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Fort Detrick, Maryland 21702-5012

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The Involvement of Human Cyr61 in Heregulin Induction of Breast Tumor Progression

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)

This fellowship initially concerned the role of the cytokine, heregulin, in the regulation of hormone receptor status in breast cancer. The mechanism by which breast cancer progresses from an ER+ to an ER- is of considerable clinical importance because some estrogen receptor-positive (ER+) breast tumors may progress to ER-negative and/or to deadly metastatic diseases. The justification for changing the project so late in the term is that Dr. Ruth Lupu, the mentor on the original award, left the LBNL shortly after I moved to the US to join the lab. Personal circumstances prevailed in my decision to change my mentor to Dr. Mary Helen Barcellos-Hoff and stay at LBNL. Recent studies in Dr. Barcellos-Hoff lab showed that depletion of transforming growth factor-beta (TGF-β), in the Tgfβ1 null mouse mammary gland leads to increased frequency of proliferating ER+ cells, and indeed increased numbers of ER+ cells as detected by immunofluorescence. Therefore, we proposed to change the focus of the award from heregulin to TGF-β, which is also known to regulate heregulin. In the last 3 months, I have shown by Western blot analysis of mammary gland extracts of wild type and Tgfβ1 heterozygotes mice that the ER level is higher in heterozygotes than wild type, consistent with the immunofluorescence data. As a continuation of this work on ER regulation, I will investigate how TGF-β1 suppresses ER using primary mouse mammary cell culture.

14. Subject Terms
breast cancer, transforming growth factor-β, estrogen receptor, ERα

15. Number of Pages
26

16. Price code
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INTRODUCTION

This report covers the period December 2001 to September 2002. This post-doctoral training grant was originally funded for the project titled “The Involvement of Human CYR61 in Heregulin Induction of Breast Tumor Progression” with Dr. Miaw-Sheue Tsai as the PI under the mentorship of Dr. Ruth Lupu. The fellowship was transferred to me in December, 2001 under the mentorship of Dr. Ruth Lupu. Because my doctoral degree is in the area of pharmaceutical chemistry, I required an introduction to the key techniques in cell culture, biochemistry, and molecular biology that are necessary to conduct research in breast cancer. Accordingly, Dr Lupu assigned me to a phytomedicines research project that was ongoing in her laboratory to enable me to master the necessary techniques while dealing with fairly familiar research questions. By so doing I would smoothly transition into the biomedical research arena. The overall goal of that ongoing project was to evaluate natural products (herbal medicines/phytomedicines) either currently in the market or about to be introduced for alternative treatment of breast cancer and menopausal symptoms [specifically for women in whom estrogen replacement therapy (ERT) is contraindicated because they have other risk factors for breast cancer].

During this training period, the experiments conducted involved testing herbal extracts for their estrogenic effects and other biological properties. I was thus able to master techniques in cell culture, as well as assays e.g. Ishikawa assay useful for evaluating the estrogenicity of a potential breast cancer chemotherapeutic agent. I also learnt several cell proliferation assays that use non-radioactive techniques for assessing cell viability & proliferation (anchorage dependent growth) as well as soft agar assay, an in vitro technique useful for evaluating anchorage independent growth of cancer cells to assess their potential tumorigenicity. Other assays learnt included ERE-luciferase reporter assay, RNAse protection assay and DPPH assay, a robust bench top assay for assessing preliminary antioxidant activity of agents. Unfortunately, my mentor Dr. Lupu left LBNL in May, 2002 but I chose to stay. Nonetheless I have summarized this project as Part I of this report.

In June 2002 I requested that my mentor be changed to Dr. Mary Helen Barcellos-Hoff at LBNL and as a consequence we requested a change in research direction. As with the original proposal, the goal of this project is study the regulation of estrogen receptor (ER) regulation in breast cancer. However I will study TGF-β rather than heregulin, because preliminary data in
mouse mammary gland have revealed a new and exciting aspect of ER regulation: the frequency
and proliferation of ER positive cells are regulated by TGF-β. Relevant to the original proposal,
TGF-β also regulates heregulin. Thus, the global goal of the proposal, i.e. to understand how
ER status is regulated, will be maintained but the focus will change from heregulin to TGF-β
and the model will change from human cell culture to mouse mammary epithelial cells. The
benefit of this project to myself as a scientist is that I have expanded my repertoire of
techniques and understanding of breast cancer biology. The benefit to the DOD Breast
Cancer Research Program is that ER populations in the human breast are known to increase
with age and in tissue at increased risk of breast cancer. Therefore understanding the
fundamental regulation of this population is imperative to understanding its dysregulation.
We requested the change in the statement of work in July, 2002. Part II of this report
summarizes the project titled “The Role of TGF-β in the Regulation of Estrogen Receptor
During Mouse Mammary Development and Carcinogenesis” and work done during the 3 months
(July –September 2002).

BODY

PART 1: Phytomedicines research project

Project: Black Cohosh (BC): A potential Herbal menopausal remedy

*Background of the research: Actaea racemosa* L., commonly known as black cohosh (BC) is a
remedy currently being taken by many women as an alternative to estrogen replacement therapy
(ERT) in order to alleviate menopausal symptoms, such as hot flashes. It is claimed to reduce the
frequency of hot flashes. However, the mechanism by which it does so is still unknown. BC has
been shown to possess estrogenic activity and recently anti-estrogenic activity was also reported
(1, 2).

We tested these extracts for estrogenic activity using the Ishikawa cell assay that measures
the estrogenic activity of compound(s) by inducing an endogenous alkaline phosphatase (AP)
enzymatic activity in the Ishikawa cell line (3). MTS cell proliferation assay was used to
determine the extracts’ effects on the *in vitro* growth of MCF-7 and MDA-MB-231 breast cancer
cells. The effects of these extracts on the anchorage-independent growth of breast cancer cells
were also assessed using the soft agar assay in which the ability of cells to form colonies would indicate tumorigenicity. At the molecular level, the ability of the BC extracts to modulate the estrogen receptor (ER) function was evaluated using the estrogen-responsive element (ERE)-luciferase reporter assay (4).

**Results:** BC extracts did not induce the transcriptional activation of the estrogen-responsive element (Figure 1), did not regulate the expression of estrogen-regulated genes in the RNase protection assay (Figure 2), had no effect on the growth of ER-positive breast cancer cells and lastly did not induce colony formation in ER-positive cells (Figure 3). These data corroborated well with the preliminary studies that had earlier been done on BC extracts in the lab. A manuscript partially based on these results was drafted and Dr Lupu is currently finalizing it for publication.

Taken together with preliminary data that had been collected earlier, this data demonstrated that no estrogen-like activity is present in any of the BC extracts tested. Therefore, BC roots and rhizomes appear safe for use as an herbal remedy for the treatment of hot flashes in menopausal women for whom estrogen replacement therapy would be contraindicated.

**Figure 1. Effects of BC extracts on Estrogen-Responsive Element (ERE)-Luciferase activity:** Cells cultured for four days in CCS were plated (100,000 per well) and allowed to attach overnight, then transfected with an estrogen-responsive luciferase reporter plasmid and an internal control plasmid PmCLV. Following transfection, cells were treated with E2 (1 nM) or BC extracts (20 μg/ml) for 24 hrs. The activity of the luciferases was measured using the Promega kit as per manufacturer’s instructions. The results are expressed as fold increase over controls.
Figure 2. a) Effects of BC extracts on ER alpha mRNA expression in MCF-7

Cells grown in CCS for 4 days were treated with the BC fractions for 24-h. Total cellular RNA was extracted using TriPure Isolation Reagent (12) and quantified by spectrometry. $[^{32}P]$-labeled ERα, pS2 or PgR riboprobes (50,000 counts/min) were hybridized with 30μg of sample, incubated at 55°C for 16h, and digested with RNAsae (10.5, 4.67, 15 μg/sample for ERα, PgR and pS2, respectively) for 30 min at 28°C. The digestion was terminated by the addition of Proteinase K (1μg/ml) and 1% SDS. Samples were then extracted with phenol, precipitated along with 10 μg yeast tRNA in absolute ethanol, and where necessary also washed with 70% ethanol. The RNA was re-dissolved in denaturing loading buffer, electrophoresed on 6% polyacrylamide gel, and the protected fragments were visualized by autoradiography.
Figure 3 (a-d): Effects of BC extracts on the anchorage-independent growth of MCF-7 (a), T47D (b) T47Dco (c) and MDA-MB-231 (d) in soft agar assay. Four days before initiation of the experiment, cells were passed into phenol red-free IMEM supplemented with 5% CCS. The assay was performed as follows. 1.5mL of 0.6% agar suspended in IMEM media was layered as the bottom layer in a 6-well plate. Cells (20,000/ml in the case of MCF-7, T47D, T47Dco, T47D V22 and MDA-MB-453, and 10,000/ml in the case of MDA-MB-231 and MDA-MB-435) were suspended in 0.35% agar mixed with BC extracts at a concentration of 20μg/ml. After an additional 14-21 days of incubation (7 days for MDA-MB-231 and MDA-MB-435) at 37°C colonies were stained with 0.5ml of 1mg/ml Crystal Violet for 24 hour, and counted using the Accu-count 2000 colony counter.
PART II “The Role of TFG-β in the Regulation of Estrogen Receptor During Mouse Mammary Development and Carcinogenesis”

Statement of Work

It is also well known that estradiol (E2) signaling through ER-α (one of the isoforms of ER) plays a central role in mammary epithelial cell proliferation. However a variety of recent studies have shown that estrogen receptor positive (ER+) cells do not proliferate. ER- cells usually proliferate and stain with proliferation markers such as Ki67 but are frequently located next to ER+ cells. Thus, although ER+ cells do not proliferate, they are necessary for proliferation, as shown by the lack of ductal outgrowth in the ER knockout mouse, and appear to regulate the proliferation of ER- cells via a paracrine mechanism.

Transforming growth factorβ1 (TGF-β) is the most potent inhibitor of human and mouse mammary epithelial cell proliferation known. Studies in our lab have shown that at estrus when there is a high rate of cell proliferation, nearly all ER+ cells co-localize with intense TGF-β staining, consistent with their non-proliferative status. It appear that TGF-β acts as a brake restraining the ER+ cells from proliferating while at the same time the ER+ cells, in response to hormonal stimulation by estrogen (E2), send out a signal to the ER- cells to proliferate. This hypothesis seems plausible given that when TGFβ1 level is reduced (as is the case in the Tgfβ1 heterozygotes), ER+ cells proliferate more as evidence by the increase in the population of ER+ positive cells compared to the same population in the wild types. Understanding the role that TGFβ plays in the proliferation of ER+ cells is important because it is known that ER+ breast cancer can progress to more aggressive ER- negative cancer that is by its nature anti estrogen resistant and is more likely to become a deadly metastatic disease. The mechanism by which breast cancer progresses from the E2-dependent phenotype to the E2-independent one is not yet fully understood and yet it is important clinically as it would help identify possible targets of intervention in the control of or halting breast cancer progression.

Specifically understanding the role of TGFβ in this process may unveil how TGFβ could be targeted in the control of breast cancer since it is well known that increased TGFβ activity is
associated with breast cancer progression (5) and can functionally mediate metastatic disease (6-8). The project will make use of Tgfβ1 heterozygote Balb/c mice as a model and will include immunohistochemistry studies, protein analysis and primary culture of mouse mammary tissue.

The specific objectives in this project are:

1) To substantiate the role of TGF-β in regulation of ER during mammary development I will determine the frequency of ER+ cells and the level of ER as a function of TGF-β activity in Balb/c mice Tgfβ1 null heterozygote and wildtype mice and compare these data to those previously obtained in the C57bl/129SV.

2) To determine whether TFGβ suppresses ER we will use primary mouse mammary epithelial and human breast cell cultures.

Preliminary work:

On embarking on this project the first task was to establish culture of MCF-7 cells in a serum free media. Our interest is to determine the level of ER in these cells and investigate how this level changes in the presence of various concentrations of TFG-β. Serum, a necessary component of regular growth media formulated to support the growth of these cells is also a rich source of TFG-β. As such it was necessary to develop a method of maintaining these cells in serum free medium in order to be able to investigate the effect of external TFG-β that will be added in the media.

I have conducted Western blot protein analysis to determine the levels of ER in TFG-β +/+ (wild type) and TFG-β+/- (heterozygotes) mouse mammary glands at different stages of the estrus cycles. In addition I have done immunohistochemistry studies to determine the distribution of proliferating and ER+ cells in wild type and heterozygotes.

Results

Total protein lysates were prepared from mammary gland (MG) samples obtained from five wild-type TGF-β +/+ Balb/c mice. Included in the analysis, as controls, were uterine tissue lysates as positive control for ER and of the intestine tissue lysates as a negative control, both
were obtained from the same a wild-type Balb/c mice. The lysates were subjected to Western blot analysis and immunodetection for ER. The results (Figure 4) show that ER in the MG was about 10× less than that found in the uterus.

In a separate experiment we compared two extraction methods that were available in the laboratory, to determine which would give a suitable protein extract in which we would be able to detect ER using the less protein than reported in most research papers. The two protocols are arbitrarily denoted Sh and R in this discussion. The extractions were performed simultaneously in duplicate tissues and, the protein concentration in the lysates, determined by Bio-Rad DC protein assay method. Comparable protein concentrations were obtained with both protocols and total yield was also the same. There was a slight difference in the protein profiles in Sh and R protocols and a marked difference in the immunodetection sensitivity for ER when smaller amounts of proteins (20μg) were analyzed, with the R protocol proteins being more sensitive compared to Sh protocol proteins (Figure 6). We will therefore use the R protocol for all future protein extracts for ER detection.
ER alpha

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<td>MG (20)</td>
<td>MG (20)</td>
<td>Int* (10)</td>
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<td>MG (20)</td>
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<td>Mice Strain</td>
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<td>Balb/c</td>
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<td>Balb/c</td>
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<td>Int*: Intestine</td>
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<td>UT**: Uterine</td>
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ER alpha in mammary gland lysates

![Graph showing intensity of bands for different samples](image)

**Figure 4** Densitometry of digitized films from Western blot analysis of Mammary Gland lysates. The mean intensity for the four Balb/c MG lysates was 1.5 X10^6 units and the standard deviation was 16% of the mean value. In this experiment, our positive control shows a band at 67kD corresponding to the ER-α. The intestine lysate used as a negative control showed no band at this position.
**ER Alpha in C57Bl6 mammary glands**

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<td>Beta Actin</td>
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**Figures 5:** Protein lysates were prepared using two different protocols denoted Sh and R. 20μg of these extracts were subjected to Western blot analysis. In Lane 1 & 3 are wild type and heterozygote lysates prepared using Sh protocol. Lane 2 & 4 are wild-type and heterozygote lysates prepared using protocol R. In both cases the level of ER is higher in the Heterozygotes than wild-type but the difference is much more pronounced in the lysates obtained using protocol R.
I confirmed that the level of ER in Tgfβ1 C57Bl6/129SV heterozygotes was higher (Figure 5) than in the wildtypes by Western blot analysis of MG lysates. This is consistent the results in the immunohistochemistry studies previously done on the mammary glands of these animals.

I have just completed my first immunostaining experiment on (TGF-β1 +/+ ) and heterozygotes (TGF-β1 +/- ) in a Balb/c background. Frozen cryosections of mammary gland on gelatin coated cover slips and were briefly brought to room temperature, fixed and blocked in supernatant from a casein/PBS solution (pH 7.4) for 60 minutes. The tissues were then incubated for a pre-determined time in primary antibodies diluted in blocking buffer after which they were sequentially incubated in secondary antibodies with fluorescein and Texas Red respectively. Nuclei are counter stained with DAPI. Images are then acquired on a Zeiss Axiovert equipped with epifluorescence.

Very few (approximately 1.5%) cells staining for ERα co-localized with Ki67 staining indicating that the ER+ cells infrequently enter the cell cycle. I will use this method in Tgfβ1 Balb/c heterozygotes to test our hypothesis that depletion of TGF-β1 leads to increased proliferation of ER+ epithelial cells.
KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research and training.

Part I of this report: Mastered the following techniques

- Ishikawa assay that can be applied to assess the estrogenicity of test agents,
- MTS cell proliferation assay, a robust non-radioactive techniques for assessing cell proliferation (anchorage dependent growth) and viability cells,
- Soft Agar assay, an in vitro technique useful for evaluating anchorage independent growth of cancer cells to assess the potential tumorigenicity.
- The ERE-luciferase reporter assays
- The RNase protection assay
- DPPH assay a robust bench top assay for assessing preliminary antioxidant activity.

Part II of this report: The ongoing research

- Established MCF-7 culture in serum free media.
- Have established a western blot protocol for the analysis of ER in mouse mammary glands and ready to go on to assessing the experimental samples.
- Have mastered the Immunohistochemistry techniques

REPORTABLE OUTCOMES:

Outcomes that have resulted from the research in Part I of this report include:

1) Manuscript titled “Black Cohosh: A Menopausal Herbal Remedy Does Not Have Estrogenic Activity” This was drafted and is currently being finalized by Dr Ruth Lupu for publication.

2) Abstract No 134: Titled “Black Cohosh (Cimicifuga racemosa) does not have any estrogenic activity” (attached as Appendix I) for a poster presented at the International Scientific Conference on COMPLEMENTARY ALTERNATIVE & INTERGRATIVE Medicine Research that took place in Boston in April 12-14, 2002 at the Boston Marriot Copley Place, Boston MA.
CONCLUSIONS

For Part I of this report: During my training period the data was generated on two Phytomedicines, namely, Black Cohosh (Cimicifuga racemosa) and on the herbal components of a modified Chinese herbal formula. These data are additions to the body of knowledge on phytomedicines which is still only scanty for many of the natural/phytomedicines already available in the market and being used by many American women as neutraceuticals or as alternative therapies for breast cancer treatment and to alleviate menopausal symptoms. Although many such products are readily available for over the counter dispensing and, in health food stores, they are not necessarily as safe as believed and any additional information that can be obtained through rigorous scientific research would be valuable in evaluating the efficacy and potential toxicity of these products.

For Part II of this report: To date, my preliminary data conforms to the hypothesis that TGF-β is restraining ER+ cells from proliferating. Further work will concentrate on specific roles of this cytokine at different stages of mammary gland development and during the initiation, and progression of carcinogenesis.

If ER-positive and anti-estrogen responsive breast tumors can spontaneously progress to an ER-negative and anti-estrogen-resistant phenotype, thereby becoming deadly metastatic diseases, then the mechanism by which breast cancer appears to progress from an ER-positive to an E2-negative phenotype is of considerable clinical importance. Our studies investigating the role of TGF-β hopefully will shed some light on this process.

At a personal level this DOD BCRP post-doctoral fellowship has enable me to learn and master skills and techniques as well as acquire knowledge in the area of breast cancer research. Specifically I have been introduced to techniques in the biochemistry, cell biology, molecular biology and animal physiology that are essential for cancer research and will greatly strengthen my capability to do research. Together with my training in pharmacognosy I hope to be able to better contribute in breast cancer research from this perspective.

Being at LBNL through the support of this fellowship has also provided me the opportunity to meet with other breast cancer researchers and learn about different areas of research that have greatly widened my knowledge in this subject.
REFERENCES


APPENDICES:

Appendix I:

Abstract: Black cohosh (Cimicifuga racemosa) does not have any estrogenic activity.

Appendix II: Curriculum vitae for the trainee: Hellen Oketch-Rabah, Ph.D
Black cohosh (Cimicifuga racemosa) does not have any estrogenic activity

HA Oketch-Rabah, L Meheini, MS Tsai, E Adas, E Kennedy, P Nuntanakorn, F Kronenberg, R Lupu
Presenting Author: HA Oketch-Rabah

Purpose: Black cohosh (BC) is currently being taken by many American women to alleviate menopausal symptoms such as hot flashes. Estrogen, the primary treatment for hot flashes, is not recommended for women at high risk for breast cancer, or for breast cancer patients and survivors. The mechanism by which BC reduces the frequency of hot flashes is still unknown. The goal of our studies was to determine whether there is any estrogen-like activity in extracts derived from BC, and to determine the safety of BC for women who should not take or choose not to take estrogen. The black cohosh extract used in these studies is currently under clinical trial for hot flashes at Columbia University. Methods: a) Extract preparation: Extracts from BC roots and rhizomes were made in hexane (BC1), ethyl acetate (BC2) and water (BC3), by sequential solvent-solvent partitioning of an aqueous-methanol BC crude extract, b) Ishikawa cell assay: Estrogenic activity was determined by the Ishikawa cell assay that measures the estrogenic activity of a compound (s) by inducing endogenous alkaline phosphatase (AP) enzyme activity, c) Transcriptional activation assay: At the molecular level, we tested the ability of the BC extracts to modulate the estrogen receptor (ER) function as evaluated using the ERE-luciferase reporter assay, d) RNAse Protection assays (RPA): We assess the extracts' ability to regulate the mRNA expression of E2-regulated genes, ER-α, PgR and pS2. These genes are regulated by synthetic estrogen and by genistein in ER-expressing breast cancer cells, e) Anchorage-dependent and -independent growth assays: We tested the ability of extracts derived from BC to induce the growth of breast cancer cells in anchorage-dependent and -independent assay. ER+ breast cancer cells were used for these assays. The assays were performed using concentrations of 0-20μg/ml. Results: In the Ishikawa cells based assay, the BC extracts did not enhance the AP activity, indicating no estrogenic activity. In addition, none of the BC extracts induced either ERE activity or regulation of known estrogen-regulated genes. By contrast, the synthetic estradiol (E2) significantly increased ERE activity and regulated the expression of E2-regulated genes in breast cancer cells that express ER. Finally, we demonstrated that neither extract of BC had estrogenic effect on the growth of ER-expressing breast cancer cells. Control E2 significantly induced cell proliferation and colony formation. Conclusion: All of our results determine that no estrogenic activity is present in any of the BC extracts tested in our laboratory. Therefore, BC roots and rhizomes appear safe for use as an herbal remedy for the treatment of hot flashes in women for whom
RESUME
Hellen A. Oketch-Rabah, PhD

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Berkeley, CA 94720, U.S.A.

Email: HAOQrabah@lbl.gov

RESEARCH INTERESTS:
My research Interest is in breast cancer research. Specifically understanding the biology of the disease and prospecting in the Plant Kingdom for natural compounds (secondary metabolites) with anticancer properties. Particularly interested in the development of partially purified phytomedicines for the treatment of breast cancer as well as pure natural compounds that could be developed into drugs for the treatment of breast cancer and female conditions such as post-menopausal symptoms.

EDUCATIONAL BACKGROUND:

1993-1996  
Philosophy Doctorate, Pharmacognosy
Royal Danish School of Pharmacy-DENMARK

Feb.-June, 1993  
Diploma in Research Methodology- DENMARK

1988-1992  
Master of Science, Plant Biochemistry and Physiology
Kenyatta University-KENYA

1983-1986  
Bachelor of Education, Science.
Kenyatta University -KENYA

1980-1981  
East African Advanced Certificate of Education
Limuru Girls' School-KENYA

1976-1979  
East African Certificate of Education
Kapsabet Girls' High School-KENYA

1 of 7
Hellen Oketch-Rabah, CV  
10/07/2002
### RESEARCH AND TEACHING EXPERIENCE:

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<td>Senior Lecturer</td>
<td>Department of Pharmacology and Pharmacognosy Faculty of Pharmacy, University of Nairobi</td>
</tr>
<tr>
<td>1997 Nov-2000</td>
<td>Lecturer</td>
<td>Department of Pharmacology and Pharmacognosy Faculty of Pharmacy, University of Nairobi</td>
</tr>
<tr>
<td>1986-1987</td>
<td>High School Teacher</td>
<td>Kisumu Girls' High School</td>
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### Teaching Experience:

2000-May 2001 **Senior Lecturer** of Pharmacognosy in the Department of Pharmacology and Pharmacognosy, Faculty of Pharmacy at the University of Nairobi.

Duties: teaching, conducting tutorial and lab classes, supervising student projects at undergraduate and graduate level, and examining Pharmacy undergraduate students in Pharmacognosy.

Administrative: Acting Chairman of Department in the absence of the Chairman. Attending faculty board meetings.

1997-May 2001 **Part-Time Lecturer** of Pharmacognosy at the Medical Training college (MTC) in Nairobi. Teaching Pharmacy Technician trainees.

1997-April 00 **Lecturer of Pharmacognosy** in the Department of Pharmacology and Pharmacognosy, Faculty of Pharmacy at the University of Nairobi.

1998-1999 External project supervisor for two Master of Pharmacy students at the Royal Danish School of Pharmacy in Denmark.

### Student Supervision:

**Undergraduate final Year B.Pharm students:**

<table>
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<tr>
<td>Catherine N. Mburu (1999)</td>
<td>A Literature Review, Activity and Composition of <em>Mondia whitei</em></td>
</tr>
</tbody>
</table>

*Hellen Oketch-Rabah, CV 10/07/2002*
RESEARCH ACTIVITIES

1997-1999
Research Project Proposal Development
Formulated several research project proposals, most of which have been funded as detailed in the sub title "RESEARCH GRANTS". We currently have a research group in the faculty of Pharmacy, constituted of members of the Department of Pharmacology & Pharmacognosy and the Department of Pharmaceutical Chemistry actively researching on Botanical antimalarials.

Research activities
Established a malaria parasite culture for use in the in vitro testing for antimalarial activity. Ethnobotanical and clinical surveys are ongoing to help select potential antimalarial plants. Several compounds with antimalarial activity have already been isolated from Maytenus senegalensis and Todalia asiatica.

1993-1996 Did my Philosophy Doctorate studies by course work and research. My research project was entitled "Antimalarial and Antileshmanial Compounds from Kenyan Medicinal Plants. The research involved, collecting ethnopharmacological information, screening of crude plant extracts of different polarities for activity against the parasites causing the two diseases. The most active extracts were subjected to bioactivity-guided fractionation to isolate the compound(s) responsible for activity. spectroscopic studies were then carried out to structurally identify the isolated compound(s). The in vitro antiplasmodial and antileishmanial activity of the isolated compounds was also determined.

1988-92 As a research Scientist at the National Museums, my duties included developing Research proposals for funding of Departmental projects. Screening plants for in vitro Antiprotozoal, antibacterial, molluscicidal and cytotoxic activity. Phytochemical screening of plant extracts using TLC and HPLC in order to identify the compounds responsible for bioactivity in the plant extracts.

RESEARCH GRANTS:

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<th>Foundation/ Year</th>
<th>Project</th>
<th>Amt. US $</th>
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<tr>
<td>EarthWatch (1999 to date)</td>
<td>Medicinal Plants of Kenya (Field work only)</td>
<td>50,000.00</td>
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<td>WHO/TDR(2000, April Year 2)</td>
<td>Botanical antimalarial drugs Research</td>
<td>84,500.00</td>
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<td>DFG German Foundation (July1999)</td>
<td>Bioactive compounds from the Kenyan Flora</td>
<td>65,000.00</td>
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<td>WHO/TDR(1999, June)</td>
<td>Botanical antimalarial drugs Research</td>
<td>78,000.00</td>
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PUBLICATIONS:


Hellen Oketch-Rabah, CV 10/07/2002


**Theses**


**Articles in Preparation**


2) Rasonaivo P. and H.A. Oketch-Rabah *"Pre-clinical considerations on antimalarial phytomedicines. Part I: efficacy evaluation". In preparation.*


Publications in Conference Proceedings


Workshops and Conferences attended & papers presented

1) Black Cohosh (*Cimicifuga racemosa*) does not have any estrogenic activity A poster presented at the International Conference on COMPLEMENTARY ALTERNATIVE & INTERGRATIVE Medicine Research that took place in Boston in April 12-14, 2002 at the Boston Marriot Copley Place, Boston MA.


3) Malaria Research and Reference Reagent Resource Center (MR4) workshop Handling and Managing Biological Materials. March 2-4,2000 in Ouagadougou, BURKINA FASO.


5) Research Initiatives on Traditional Antimalarial Medicines (RITAM), 8th-11th December 1999 in Moshi, Tanzania.TARGETS FOR RESEARCH: Old medicines a potential source of new drugs *Representing Botanical Antimalarial Drug Development- the Kenya Group.*


9) 8th Natural Products Research in East and Central Africa, NAPRECA Symposium, 8-13th August, ’99, Gabarone, BOTSWANA. Preliminary in vitro antiplasmodial activity of some plants extracts used traditionally in Kenya for the treatment of malaria

10) IDRC workshop, “What works in Development”, April, 1999, Nairobi, KENYA. What can the Research Scientists Contribute in the developing countries?


12) IIIrd Pan-African Malaria Meeting, Nairobi, Kenya, 21-14 June 1998., Nairobi, KENYA. Medicinal Plants and Traditional Medicines: Can they contribute in the malaria control?


15) Regional Workshop on Medicinal Plants and Traditional Medicine in Cape Town, South Africa, 14-18 April, 1998. Participatory Research and Involvement of local Communities and Traditional Healers: Potential benefits and pit falls.

16) Ph.D. Lecture (viva) at The Royal Danish School of Pharmacy, 5 December 1996. Kenyan Medicinal Plants: A source of new Antiprotozoal Compounds.

17) Lecture at Copenhagen Drug Resistance meeting, September 1996. Antimalarial compounds from Kenyan Medicinal Plants.


19) Presented at Scientific meetings at the Copenhagen University Hospital in July and August 1996. Antileishmanial and Antiplasmodial activity of compounds from Asparagus africanus and Vernonia brachycalyx

20) Lecture at NAPRECA Summer School in Madagascar, September 1995. A search for Antileishmanial compounds from medicinal plants.

OTHER COURSES:

1) 1993-1996
   (DENMARK)

   Courses during the Ph.D. study at the Royal Danish School of Pharmacy

   Department of Medicinal Chemistry
   i) Interdisciplinary course on research theory and research methods
   ii) Sample preparation and separation techniques in bio-analytical chemistry
   iii) Teaching and learning: Theory and practice

   6 of 7
iv) Academic writing in English

2) Mar-June 1993 Diploma in Res. Methodology at Danish Bilharziasis Laboratory (DBL) in Denmark.

i) Epidemiology and Control of Tropical Vector-borne diseases
Research design, implementation and evaluation.

ii) Project Management: Capabilities for project planning and reporting with emphasis on Logical Framework Approach (LFA); Introduction to personal computers (word processing and graphic programs); literature search (computerized literature databases and handbooks; budgeting, contract design and book keeping, project budgets); report preparation; oral presentations.

iii) Data management and statistics: Theory and applications of statistical methods in handling of scientific data; use of SPSS/PC+ and other computer graphic packages.

iv) Research program planning: Preparation of project proposals and project implementation plans (objectives, methods, sample size, recording forms, budgets, etc.)


Membership to SOCIETIES
1. Member of American Society of Pharmacognosy (ASP).
2. Third World Organization for Women in Science (TWOWS).

REFERENCES

Available on request
centrations was used as a positive control. After 48 hours, cellular proliferation was assessed by measuring the conversion of formazan dye from a tetrazolium salt by mitochondrial dehydrogenases in the cells. Both cell lines and RYR extract with significant inhibition of proliferation (p < 0.01). We conclude therefore Chinese Red Yeast Rice has the potential to have anticancer activity when used as a dietary supplement.

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151 Pharmacological actions of a Chinese herbal formula used for seasonal allergic rhinitis (SARF) GB Lenon, Xue CCL, Xue CG Li
Presenting Author: GB Lenon
A Chinese herbal medicine formula has been proven its effectiveness in relieving symptoms of seasonal allergic rhinitis SAR during randomised clinical trial (Xue et al., 2000). However, the mechanism of the actions of this SARF formula (SARF) has not yet elucidated. In this study, we investigated the effect of SARF on responses induced by various agents in vitro. In isolated tracheal preparations from rat or guinea pig, the responses to acetylcholine (10 μM), carbachol (1μM), substance P (0.1-10μM), 5-HT(μM), prostaglandin E2, leukotriene C4 or histamine (0.1-30μM) were significantly affected by SARF (0.04-1.0ng/ml). In contrast, contractions elicited by compound 48/80 (25μg/ml) in both tissues were significantly inhibited by SARF. The responses in the presence of SARF (0.4 μg/ml) were reduced to 36.4 ± 25.9% (n=5) and 36.4 ± 25.9% (n=5) for rat and guinea pig, respectively. In isolated rat aortic ring preparations, responses to endothelium dependent and independent relaxants acetylcholine and nitric oxide (NO) derived from nitroprusside (SNP) respectively were not significantly affected by SARF. However, the release of leukotriene B4 (LTB4) induced by calcium ionophore in porcine neutrophils was also significantly inhibited by SARF (the release of LTB4 was reduced to 17.9 ± 4.1% (n=5) compared to the control response of 29.2 ± 5.7% (n=12). In addition, the release of leukotriene B4 (LTB4) induced by calcium ionophore in porcine neutrophils was also significantly inhibited by SARF (the release of LTB4 was reduced to 17.9 ± 4.1% (n=5) compared to the control response of 29.2 ± 5.7% (n=12). The addition of 10% LTB4 did not change the control response of 29.2 ± 5.7% (n=12). In addition, the release of leukotriene B4 (LTB4) induced by calcium ionophore in porcine neutrophils was also significantly inhibited by SARF (the release of LTB4 was reduced to 17.9 ± 4.1% (n=5) compared to the control response of 29.2 ± 5.7% (n=12). The addition of 10% LTB4 did not change the control response of 29.2 ± 5.7% (n=12). The addition of 10% LTB4 did not change the control response of 29.2 ± 5.7% (n=12).

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Black cohosh (cimicifuga racemosa) does not have any estrogenic activity
HA Okech-Babah, I. Memehi, MS Tsai, E. Atlas, E. Keminly, P. Nuanamfon, F. Kronenberg, R. Lupu
Presenting Author: HA Okech-Babah
Purpose: Black cohosh (BC) is currently being taken by many American women to alleviate menopausal symptoms such as hot flashes. Estrogen, the primary treatment for hot flashes, is not recommended for women at high risk for breast cancer, or for breast cancer patients and survivors. The mechanism by which BC cells at the frequency of hot flashes is still unknown. The goal of our studies was to determine whether there is any estrogen-like activity in extracts derived from BC, and to determine the safety of BC for women who should not take or choose not to take estrogen. The black cohosh extract used in these studies is currently under clinical trial for menopausal hot flashes in lipids and cholesterol levels in a Canadian University. A) Extract preparation: Extracts from BC roots and rhizomes were made in hexane (BC1), ethyl acetate (BC2) and water (BC3), by sequential solvent-solvent partitioning of an aqueous-methanol BC crude extract. B) Ishikawa cell assay: Estrogenic activity was determined by the Ishikawa cell assay that measures the estrogenic activity of a compound (s) by inducing endogenous alkaline phosphatase (AP) enzyme activity, C) Transcriptional activation assay: At the molecular level, we tested the ability of the BC extracts to modulate the estrogen receptor activation (ER) function as evaluated using the ER-luciferase reporter assay, D) RNase Protection assay (RPA): We assess the extracts' ability to regulate the mRNA expression of ERα-regulated genes, ERα, Pgr, and PR. These genes are regulated by estrogenic activity and by genistein in ER-expressing breast cancer cell lines. The estrogenic activity in this assay was normalized to the ability of extracts derived from BC to induce the growth of breast cancer cells in anchorage-dependent and -independent assay. ER+ breast cancer cells were used for these assays. The assays were performed using concentrations of 0-200ng/ml. Results: In the Ishikawa cells based assay, the BC extracts did not enhance the AP activity, indicating no estrogenic activity. In addition, none of the BC extracts induced either ERα activation or regulation of known estrogen-regulated genes. By contrast, the synthetic estradiol (E2) significantly increased ERα activity. In our regulation assay, the ERβ activity is present in any of the BC extracts tested in our laboratory. Therefore, BC roots and rhizomes appear safe for use as an herbal remedy for the treatment of hot flashes in women for whom estrogen therapy presents a risk.

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155 Electrocupuncture stimulation of hindlimb acupuncture points induces expression of c-fos protein in the brain pathways
Xiao-Xue Zhang, Sheng-Xiong Ma, Xue-Yi Li
Presenting Author: Sheng-Xiong Ma (UCLA School of Medicine)
Purpose: The expression of immediate early gene, c-fos, has been used to map the distribution of brain neurons activated by stimulation, and Fos-like immunoreactivity (FLI) serves as a marker of neuronal activity to trace the neuronal pathway. We have recently observed that the neuronal nitric oxide synthase expression is predominantly increased in the gracile nucleus with electrosensory (EA) stimulation of hindlimb acupuncture points (acupoints) in rats. The gracile nucleus receives peripheral somatosensory afferent inputs projecting from the hindlimb. Gracile-basal ganglia pathway plays an important role in the central modulation of some sympathetic cardio vascular functions. In the present study, we examined the influence of EA stimulation on the expression of c-fos in the brainstem, thalamus, and cortex by using an immunohistochemical technique. Methods: Low-frequency EA stimulation (3 Hz) was applied to the hindlimb acupuncture points (Jinggou and Shugu (BL 64-65), in rats anesthetized with ketamine. Rats in the sham-treated group received surgical and EA needle electrode placements, but not the acupuncture points without performing the stimulation. After the photocell stimulation and sham-treatment, the animals were perfused with 4% paraformaldehyde. Sections of rat brain were examined by immunolabeling with a polyclonal antibody directed against c-fos. Results: Unilateral EA stimulation of hindlimb acupuncture points (acupoints) produced a significant increase in the number of c-fos immunopositive cells in the gracile nucleus, but not in the substantia nigra, ventral tegmental area (VTA), or hypothalamus. The number of c-fos immunopositive cells in the gracile nucleus was significantly higher in the EA stimulation group than in the sham-treatment group (P<0.05, n=4). The number of c-fos immunopositive cells in the substantia nigra, ventral tegmental area (VTA), and hypothalamus was not significantly different between the EA stimulation and the sham-treatment groups. Conclusion: EA stimulation of hindlimb acupuncture points (acupoints) produced a significant increase in the number of c-fos immunopositive cells in the gracile nucleus, but not in the substantia nigra, ventral tegmental area (VTA), or hypothalamus. The number of c-fos immunopositive cells in the gracile nucleus was significantly higher in the EA stimulation group than in the sham-treatment group.