luciferase promoters. After the DNA was transfected into the cell, I tested a drug profile of ethanol, estradiol, 4OH-tamoxifen, ICI, fulvestrant, genestein, estren, and GW7604. This project was extremely relevant to breast cancer because the SKBR3 line is a great example of breast cells with aggressive malignancy. By doing all of these experiments with different promoters in this aggressive cell line, we could see which drug(s) killed more cells and therefore how we could treat breast cancer more effectively depending on cell and promoter type.

Megan Fitzgerald. This summer my project involved chemical synthesis. I utilized two different reactions in order to form the TPEE compounds I was trying to synthesize. TPEE compounds are of interest due to their structural similarity to Tamoxifen. The hope is that they may have the positive effects of Tamoxifen on the breast and bone without the unwanted effects on the uterus, cardiovascular system, and the unwanted hot flashes. I gained experience with the McMurry coupling, ether formations, the Grignard reaction, and the Friedel-Crafts reaction. I was able to make 7 compounds successfully, and I then performed further assays on them. I performed fluorescence polarization assays and a luciferase assay. The fluorescence polarization assay was to test the binding ability of four of the compounds I had made to ER alpha, and I found that my compounds bind nicely. In the luciferase assay, I was testing to see if the compounds turned on ER transcription. I found that three of the compounds were agonist while one was an antagonist. Further testing is being done in Virginia with a collaborator of Dr. Weatherman's to test their affect on blood clotting. Also two more compounds still must be synthesized, and the assays run again to compare data.

Mohammad Hassan. The ErbB family receptor includes ErbB1 (EGFR/HER1), Erb2 (HER2/Neu), ErbB3 (HER3), and ErbB4 (HER4). It has been shown that ErbB4 signaling is coupled to growth arrest and loss of ErbB4 signaling contributes to tumorigenesis. ErbB4 over-expression in breast cancer patients correlates with markers of a more favorable prognosis. One way to further verify that, and find more about the intercellular signaling, is to develop an adenoviral system that is able to deliver ErbB4 gene to breast cancer cells. To explore the structure-function relationships within ErbB4, I conducted site-directed mutagenesis experiments specifically the Q646C mutant, and I then transfected breast-cancer cells with the gene for the mutant and observed growth arrest of the cells.

Kyle Hodges. This summer I worked in the V. Jo Davisson lab. One of the drugs the lab was working with was Geldanamycin, a natural product that inhibits Heat Shock Protein 90. Specifically, the lab is working to learn more about the nature of the binding complex formed between Geldanamycin, HSP90, and various other chaperone proteins. As a part of this effort, I spent the majority of my time evaluating the use of drug-affinity resins in drug elutions used to study the Hsp90/GA complex in the GA sensitive cell line, MCF-7. I compared pull-downs done with seven different lysis recipes and also compared the effects of using different volumes of elution and incubation times. Changing these conditions affected the bands of the proteins that could be seen on electrophoresis gels and Western blots.

Steve Kaverman. This summer I worked in the lab of Dr. Riese. The lab deals a great deal with the protein ErbB4, a receptor tyrosine kinase of the EGFR family. ErbB4 is a protein that is present in normal breast tissue as well as breast cancer tissue. The ErbB4 protein has been observed to exhibit properties of a tumor suppressor. This could potentially be useful in many breast cancers. My project dealt with this protein. ErbB4 contains a Bcl2 Homology 3 (BH3) domain. This domain binds pro-survival proteins and therefore can induce apoptosis. I made four mutations this summer of ErbB4. I mutated ErbB4 at amino acid 985 and also at 990. These mutations were done in both a wild type ErbB4 background as well as a constitutively active

mutant developed by the lab. The mutations were induced using a primer for the reverse and forward direction. These primers were incubated with parental DNA of either wild type or Q646C (the constitutively active mutant) background. The DNA and primers were then run through a PCR reaction to amplify the mutated DNA. Once the mutated DNA was obtained the DNA was transformed into chemically competent E. coli. The DNA was then purified using a large-scale plasmid prep. The DNA was then sequenced to insure no point mutations occurred during the PCR reaction. The DNA was subcloned and again purified by large scale plasmid prep. Once pure DNA was obtained the DNA was transfected into Ψ2 cells. These cells were then put into selection. The DNA transfected also encodes for a drug resistance gene. When the cells are cultured in drug the cells that take up the DNA survive and those that don't die. After selection ecotropic retrovirus was harvested and cells frozen back. The retrovirus was then used to infect PA317 cells and put in selection. After selection, amphitropic retrovirus was harvested and cells frozen back. The amphitropic retrovirus was used to infect both normal and breast cancer tissue. Colony formation assays were done.

Jack Laney. This summer I worked on synthesizing small molecule inhibitors of SH2 domains. My work was strictly organic synthesis directed towards a small molecule library of inhibitors of SH2 domains. Accordingly, I ran several syntheses in parallel, so that I could hopefully wind up with several product molecules by summer's end. I got approximately half way through each synthesis, and got pure product and good product yields that my mentor (a graduate student in the lab) will be able to use later in her research. I also discovered, in literature, a new route for one of the steps in the synthesis that we had trouble with. I was not able to test the route because I found it days before leaving, but the graduate student I worked with is going to test this route to see if it works as well as the literature reported. The research that I did ties into breast cancer through SH2 domains. By inhibiting SH2 domains in breast cancer cells, the growth of the cancer is stopped and the tumor is killed. The inhibitors that we are creating in the lab will be (if they work as expected) hypoxic selective cytotoxins—meaning they will only be active in cells that lack oxygen. Since breast cancer tumors are most often solid tumors, they are a good target for hypoxic selective cytotoxins (solid tumors are hypoxic). So, ideally, the molecules I helped build this summer would bind selectively to SH2 domains in breast cancer cells as opposed to any other cells in the body.

Michael Placzek. This summer I worked in Dr. Cushman's lab with graduate student Andrew Morrell. Working with Andrew was an experience of a lifetime. I performed research on topoisomerase I inhibitors. Although the topoisomerase I inhibitors we worked with are not directly related to breast cancer, they may provide knowledge to that field. I conducted numerous experiments allowing me to gain experience and understanding of life as a graduate student in medicinal chemistry. The research I conducted was mainly organic synthesis with little biological experiments being performed. I successfully synthesized eight compounds that are being sent for top1 inhibition testing. Andrew gave me insight into organic synthesis and helped me gain the skills I need for graduate school. Through attending and speaking in journal club I was able to gain information on current breast cancer drugs such as Hsp90 inhibitors. This summer has finalized my decision on attending graduate school and has turned my area of graduate study towards medicinal chemistry. This experience has truly has changed my life.

Jonathan Ritter. Because of my interest in both chemistry and biology, I had the opportunity to work in conjunction with the Gibbs and Harrison labs. Farnesyl transferase inhibitors (FTI's) incorporated onto the Ras protein causes the protein to function differently within the cell. Ras is a protein associated with most cancers including breast cancer. Ras proteins are small GTPases that function as on-off switches, regulating cellular functions such as proliferation,

differentiation, cell growth and cellular signal transduction. Ras is mutated in 15% of cancers. These mutated Ras bind GTP and remain constitutively active. The Ras molecule remains in an "on" position. It is believed that this constant activation leads to the development to certain types of cancer. In the Gibbs lab I performed kinetic analysis on a FPP analog, "3-Methylbutenyl." This analog acts as a substrate within the farnesyl transferase enzyme (FTase). First, I elucidated the enzyme kinetics for endogenous FPP (farnesyl pyrophosphate) and later compared the 3-Methylbutenyl to my data from my previous experiment involving FPP. I found that 3-Methylbutenyl does indeed act as an efficacious substrate when compared to FPP, with approximately equal K_m values. In addition, I had the opportunity to perform some organic synthesis directed towards the preparation of a *p*-biphenyl modified FPP analogue. In Prof. Harrison's lab I was testing the ability of 3-Methylbutenyl-FPP to act as a substrate with FTase within a cellular environment. NIH3T3 (mouse fibroblast) cells were used for our experiments. First, we had to over-express the protein Ras within our cells and obtain as much farnesylated Ras a possible within our cell lines. This was done by inducing the cells with IPTG; which causes the transcription of the Ras protein. We analyzed the farnesylation of Ras through western blots and antibody recognition through fluorescence analysis. When we found our optimum concentration of IPTG where we were looking for we treated the cells with statins. Statins inhibit the formation of endogenous FPP within our cell lines. If time had allowed during the fellowship I would have been able to treat the cells with lovastatin, finding the optimum concentration where we got all unfarnesylated Ras. I would have then been able to analyze 3-Methylbutenyl-FPP by adding the analog to our cells and looking at farnesylation and thus incorporation of the analog. The research I conducted this summer has several potential pharmacotherapeutic implications for cancer treatment strategies, because Ras is a protein mutated and over-expressed in my patients with cancer. If we can stop the formation of Ras or cause it to function differently within a cancerous cell then we may be able to decrease the proliferation of the cancer cells and perhaps cause existing tumors to shrink and possibly go into remission.

Katie Seipel. In Dr. Gibbs' lab at Purdue University, I worked closely with graduate student Amanda Krzysiak in a biochemistry research area. The focus of our research was whether the substrate activity of peptides could be modulated by FPP (farnesyl pyrophosphate) analogs. One type of post-translational modification is the addition of a lipid group to a protein. An example of lipidation is the addition of the fifteen carbon isoprenoid group FPP. The addition of this group allows certain proteins to be localized to the plasma membrane, where they are involved in growth regulation in cells. This growth regulation is often out of control in cancer cells, and the hope is that by developing inhibitors or alternate substrates for the FPP enzyme, this growth regulation can be controlled. This then is expected to help stop the spread of cancer. The lab had already generated a number of analogs before I joined them in late May. My initial goal for the summer was to synthesize two alanine CAAX box analogs, CVLA and CIVA, and then screen them against the existing FPP analog library to compare the activity of the analogs with that of FPP. I began the synthesis immediately of my peptides using Solid Phase Peptide Synthesis. In the meantime, I screened the peptide fragments CVVM or N-Ras and CCVM or PRL-3 against the analog library. First, I determined whether the analogs I screened were better or worse substrates for the peptides using a substrate assay. I noted which analogs could be alternate substrates. I also performed an inhibitor assay to more closely study the inhibitor properties of certain FPP analogs which did not show substrate activity. During and after these screenings, I attempted the Solid Phase Peptide Synthesis of my alanine peptides two more times. I was unsuccessful in these attempts. However, the data from my assays of CVVM and CCVM were useful.

Zeina Shtaih. These past three months have been a period of immeasurable growth for me both intellectually and personally. The opportunity to work as an undergraduate research scientist has allowed me to learn how far one can go venturing outside his/her comfort zone and thinking and searching outside the "known" and into the "unknown." My project, this summer, entailed understanding the physical and functional interaction between Caspase-3 and ATF2. Caspase-3 is a cysteine protease involved in the apoptotic response and is a major player in committing the cell to apoptosis. It is predominately found in the cytoplasm of the cell, until it is cleaved into its active cleavage products, 17 KDa and 12 KDa subunits, which will then gain access into the nucleus and initiate DNA fragmentation and the like. As for ATF2, it stands for Activating Transcription Factor 2 and is a member of the bZip family of proteins, meaning that it has a basic rich region through which it binds the DNA, as well as a leucine zipper required for dimerization. This dimerization is necessary for it to bind the DNA and activate or repress transcription. ATF2 regulates cellular growth, differentiation, stress responses, apoptosis and transformation. While ATF2 has been shown to shuttle continuously between the cytoplasm and nucleus and be sequestered in the nucleus when bound to c-Jun, a binding partner, overexpressed ATF2 has been found to be localized mainly in the cytoplasm and lead to chemoresistance. With this information we thought of a possible interaction between ATF2 and Caspase-3. This binding may lead to two possibilities, either, sequestering both ATF2 and Caspase-3 outside of the nucleus preventing them from entering the nucleus to continue their apoptotic role, or disabling the cleavage of Caspase-3 into its active products. Both will lead to hindering apoptosis. My first goal was to test the localization of the full length Caspase-3 and its two cleavage products. To accomplish this I designed and constructed three plasmids, full length Caspase-3, 17 KDa Caspase-3 and 12 KDa Caspase-3 all fused to a fluorescent protein Venus. I then transfected Cos-1 cells with these plasmids and checked their localization at 22 hours post-transfection. I found that Caspase-3 was located in the cytoplasm and its cleavage forms located diffusely between both the cytoplasm and nucleus. The second goal was to answer the question, "Do full length Caspase-3 and its cleavage products interact with ATF2? If so, where is the interaction localized in the cell?" In order to attain interaction data between Caspase-3 and ATF2, I needed to utilize a unique tool to visualize protein-protein interaction in cells. I used the Bimolecular Fluorescence Complementation Assay (BiFC), developed by Dr. Chang-deng Hu. Using this technology, I constructed three plasmids, full length Caspase-3 and its two cleavage products 17 KDa and 12 KDa all fused to a fusion protein VN or VC. I then co-transfected Cos-1 cells with each of the constructs and ATF2 also fused to VN or VC and analyzed the interaction using BiFC at 22 hours post-transfection. The transfection results indicated interaction during cytoplasmic localization. My third goal was to see what happens to this suspected interaction when apoptotic conditions are imposed on cells. The condition we used to induce apoptosis was through the addition of hydrogen peroxide. Here we again transfected Cos-1 cells with Caspase-3-Venus, ATF2-Venus, and co-transfected ATF2-YC155 with Caspase-3-VN173. We waited 22 hours and then added 3% H_2O_2. Following four hours post-treatment, we checked florescence to find that now both Caspase-3 and ATF2 localized to the nucleus, whereas, prior to treatment they were in the cytoplasm. However, the BiFC analysis of the co-transfection of ATF2 and Caspase-3 after treatment remained localized in the cytoplasm with no movement into the nucleus. These results are very interesting as they show that this interaction is preventing the movement into the nucleus of both ATF2 and Caspase-3 during apoptosis, thus, greatly hindering the apoptotic response as both proteins are needed in the process. These preliminary results will need to be further confirmed using co-immunoprecipitation to see whether this interaction is true. I am currently performing the co-immunoprecipitation and western blot analysis in order to confirm the interaction through co-immunoprecipitation and study the activation of Caspase-3 in the absence and presence of ATF2 overexpression. I plan to

continue my work on this project throughout the school year and hope to gather exciting results that will support our hypothesis.

Reportable Outcomes

- Ten undergraduate students, each working with one of eight mentors (and two mentors in one case), carried out breast cancer research in the Summer of 2005, bringing the total number of students sponsored by the program over four years to 42.
- Two of the students were supported by the Office of the Dean of Pharmacy or individual research grants.
- All students provided favorable reviews of the program.
- Of the 42 participants for the first two years, 24 have been women. Women are underrepresented in the life science academic and research community.
- Several meeting presentations have resulted from the work of program participants:
- No new publications or presentations were reported this year, although three papers have been submitted.
- Professor David Riese II, the co-PI of this grant, attended the 4th Era of Hope meeting held June 8–11, 2005, in Philadelphia, Pennsylvania.
- As noted in last year's report, unused travel funds were re-budgeted for student support and a one-year no-cost extension of this grant has been obtained. This allowed us to offer this program for an additional year. The travel of two students was also supported by the re-budgeted funds.