AD_____

Award Number: DAMD17-99-1-9143

TITLE: Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

PRINCIPAL INVESTIGATOR: Dr. Robert Wells

CONTRACTING ORGANIZATION: Colorado State University
Fort Collins, Colorado 80523-2002

REPORT DATE: September 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Annual (1 Sep 00 - 31 Aug 01)

4. TITLE AND SUBTITLE
Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

5. FUNDING NUMBERS
DAMD17-99-1-9143

6. AUTHOR(S)
Dr. Robert Wells

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Colorado State University
Fort Collins, Colorado 80523-2002

E-Mail: robwells@colostate.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Melatonin and breast cyst fluids (BCFs) both exert antiproliferative effects on breast cancer cells that may be mediated by growth factors. The primary objectives of this study are to establish a clinical BCF sample bank among patients with gross cystic breast disease to study of the relationship between this disease, melatonin and related growth agents in BCF, and breast cancer risk. BCF samples from participating patients will be used to elucidate the contribution of melatonin and related growth agents (EGF, TGF-beta, DHEA-S) to the oncostatic effects of BCFs on MCF-7 human breast cancer cells. Our progress to date includes the establishment of a BCF sample bank and continued patient enrollment in year 2. Assay procedures were established for each biochemical analyte specified in the statement of work and BCF samples were analyzed for: melatonin, sodium, potassium, TGF-beta 1, TGF-beta 2, EGF, and DHEA-S. Preliminary time course, dose-response, and repeatability experiments were initiated using BCF to treat MCF-7 cells. A protocol was established for exposing MCF-7 cells with BCF and treatments were performed using samples from recruited patients. In summary, cell culture experiments using BCF samples and corresponding biochemical composition assays continued in year 2 and will progress into year 3.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Melatonin, Gross Cystic Breast Disease, MCF-7 Cells	116
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Long term exposure of breast tissue to estrogen plays a major role in breast tumor formation, although risk factors linked with chronic estrogen exposure only account for a portion of disease occurrence. Other hormones and growth factors are likely to be related to unidentified breast cancer risk factors. The pineal hormone, melatonin, exerts antiproliferative and anticarcinogenic effects via several proposed mechanisms, including the modified secretion of growth factors that are critical for breast tumor development. Prior research suggests that women with high endogenous melatonin levels may have lower breast cancer risks and that melatonin may be an effective therapeutic agent for breast cancer treatment. Women with palpable breast cysts have a 2-6 fold increased risk of developing breast cancer. Despite the identification of several growth agents in breast cyst fluid (BCF) that appear to segregate based on electrolyte (Na,K) composition, it is still unclear what role BCF plays in breast tumor development. BCF exerts an inhibitory effect on the proliferation of breast cancer cells *in vitro*. We hypothesize that this effect is due to the presence of melatonin in BCF, which stimulates production of the growth inhibitory cytokine, transforming growth factor-beta (TGF-beta), in human breast cancer cells. The two major goals of this study encompass clinical and laboratory-based activities. The clinical effort will establish a cyst fluid sample bank to evaluate the relationship between melatonin in BCF and breast cancer risks. The laboratory effort will use the BCF samples to elucidate the contribution of melatonin to the oncostatic effects of cyst fluids in a breast cancer cell model. The relationship between melatonin and specific growth factors in BCF will also be assessed. The specific aims are to: 1) create a BCF sample bank among patients with gross cystic breast disease in order to perform a prospective study of the relationship between gross cystic breast disease, melatonin and related growth agents in BCF, and breast cancer risk; 2) determine melatonin, TGF-beta, epidermal growth factor (EGF), dehydroepiandrosterone-sulfate (DHEA-S), and electrolyte (Na,K) concentrations in BCF samples; 3) perform cell proliferation studies in MCF-7 breast cancer cells treated with BCF and compare growth rates with melatonin and growth factors in BCF; 4) determine whether TGF-beta synthesis is enhanced in BCF-treated MCF-7 cells and whether the enhancement is related to melatonin levels in the BCF; 5) determine the relationship between melatonin and growth agents (EGF, TGF-beta, DHEA-S) in BCF; 6) quantify Na and K concentrations in BCF to determine whether low breast cancer risk BCF (high Na:K ratio) contains

elevated levels of melatonin and TGF-beta, and reduced concentrations of EGF and DHEA-S; and 7) determine whether melatonin in BCF is associated with known risk factors for breast cancer in patients with gross cystic breast disease.

BODY

The statement of work for this project includes four tasks. The major objectives of each task are as follows:

> Task 1: Establish a sample bank for breast cyst fluids in patients with gross cystic breast disease (Specific Aim 1);
>
> Task 2. Determine melatonin, TGF-beta 1 and 2, EGF, DHEA-S, and electrolyte (Na,K) concentrations in breast cyst fluid samples (Specific Aim 2);
>
> Task 3. Perform cell proliferation studies in MCF-7 breast tumor cells treated with breast cyst fluids in order to compare cell growth effects with melatonin and growth factors in BCF (Specific Aims 3 and 4);
>
> Task 4. Perform biostatistical data analyses to: determine the relationship between melatonin and growth-related substances in BCF (Specific Aim 5); determine whether low breast cancer risk (high Na:K ratio) BCFs contain higher levels of melatonin and TGF-beta and lower levels of EGF and DHEA-S (Specific Aim 6); and determine whether melatonin in BCF is associated with known risk factors for breast cancer in patients with gross cystic breast disease (Specific Aim 7).

Each of the four tasks described above has been initiated and is on-going according to the study design. A sample bank for breast cyst fluids from patients with gross cystic breast disease has been established (Task 1). Biochemical protocols for each BCF analyte have been established and BCF assays are on-going (Task 2). Cell culture conditions for treatment of MCF-7 cells with BCF samples have been determined and laboratory experimentation using BCF is on-going (Task 3). Tasks 1-3 will remain on-going until patient recruitment, sample collection, and laboratory experimentation has been completed. Task 4, which focuses statistical data analyses, has also been initiated. Computerized data bases have been developed that compile BCF biochemical assay and cell proliferation results as well as questionnaire data. To summarize, each task described in the statement of work has been initiated and specific subtasks have either been

completed or remain on-going in accordance with the study design. A more detailed description of work completed in Year 2 is provided below.

TASK 1

There were four subtasks established under Task 1 (1A-1D). The objective of Task 1A was to obtain final approval from institutional review boards (IRBs) for proposed human informed consent procedures. Approval of the informed consent procedures for this study from the USAMRMC RCQ, Colorado State University, and the University of Washington Medical School (UW) was obtained and patient recruitment was initiated in Year 1. Task 1B required the establishment of protocols for BCF handling, storage, shipment, and record keeping. This task was completed in Year 1. Tasks 1C and 1D called for the initiation of patient recruitment and shipment of BCF samples to CSU. Both of these tasks were initiated in Year 1 and remain in progress as described below.

It was apparent in Year 1 that subject recruitment and BCF sample collection was not proceeding at an acceptable rate. Therefore, we identified two additional subject recruitment sites, the University of Colorado Health Sciences Center (UCHSC) in Denver, Colorado (Tina Finlayson, MD, On-site Coordinator) and the Breast Diagnostic Center at Poudre Valley Hospital (PVH) in Fort Collins, Colorado (Winfield Craven, MD, On-site Coordinator). Consent forms for these sites were approved by the UCHSC, PVH, and CSU IRBs by November, 2000. We then contacted the USAMRMC RCQ for instructions on how to modify our human subjects protocol to add the new recruitment sites. In accordance with their instructions, a request to amend the human subjects protocol by adding two new recruitment sites was submitted to the USAMRMC RCQ for approval in December, 2000 and again using a slightly different format (addendum) in January, 2001. In February, the USAMRMC RCQ requested an entirely new human subjects protocol, modified to a) include the addition of the two new sites, b) answer questions and provide clarifications on the existing, active protocol, and c) modify the consent forms that were already approved by the other IRBs. We contacted our grant administrator at the USAMRMC to ask that the requirement for a new, revised human subjects protocol be waived since: a) the project was on-going, b) the human subjects protocol had already been approved, c) we needed to accelerate our schedule and revising the protocol would cause a delay, d) the requested modifications were simply to add recruitment sites and did not change the study design, e) the requests for the

additional sites were readily accepted by the other IRBs, and f) the project has been designated as a minimal risk study. We were instructed not to respond to the RCQ's request until a meeting was held between our grant administrator and the USAMRMC RCQ to resolve this issue. According to our grant administrator, the RCQ did not respond to repeated telephone and e-mail requests for a meeting. Finally, we submitted our response to the RCQ with a revised protocol on September 6, 2001 (Appendix A). In the interim, the IRBs at UW and UCHSC conducted their annual project reviews and made some modifications to their respective consent forms. Those modifications were submitted to the RCQ for approval along with the revised protocol. We are still awaiting a response from the RCQ regarding our revised protocol. Without approved consent forms, we have been forced to suspend patient recruitment. Also, two part-time laboratory technicians are no longer employed by this project. We have been informed that several breast cyst aspirations occur each week at each of the two new recruitment sites. Unfortunately, those BCF samples are not accessible to this study and are discarded. Thus, despite our efforts to accelerate recruitment, we have encountered unforeseen delays in obtaining USAMRMC RCQ approved consent forms. Despite these delays, we are still optimistic that once approved consent forms are in place, the objectives of this task can be met.

TASK 2

Task 2 focuses on the determination of melatonin, electrolytes (Na,K), and three other growth related agents (EGF, TGF-beta, and DHEA-S) in BCF samples. The specific subtasks were defined as follows: 2A) establish baseline assay conditions and quality control criteria for radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs); 2B) perform biochemical assays of melatonin and growth-related agents; and 2C) determine ionic composition (Na,K) of BCF samples by atomic absorption (AA) spectroscopy. Assay methods have been established for determination of melatonin, electrolytes (Na,K), EGF, TGF-B1, TGF-B2, and DHEA-S in BCF samples. The assay protocols are presented in Appendix B. Biochemical assays have successfully been performed for each of these analytes in most BCF samples collected to date (Table 1). Assays will be performed in the remaining (low volume) BCF samples next month. In summary, Task 2A has been completed and biochemical assays of electrolytes, melatonin, and related growth agents in BCF (Tasks 2B and 2C) are on-going as specified in the statement of work.

TASK 3

Task 3 delineated the following subtasks: 3A) determine initial dose-range toxicity and proliferation response relationships in MCF-7 cells, 3B) perform cell proliferation and TGF-beta response studies with BCF samples, and 3C) calculate growth indices for each cell culture experiment. Cell culture experiments to address Task 3 objectives were initiated in year 1 and continued in year 2. Year 2 work on Task 3 focused on establishing cell culture conditions for assessing the growth-related effects of BCF treatments in MCF-7 cells. Experiments were performed to: 1) assess the time course of MCF-7 cell growth in serum containing and serum free medium, 2) evaluate the concentration dependence of BCF treatments, 3) determine the repeatability of BCF treatment effects on growth, 4) assess the effect that rinsing serum from cell cultures after initial (24-hour) plating has on cell growth, 5) determine the composition of the serum used for initial plating and growth of MCF-7 cells prior to treatment with BCF with regard to the presence of melatonin, EGF, TGF-B1, TGF-B2, and DHEA-S, and 6) evaluate the relationship between cell culture passage number and MCF-7 cell proliferation in serum free and serum containing medium.

Figure 1 presents a growth curve of MCF-7 cells in serum free (SFM) or serum containing medium (regular or RM). Since fetal bovine serum (FBS) contains many of the growth promoting and inhibiting factors of interest for this study, we investigated the growth of the cells in their absence, i.e. in serum free medium. It was important to establish that cells could be grown for a time in serum free medium, thus insuring that the effect of exogenously added BCF could be measured in the absence of the complicating factors found in FBS. This graph shows that MCF-7 cells can be cultured in SFM out to five days without any adverse effects. Exponential growth was observed up to around five days of treatment. At seven days however, medium depletion is evident in SFM cultures, but not in RM cultures.

Figure 1 also addresses the effect of rinsing of the cultures on subsequent cell growth. The graph shows that rinsing after the initial 24-hour plating in RM (Day 0) has little, if any effect on cell growth of the SFM cultures out to five days. We conclude that MCF-7 cells can be treated for 5 days in serum free conditions and that rinsing can be used to further remove any complicating factors found in fetal bovine serum prior to treatment of the MCF-7 cells with BCF. Thus, the effects of BCF on cell proliferation alone can be isolated under these conditions.

Another important factor to consider was the concentration dependence of BCF on cell proliferation. In some cases, BCF sample volume is small and we needed to determine the minimum concentration needed to support the growth of the cells. Figure 2 shows the results of an experiment designed to find the optimal concentrations using three different BCF samples. Cell number was measured after five days of treatment in 5%, 10%, and 16% BCF. For each sample, cell growth was inhibited after five days at all concentrations used. Furthermore, growth inhibition was maximized at 10% BCF. Using the same culture conditions, the experiment was repeated using 10% BCF and virtually identical cell counts were obtained for each BCF sample after 5 days of treatment.

We also performed experiments to monitor the relationship between passage number and cell growth. MCF-7 cells are notorious for changing growth dependency as the cells age during regular culturing of the cells. Results presented in Figure 3 indicate that at higher passage numbers, the growth of the cells may be inhibited. As a result of these experiments, we have decided to use the cells only up to passage 161. After that time, the cell properties may change enough to be reflected in a reduced growth rate.

Based on information gathered in the preceding experiments, a final protocol for cell culture conditions and BCF treatment of MCF-7 cells was developed (Task 3A) (See Appendix C). The protocol has been used to assess the growth regulatory properties of BCF samples collected to date (Tasks 3B and 3C) (see Table 1). In summary, laboratory efforts in year 2 established cell culture parameters and a protocol for the treatment of MCF-7 breast cancer cells with BCF. Assessment of the growth regulatory properties of incoming BCF samples will continue in Year 3.

TASK 4

The objective of Task 4 is to perform biostatistical data analyses to: determine the relationship between melatonin and growth-related substances in BCF; determine whether low breast cancer risk (high Na:K ratio) BCFs contain higher levels of melatonin and TGF-beta and lower levels of EGF and DHEA-S; and determine whether melatonin in BCF is associated with known risk factors for breast cancer in patients with gross cystic breast disease. Because data accrual is still on-going, it would have been impractical to conduct extensive biostatistical data

analyses. However, some activities in support of Task 4 were performed in year 2. A computer data base was developed and entry of biochemical BCF constituents data was initiated (Task 4A) (see Table 1). Other pertinent information, such as sample volume, date and time of collection, and number of previous aspirations, has also been included in the data base for more in depth analyses in conjunction with biochemical data and breast cancer risk factors. A data entry form was developed for the questionnaire using the Access computer software package and breast cancer risk factor data was digitally archived. Since Task 4 focuses on data analyses, the bulk of these activities will be performed in the final phase of this study. Computerized data bases required for the statistical data analyses required by this task were initiated in year 2 and remain on-going.

KEY RESEARCH ACCOMPLISHMENTS

- Patient enrollment continued in year 2; the addition of two new recruitment sites is forthcoming.
- Assay protocols have been established for each of the biochemical determinations to be made in BCF samples including: melatonin, sodium, potassium, TGF-beta 1, TGF-beta 2, EGF, and DHEA-S.
- Biochemical assays for each analyte have been performed on available BCF samples.
- Cell culture experiments were conducted to evaluate the time course, concentration dependence (dose-response), and repeatability of growth effects observed in BCF-treated MCF-7 cells.
- A protocol was established for the treatment of MCF-7 human breast cancer cells with BCF.
- BCF samples from recruited patients were used to treat MCF-7 cells in culture.
- Computer data bases were initiated for biochemical constituents in BCF samples and for breast cancer risk factor data obtained via questionnaire.

REPORTABLE OUTCOMES

- The human breast cyst fluid sample bank, established in year 1, was expanded in year 2 and serves as a biological resource for performing studies on the relationship between breast cyst fluid composition, gross cystic breast disease, and breast cancer risk in women.
- A manuscript was published on the anti-proliferative properties of melatonin in MCF-7 breast cancer cells (see Appendix D).

CONCLUSIONS

Breast cancer remains a leading cause of cancer mortality in women worldwide. Because the known risk factors for breast cancer only account for a portion of the disease occurrence, research is needed to determine the role of hormones and related growth agents other than estrogen that are likely to be associated with as yet unidentified breast cancer risk factors. Women with palpable breast cysts have a 2-6 fold increased risk of developing breast cancer, although studies indicate that breast cyst fluid has antiproliferative properties. Further research is needed to evaluate the role of cyst fluid composition in breast tumor development. Our research has for the first time identified the presence of the oncostatic hormone, melatonin, in breast cyst fluid and we hypothesize that melatonin is responsible for the previously observed antiproliferative effects of breast cyst fluids *in vitro*.

The primary objectives of this study are to establish a clinical breast cyst fluid sample bank among patients with gross cystic breast disease in order to study of the relationship between gross cystic breast disease, melatonin and related growth agents in BCF, and breast cancer risks in women. BCF samples from this repository will be used to elucidate the contribution of melatonin and related growth agents to the oncostatic effects of cyst fluids in a human breast cancer cell model. This resource coninues to grow as long as patients continue to be enrolled and breast cyst fluid samples are collected. In the laboratory, assay procedures were established for biochemical analytes specified in the statement of work and BCF samples were analyzed for: melatonin, sodium, potassium, TGF-beta 1, TGF-beta 2, EGF, and DHEA-S. Preliminary time course, dose-response, and repeatability experiments were completed using BCF to treat MCF-7 cells. A protocol was established for the treatment of MCF-7 human breast cancer cells with BCF and MCF-7 treatments were performed using BCF samples from recruited patients. In summary, cell culture experiments using BCF samples and corresponding biochemical composition assays were performed in year 2. Results obtained from this study will add to our understanding of breast cancer biology and help elucidate the mechanism whereby BCF and melatonin inhibit breast cancer cell growth. Information resulting from this project will be valuable for evaluating the potential predictive and therapeutic role of BCF constituents, including melatonin, in breast cancer risk.

Table 1. Biochemical Concentrations and MCF-7 Growth Indices for Breast Cyst Fluid Samples

Cyst Fluid ID#	Melatonin (pg/ml)	Na (ppt)	K (ppt)	Na:K	TGF-B1 (pg/ml)	TGF-B2 (pg/ml)	EGF (pg/ml)	DHEA-S (pg/ml)	Growth Index: CF/SFM	Growth Index: CF/RM
UW-002	73.17	14.00	16.11	0.87	23.7	20,785.8	1106.48	103.89	0.36	0.14
UW-003A	74.69	3.00	8.01	0.37	BDL	32,688.1	213.62	98.61	0.32	0.10
UW-003B	57.58	4.00	NA	NA	38.7	35,902.5	272.78	76.17	0.64	0.24
UW-004	53.25	8.00	6.00	1.33	4.1	32,778.0	31.93	63.96	0.63	0.25
UW-005	33.73	11.00	3.00	3.67	BDL	32,103.6	40.00	23.35	0.39	0.16
UW-006	29.13	13.00	9.24	1.41	54.6	57,155.0	25.39	35.15	2.42	0.43
UW-007A	25.71	9.00	2.00	4.50	78.0	8,827.3	285.37	6.17	1.31	0.07
UW-009A	38.52	20.00	5.58	3.58	20.9	3,634.7	18.68	17.76	1.45	0.51
UW-010A	32.56	2.00	0.70	2.86	167.6	1,589.3	19.55	46.20	0.44	0.17
UW-013A	93.81	3.00	NA	NA	292.4	44,270.2	0.58	41.65	1.51	0.14
UW-014A	73.37	22.00	NA	NA	BDL	1,461.1	9.20	130.00	0.82	0.14
UW-015A	111.03	17.00	NA	NA	325.4	48,611.8	188.02	35.31	1.53	0.26
FBS	1.50	NA	NA	NA	13.2	NA	BDL	0.07	NA	NA

TGF-B: Transforming Growth Factor-Beta EGF: Epidermal Growth Factor DHEA-S: Dehydroepiandrosterone-Sulfate BDL: below detection limit pg/ml: picograms per milliliter ppt: parts per trillion NA: not available FBS: fetal bovine serum CF/SFM: Total cell counts for cyst fluid treated cells divided by total counts in cells treated with serum free media CF/RM: Total cell counts for cyst fluid treated cells divided by total counts in cells treated with regular media

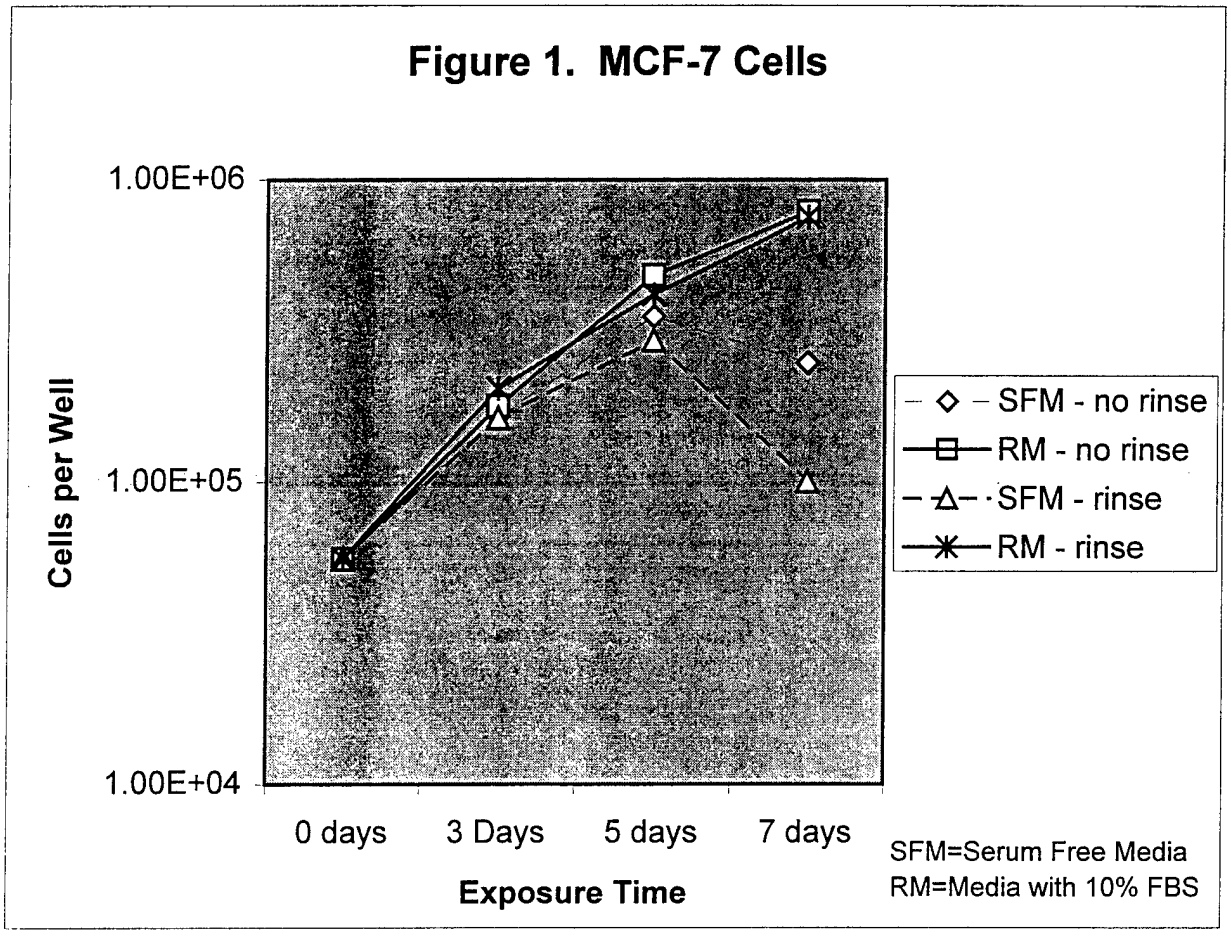

Figure 1. Growth of MCF-7 cells assessed as a function of treatment time and rinsing. Cells were plated in complete medium (RM) in six well plates at a density of 1.5 X 10^5 cells per well. 24 hours later the medium was removed, and the cultures were either rinsed or not rinsed. Serum free medium (SFM) or medium containing 10% fetal bovine serum (RM) was added back, and the cultures were incubated for the times indicated.

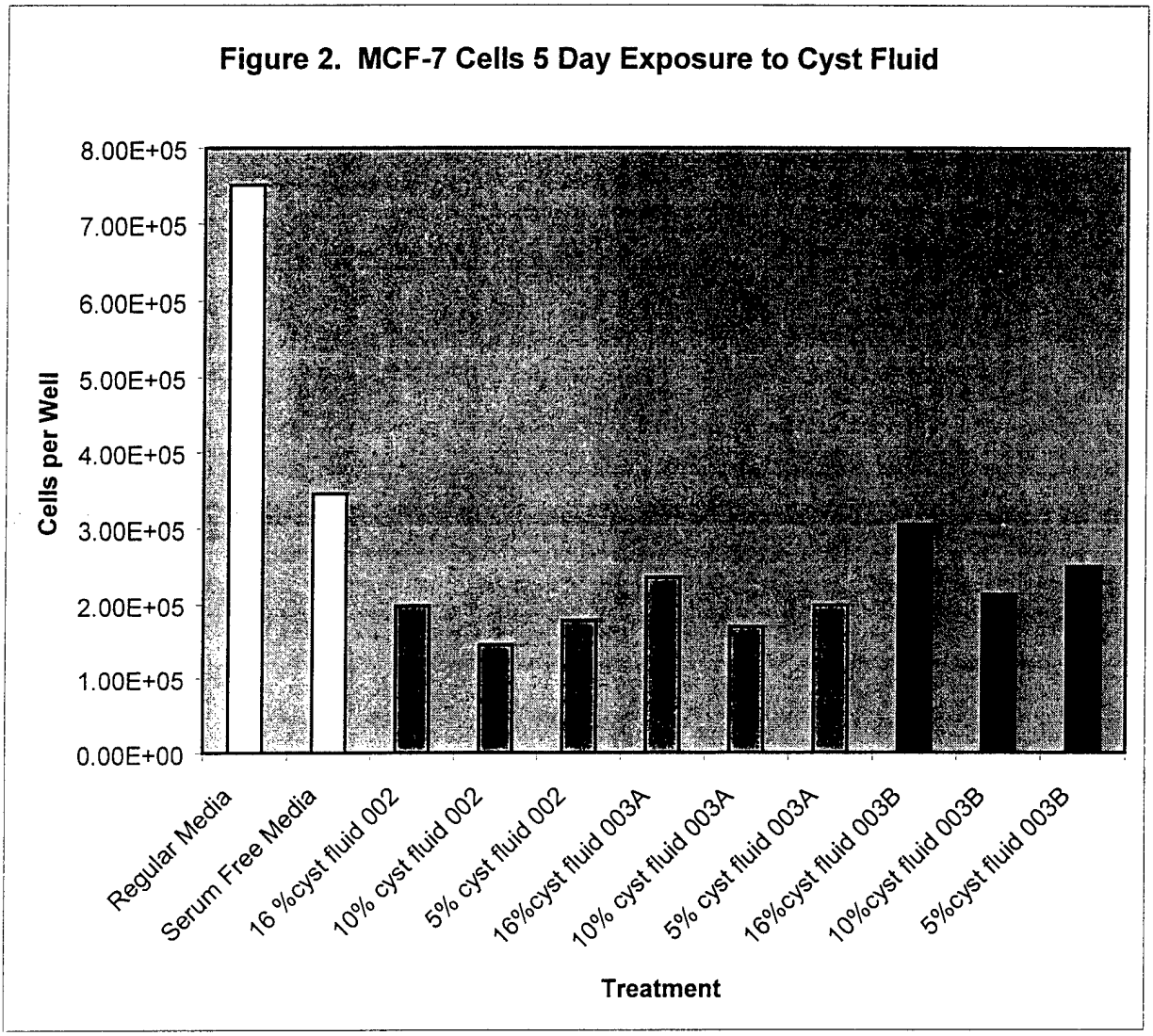

Figure 2. Concentration dependence of Breast Cyst Fluid (BCF) treatment. MCF-7 cells were plated as described in Fig. 1. 24 hours later the medium was changed (no rinse) to serum free medium (SFM) or medium containing the indicated concentrations of BCF. After five days of growth at 37° C, cell were counted.

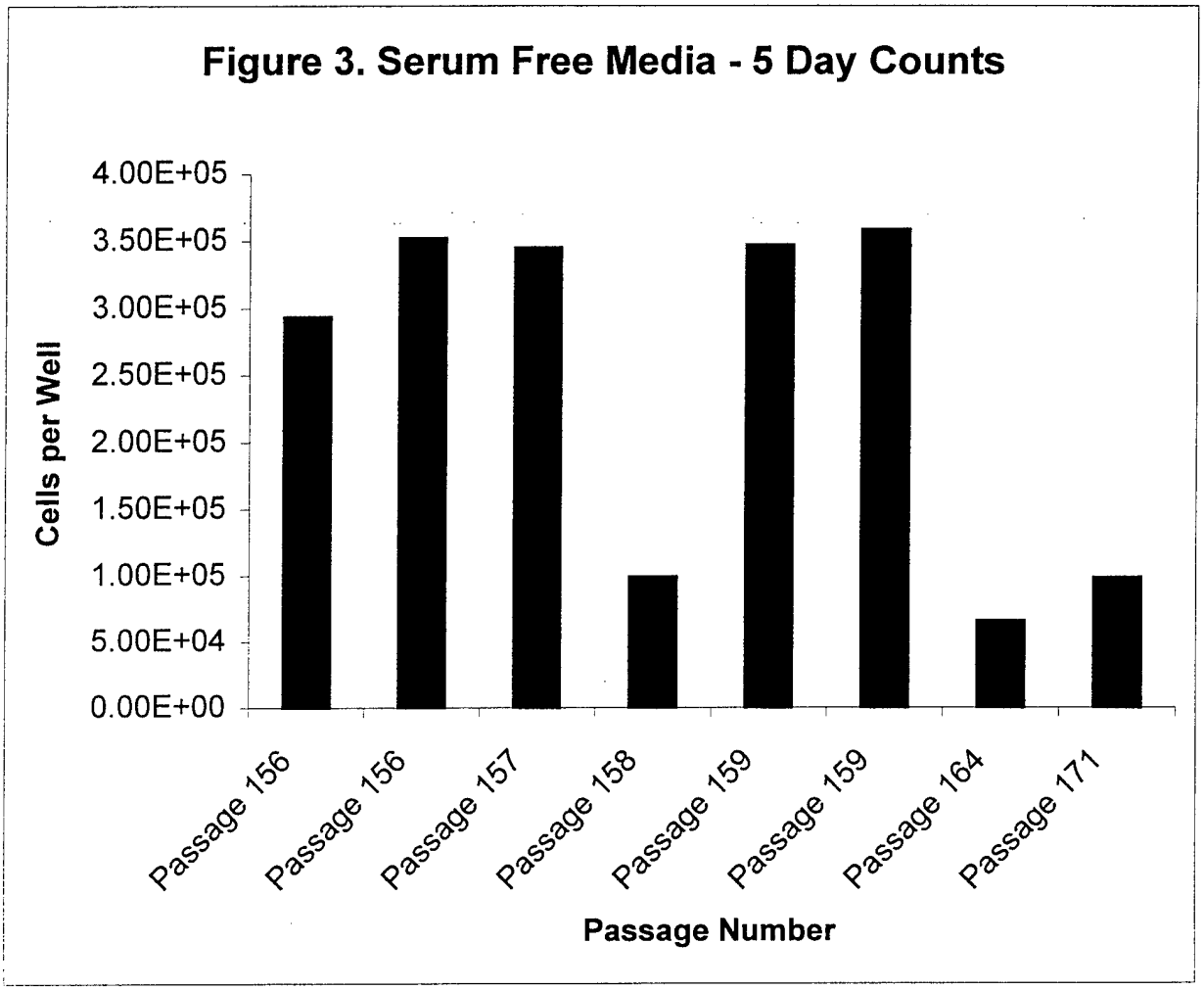

Figure 3. Total number of MCF-7 cells per well as a function of passage number. A passage is equal to a week of incubation and subsequent trypsinization and 1/5 dilution to a fresh T75 flask. Cells were plated as described in Fig. 1.

APPENDIX A

Revised Human Subjects Protocol

Colorado State University

Department of
Environmental Health
College of Veterinary Medicine
and Biomedical Sciences
Environmental Health Building
Fort Collins, Colorado 80523-1676
(970) 491-7038
FAX: (970) 491-2940

MEMORANDUM

TO: U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RCQ-HR/Ms. Jarsie Weeks
504 Scott St.
Fort Detrick, MD 21702-5012

FROM: Robert Wells, Ph.D.
Associate Professor
Radiological Health Sciences
Colorado State University

James Burch, M.S., Ph.D.
Assistant Professor
Department of Environmental Health
Colorado State University

DATE: September 6, 2001

SUBJECT: Revised Human Subjects Protocol for "Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease"

As requested, we have revised our human subjects protocol to provide additional information requested in your Memorandum of Record (A-8953). Our itemized responses to your memo are provided below. Attached for your review are: our new human subjects protocol (Attachment A), the revised consent forms, and related materials.

As part of this submission, we are requesting the addition of 2 new patient recruitment sites, the University of Colorado Health Sciences Center (UCHSC) (Christina Finlayson, M.D., On-site Coordinator), and the Poudre Valley Hospital (PVH) Harmony Imaging Center (Winfield Craven, M.D., On-site Coordinator) to this study in order to meet recruitment goals. The inclusion of these sites is critical to the success of this project and does not alter the study design, which has been designated as minimal risk by the HSRRB. We have attached letters of support from the new on-site

coordinators at PVH and UCHSC and copies of their CV's (Attachment B). Please contact either Dr. Burch (970 491-6178) or myself (970 491-1087) if there are any questions.

Comment 15.a.(1): Amended Human Subjects Protocol.

Our human subjects protocol was submitted to the USAMRMC on June 30, 1999. On October 21, 1999, we responded to recommendations made by the USAMRMC on August 26, 1999. The protocol and revised consent form were approved by the USAMRMC with a minimal risk designation on October 27, 1999. Thus, the latest version of the protocol was submitted on June 30, 1999. Revisions submitted after October 27, 1999 are all requests to add two new recruitment sites using the various formats requested by your office. We have now completely rewritten the protocol to address questions and clarifications requested in your memorandum (see Attachment A, protocol).

Comment 15.a.(2): University of Washington Consent Form.

The consent form approved by the University of Washington (UW) on April 7, 2000 was used in year 1. Without notifying anyone, the former research coordinator at UW apparently made some slight modifications to the consent form originally approved in October, 1999. That research coordinator was subsequently terminated. We have attached copies of the April 7, 2000 and October, 1999 consent forms and have highlighted the wording differences between the forms (Attachment C). There do not appear to be any substantive differences between these two forms.

The consent form used at the UW was recently revised as part of the routine Year 2 UW IRB re-evaluation. It was modified to incorporate new terminology required at the UW, identify currently participating physicians, and incorporate modifications that were added to consent forms for the other recruitment sites. In addition, the UW and Colorado State University (CSU) IRB's determined that the sample donation form contained exculpatory language prohibited under 45 CFR 46.116, and thus the donation form contains modified text. The revised UW consent form is attached for review and approval by the USAMRMC (see Attachment D). Recruitment of participants at UW has been suspended pending USAMRMC and CSU approval of the revised UW consent form.

Comment 15.a.(3): Information regarding the BERNI data base.

The BERNI database has a separate UW IRB approval that delineates who has access, how the database is established and maintained, and how data are disposed of (see Attachment E). Because all breast cyst fluid (BCF) samples will be analyzed at CSU, no data concerning cyst fluid constituents will be entered into the BERNI database. Only the questionnaire data will be entered into the UW

BERNI database, as it would be for any woman attending that clinic, regardless of whether she is a participant in the CSU study. The UW consent form states that data in the BERNI database will be maintained indefinitely. CSU investigators have no control whatsoever over the BERNI database. The UW investigators are providing CSU with: BCF samples, and copies of the completed questionnaires and consent forms.

Samples are shipped to CSU after collection and analyzed at CSU within several weeks to several months. Data from the biochemical analyses and questionnaires are entered into the CSU database with a subject identification number. No names or personal identifiers are entered into the CSU database. The list linking subject identification numbers with personal identifiers is maintained by a study investigator and kept in a locked file as designated in the protocol. Women who choose to terminate their participation in the CSU BCF study can contact Dr. Robert Wells (PI) or the local study coordinator (telephone numbers listed on the consent form) and request that their participation in the CSU study be terminated. If a subject asks to be withdrawn from the study, the link between the subject's name and the study data is destroyed. If any samples are remaining (i.e., they haven't already been analyzed), they are removed and discarded. The individual is also excluded from any follow-up (see protocol section 9 (a) in Attachment A).

Comment 15.a.(4): Surgical Consent Form.

The surgical procedure for the collection of cyst fluids is not part of this study design. Cyst fluid removal will occur regardless of subjects' participation in this study. Therefore the consent form for the surgical procedure for cyst fluid removal is solely the concern of the hospital or clinic where the procedure is performed, the physician performing the procedure, and the patient, and is not germane to this study. The purpose of this study is to collect and analyze cyst fluids that would otherwise be discarded following aspiration. The surgical/procedural consent forms from each recruitment site are attached (see Attachment F).

Comment 15.a.(5): Recruitment materials.

A copy of the recruitment poster that has been approved for use at UW is attached (see Attachment G). No recruitment materials are currently planned for use at the other sites. If such materials are used, the attached recruitment poster will be used with site-specific names and telephone numbers.

Comment 15.a.(6): Documentation related to continuing review of research.

(a) 18 subjects were recruited as of May 1, 2001.

(b) Subject recruitment has been suspended due to delays in obtaining IRB approvals. There have been no adverse events or other unanticipated problems.
(c) No subjects have withdrawn and no complaints have been received.
(d) Our year 1 annual report was submitted to the USAMRMC in September, 2000 (Point of Contact: Judy Pawlus 301-619-7322). A summary of the year 1 annual report is attached (see Attachment H).

Comment 15.a.(7): Wait for HSRRB approval prior to providing IRB documentation.

We will wait for HSRRB approval prior to providing IRB documentation from CSU and the other recruitment sites. Please note that these instructions are the complete opposite of instructions originally given to us by the HSRRB office. Accordingly, we spent several months obtaining consent form approvals at the new recruitment sites and at CSU's IRB prior to submitting them to HSRRB for approval. Additionally,

> *"This policy directly conflicts with CSU's policy to review PROPOSED research. CSU requires that approval be obtained before the protocol is submitted, or at the latest, within one month of submission of a protocol for funding... [HSRRB] approval does not mean that the study will automatically be approved by the sites...since the local IRBs have the prerogative to be more stringent than the federal regulations." Celia S. Walker, Director, Regulatory Compliance Office, CSU.*

15.b. Revisions to be made to the protocol

A completely revised protocol is attached (Attachment A). Revisions are presented **in bold type.**

15.b.(1) Informed consent procedures

The informed consent procedures are presented in section 8 of the revised protocol (Protocol Design, Attachment A).

15.b.(2) Risk analysis

This study has been designated by the HSRRB as a minimal risk study (see Attachment I). The study is directed toward patients having breast cyst aspirations, not biopsies for solid tumors. The needle aspirations of breast cysts are ongoing procedures that would be performed in these patients regardless of their status as a study participant. Risks associated with the breast cyst aspiration, such as pain, bleeding and infection, are not relevant to the study design because patients will have already consented to the removal of their cyst fluid. No added risk or

discomfort is anticipated from donating a BCF sample for research. The cyst fluids would otherwise be discarded. The risks associated with the study are described in section 9 of the revised protocol (Risks/Benefits Assessment, Attachment A).

Minimal risk is expected from completing the questionnaire. There is a possibility that subjects may find one or more questions objectionable. If a participant finds a question objectionable, they are instructed that they may skip the question.

The principal risk to the subjects is the potential for a breach of confidentiality. Each subject will be assigned an identification number. Data will be entered into the study database with only the identification number. The list linking personal identifiers with the identification number will not be entered into the study database. The list and study consent forms will be kept in locked storage in the custody of a study investigator.

15.b.(3) Inclusion and exclusion criteria

The inclusion and exclusion criteria are described in section 7 of the revised protocol (Study Population, Attachment A).

15.b.(4) Study personnel

A description of the roles and responsibilities of study personnel is provided in section 14 of the revised protocol (Roles and Responsibilities of Study Personnel, Attachment A).

15.c. PVH and UW consent form revisions

The principal risk to the subjects in the CSU BCF study is the potential for a breach of confidentiality. The breast cyst aspiration would be performed regardless of whether the patient chooses to participate in this study. This study will use breast cyst fluid that would otherwise be discarded. In the UCHSC consent form, the description of risks associated with the breast cyst fluid aspiration procedure, which is not part of the CSU study design, was required by UCHSC's COMIRB. This section of the UCHSC consent form has been modified to clarify this. We started out submitting consent forms with the exact wording to each site-specific IRB and ended up with slightly different wording in some cases. Consent forms must be concisely worded so as not to impede recruitment. The risks associated with aspiration are not part of the CSU study design. It is not essential, nor should it be necessary, for the unique requirements of the COMIRB to govern the content of consent forms already approved by the IRBs at PVH or the UW. Multi-institutional projects are critical to obtaining

sufficient numbers of patients to evaluate complex disease processes such as breast cancer. It is important to recognize that projects with multiple IRB approvals have unique administrative requirements that require a certain amount of co-operation among the institutions involved. Flexibility in obtaining multiple IRB approvals should be part of that process.

15.d.(1) Revisions to the PVH and UCHSC consent forms

A description of the storage of samples for future use and withdrawal of participants is presented in section 8 of the revised protocol (Protocol Design, Attachment A).

The aim of the currently funded study is to recruit 150 patients and analyze donated breast cyst fluids. Information obtained from the currently funded study will be used as the basis for requesting future funding to perform a follow-up prospective study of BCF composition and breast cancer risk in a population of approximately 800-1500 women (1500 maximum) including those enrolled in the current study. The UCHSC consent form has been modified to reflect this.

Samples arriving at CSU are analyzed within several months. Any remaining sample is stored at CSU for a future follow-up study. If the subject chooses to terminate participation, they can contact a study investigator listed on the consent form. Samples not already analyzed for the biochemical parameters described in the study protocol would then be removed from storage and destroyed. The patient's name and personal identifiers would be removed from files and their name deleted from the list linking participants and their subject identification numbers. Sentences have been added to the revised consent forms to reflect changes concerning withdrawal from the CSU study (see Attachment D).

The PVH consent form indicate that we will include cytopathology data for a patient's cyst fluid sample in our analysis, if the information is available from PVH as secondary data. No additional personal information about a patient's case status would be provided by consenting PVH participants. The other recruitment sites do not routinely collect cytopathology data thus such wording does not appear in the UW and UCHSC consent forms.

15.d.(2) Colorado State Cancer Registry

The BCF and questionnaire data will be maintained by the CSU investigators. The Colorado State Cancer Registry is under the control of Colorado State Department of Public Health and Environment. The CSU investigators have no direct access to data in this or any other cancer registry. With patient consent, epidemiologists may apply to the State and retrieve vital

records from the Registry. We are seeking permission to access a cancer registry in a future follow-up study as part of our informed consent process. If a subject chooses to terminate their participation in the study, their Registry data will not be accessed for a future follow-up study, as is described in the Protocol (Section 8, Attachment A).

15.e.(1) Revisions to the UCHSC consent form

The UCHSC consent form has been modified to reflect the information described in Section 7 of the revised protocol (see Attachment D).

15.e.(2) The benefits section has been edited.

LIST OF ATTACHMENTS

ATTACHMENT A. REVISED PROTOCOL

ATTACHMENT B. LETTERS OF AGREEMENT AND CV'S FOR NEW SITES

ATTACHMENT C. OLD CONSENT FORMS

ATTACHMENT D. REVISED CONSENT FORMS

ATTACHMENT E. UNIVERSITY OF WASHINGTON IRB APPROVAL FOR BERNI DATABASE

ATTACHMENT F. SURGICAL/PROCEDURAL CONSENT FORMS

ATTACHMENT G. RECRUITMENT POSTER

ATTACHMENT H. SUMMARY OF THE YEAR 1 ANNUAL REPORT

ATTACHMENT I. HSRRB APPROVAL AT THE CURRENT STUDY SITE (UW)

ATTACHMENT A. REVISED PROTOCOL

DEPARTMENT OF DEFENSE BREAST CANCER RESEARCH PROGRAM

Human Subjects Protocol

1. **Project Title:** Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

2. **Phase:** Not Applicable

3. **Principal Investigator:** Robert Wells, Ph.D.
Associate Professor
Radiological Health Sciences
Colorado State University
Fort Collins, CO 80523

Co-Investigator: James Burch, M.S., Ph.D.
Assistant Professor
Department of Environmental Health
Colorado State University
Fort Collins, CO 80523

On-Site Coordinator: Ben Anderson, M.D.
Medical Director
BioClinical Breast Care Program
Associate Professor of Surgery
University of Washington, Box 356410
Seattle, WA 98105

On-Site Coordinator: Christina Finlayson, M.D.
Assistant Professor
GI, Tumor and Endocrine Surgery
4200 E. 9th Ave. Box C-311
University of Colorado Health
Sciences Center
Denver, CO 80262

On-Site Coordinator: Winfield Craven, M.D.
Medical Director
Harmony Imaging Center
Poudre Valley Health System
2127 E. Harmony Rd., Ste. 130
Fort Collins, CO 80528

Consulting Toxicologist: Greg Cosma, Ph.D.
Pharmacia & Upjohn
7000 Portage Rd.
Kalamazoo, MI 49001-0199

Consulting Statistician: Thomas Keefe, Ph.D.
Professor
Department of Environmental Health
Colorado State University
Fort Collins, CO 80523

4. Location of Study: Patient recruitment will be performed at the University of Washington School of Medicine (UW) (Ben Anderson, M.D., On-site Coordinator), **the University of Colorado Health Sciences Center (UCHSC) (Christina Finlayson, M.D., On-site Coordinator), and the Poudre Valley Hospital (PVH) Harmony Imaging Center (Winfield Craven, M.D., On-site Coordinator).** Biochemical and cell proliferation assays, and data analyses will be performed at the Department of Environmental Health, Colorado State University, Fort Collins, CO.

5. Time Required to Complete: Start Date: 09/01/99
End Date: 08/30/02

6. Objectives: The central goals of the proposed studies are two-fold. The first objective is to explore oncostatic effects of cyst fluids in relation to their melatonin levels, as well as correlate melatonin levels with two growth factors (EGF and TGF-beta) and another hormone (DHEA-S), which have previously been identified in breast cyst fluids. The second objective is to establish a breast cyst fluid sample bank in order to evaluate the relationships between gross cystic breast disease, melatonin, other growth regulatory agents, and known risk factors for breast cancer in a cohort of patients with gross cystic breast disease.

7. Study Population:

(a) The target population is women with cystic breast disease. The aim of the currently funded study is to recruit 150 patients and request that they donate their breast cyst fluid for analysis. The subjects will be a convenience sample recruited from all women having needle aspirations on breast cysts at the three sites. These procedures will be performed at the UW School of Medicine Breast Health Center, the UCHSC, and the PVH Harmony Imaging Center. Names and addresses of on-site coordinators are provided in Section 3. Information obtained from the currently funded study will be used as the basis for requesting

future funding to perform a follow-up prospective study of BCF composition and breast cancer risk in a population of approximately 800-1500 women (1500 max.) including those from the current study.

Over 90% of the patient population at these sites is white. Patients will be recruited by the attending physician or their nurse immediately prior to receiving their aspiration or during their consultation prior to the procedure. Participants will be selected without regard to race, religion, or ethnic origin. **Any adult (18 years and older) female patient receiving cyst aspiration by their attending physician will be considered eligible to participate, including postmenopausal and pregnant women. No other inclusion or exclusion criteria, such as previous breast augment or reduction, will be applied.**

(b) Breast cyst fluid (BCF) samples to be analyzed in this study will be discard cyst fluids collected from patients receiving needle aspiration of breast cysts. Patients will be recruited for participation in this study and consent will be requested for linking measured levels of melatonin and other growth-related agents in BCF with information on individual risk factors for breast cancer to be obtained via a questionnaire.

Information obtained from the currently funded project will be used as the basis for requesting future funding to perform a follow-up prospective study of breast cancer risk. To incorporate current participants into the follow-up, we are requesting consent to use the patient's name, social security number, and date of birth for linkage to a cancer registry at the end of a 6 to 10 year follow-up period. In this manner, there will be no need to re-contact consenting participants for inclusion in the follow-up study.

(c) Pregnant women are not excluded from this minimal risk study.

8. Protocol Design:

a. Subject identification

Subjects will be assigned a code number after consenting to participate in the study. A list will be kept linking the code number to the subject's personal identifiers. Personal identifiers will not be entered into the study database. Consent forms and the list will be kept in locked storage in the custody of a study investigator.

b. Description of the recruitment process

Patient recruitment will be performed at the breast health clinics where the cyst aspirations are performed. Participant consent and collection of questionnaire data will both be obtained by the on-site coordinators or their medical staff during a patient's routine office visit for medical treatment or consultation at the time of needle aspiration. See Attachment I for recruitment materials.

c. Description of the Informed Consent process

The study rationale, informed consent, and each subject's involvement will be described. Patients will then be asked to read the consent form and questions or clarifications will be solicited afterwards. The consent form contains a written explanation of the purpose and methods of the project, its voluntary nature and the option for withdrawal at any time. The patient will be able to take as much time as they want to read the consent form and ask questions. If the subject agrees to continue, she will sign the Consent Form and a signed copy will be provided for the participant's files (see Attachment D).

d. Subject assignment (randomization)

This study will obtain a cross-sectional convenience sample, no randomization will take place.

e. Evaluations prior to entry

Patients will have already consented, using standard, site-specific surgical/procedural consent forms, to undergo needle aspiration of breast cysts before becoming eligible to participate in this study. This study uses breast cyst fluids that would otherwise be discarded.

f. Evaluations to be made during the conduct of the study

Participants will be asked to complete a breast cancer risk factors questionnaire. The questionnaire solicits information on known risk factors for breast cancer including age, race, body weight, menopausal

status, reproductive history, and the use of certain medications (estrogen replacement, oral contraceptives). The amount of time required to complete the questionnaire is approximately 10 minutes. The additional time commitment for this study is expected to be approximately 15 minutes or less.

Breast cyst fluid aspirations will be performed during normal (daytime) office hours as they would have been were this study not taking place. Breast cyst fluid samples to be analyzed in this study will each be assigned an anonymous identification number that will be used to track samples throughout the study. Samples will be aliquoted, frozen at -80 C°, and express mailed on dry ice to the laboratory of Dr. Robert Wells at Colorado State University, where they will remain in cold storage (-80 C°) until analysis. The date and time of collection will be recorded for each sample. Analyses to be performed on BCF samples will include the hormones, melatonin and DHEA-sulfate, and the growth factors, EGF and TBF-beta1 and 2. In addition, electrolyte (sodium and potassium) concentrations will be determined. Results from these analyses will be used to correlate hormones, growth factors and electrolyte levels. Samples will also be used to assess their effect on proliferation of MCF-7 breast cancer cells. Subsequent analyses will correlate levels of these agents with known risk factors for breast cancer. Biochemical and data analyses will be performed at the Department of Environmental Health, CSU, Fort Collins, CO.

Permission is being requested in the consent form to use the patient's name, Social Security number, and date of birth for linkage to a Cancer Registry at the end of a 6 to 10 year follow-up period. **The currently funded study will be used as the basis for requesting future funding to perform a follow-up prospective study of breast cancer risk and breast cyst fluid composition in a population of 800-1500 women (1500 maximum), including those in the current project. Sample size calculations will be performed to determine the number of subjects needed in the follow-up study. Funding for that study, a new consent form, and IRB approval will be sought at an appropriate time in the future.**

g. Clinical assessments

No additional clinical assessments will be made as a consequence of participation in this study

h. Research activity experienced by the subject

After informed consent, participants will be asked to complete a breast cancer risk factors questionnaire. The amount of time required to complete the questionnaire is approximately 10 minutes. When the questionnaire is complete, the participant undergoes the scheduled breast cyst fluid needle aspiration procedure. Instead of being discarded, the fluid is transferred to a container to be treated as explained in Section 8 (f). The patient has no further contact with the study.

9. Risks/Benefits Assessment:

a. Describe risks

The principal risk to the subjects is potential harm resulting from a breach of confidentiality. Personal medical information is extremely confidential and requires careful consideration of data handling and record keeping procedures to ensure inadvertent disclosure of this information. **To minimize this risk, subjects will be assigned a code number after consenting to participate in the study. A master list will be kept which will link the code number to the subject's personal identifiers. The code number will be used to enter data into the data base for analysis. Personal identifiers will not be entered into the study database. Signed consent forms and the linking list will be kept in locked storage in the custody of a study investigator.** Statistics derived from confidential data will be reported without disclosure of individual subjects' identities. The final report and all reports made available to the public will not contain test results or findings that will identify an individual subject.

Minimal risk is expected from completing the questionnaire. There is a possibility that subjects may find one or more questions objectionable. If a participant finds a question objectionable, the participant may skip the question. Refusing to answer a question does not affect the subject's participation in the study. Subjects may terminate participation at any time. Women who choose to terminate their participation in the CSU BCF study can contact Dr. Robert Wells (PI) or the local study coordinator (telephone numbers listed on the consent form) and request that their participation in the CSU study be terminated. If a subject asks to be withdrawn from the study, the link between the subject's name and the study data is destroyed. Samples in

frozen storage, if any, are removed and discarded, and the individual is excluded from any follow-up.

The needle aspirations of breast cysts are ongoing procedures that would be performed in these patients regardless of their status as a study participant. Because patients will have already consented to the removal of their cyst fluid, no added risk or discomfort is anticipated from donating a sample for research. **The cyst fluids would otherwise be discarded.**

BCF samples will be collected via needle aspiration by licensed physicians with assistance from their attending medical staff, as they would have been were this study not taking place. The analyses to be performed in the BCF samples are experimental in nature and have no known clinically relevant diagnostic or prognostic value. Subjects will be informed that they are not required to participate in the study and that they can terminate their participation at any time. Consent for future access to a cancer registry will be obtained prior to the onset of breast or other forms of cancer and patients will not be re-contacted following this initial consent. Thus, no additional physical or emotional stress is anticipated from this aspect of participation. The consent form states that participants will not be re-contacted by CSU investigators. Patients will not receive monetary compensation for their participation.

(b) Describe benefits

Subjects are not likely to benefit directly from participation. Because patients with gross cystic breast disease have a 2 to 4 fold increased risk of developing breast cancer, patients with this disease may benefit from research on the mechanisms of breast cancer onset and development. Approximately 60% of breast cancer risk remains unexplained. Thus, research focusing on the biochemical mechanisms underlying breast carcinogenesis and the elucidation of unexplained risk factors is essential for prevention and management of this disease. The hormone melatonin appears to play a protective role in the development of breast cancer. If melatonin is associated with reduced breast cancer risk, then research involving melatonin could lead to novel approaches in identifying those at risk and better disease prevention and control strategies. Translational research strategies incorporating melatonin in clinical trials for women with cystic breast disease or other high risk groups may be suggested by this research. Information obtained from this research may lead to better strategies for the prevention and treatment of breast cancer, which could reduce societal medical costs and years of productive life lost due to this disease.

10. Reporting of Serious and Unexpected Adverse Events:

Serious and unexpected adverse experiences will be immediately reported by telephone to the USAMRMC Deputy Chief of Staff for Regulatory Compliance and Quality: (301) 619-2165. During non-business hours, call (301) 619-2165 and send information by fax to (301) 619-7803. A written report will follow the initial telephone call within 3 working days. Written reports will be addressed to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RCQ-HR, 504 Scott Street, Fort Detrick, MD 21702-5012.

11. Description of Protocol Drug(s) or Device(s):

Not Applicable.

12. Disposition of Data:

Project records will be maintained by Dr. Wells and stored in his office in the Department of Radiological Health Sciences (Room 437, MRB Bldg) in a locking file cabinet. The file cabinet and room will be closed and locked when not in use. After the study is complete, data files will be archived for at least three years in the Department of Environmental Health in Room 124 of the Environmental Health Building in a locked file cabinet.

13. Modification of the Protocol:

Significant protocol modifications will be submitted to the U.S. Army Medical Research and Materiel Command as well as to the Institutional Review Boards of the University of Washington, **The University of Colorado Health Sciences Center**, **Poudre Valley Hospital**, and Colorado State University for approval.

14. Roles and Responsibilities of Study Personnel (names and addresses are presented in Section 3 of the protocol):

As Principal Investigator, Dr. Robert Wells has overall responsibility for the design and conduct of the study. He oversees all cyst fluid studies with respect to laboratory analyses and experimental design, and will prepare reports and manuscripts that result from this work. In addition, he oversees the work of other study personnel in the completion of the cell proliferation studies. Dr. Greg Cosma serves as a consultant to the project and provides input on the conduct of the study. Dr. James Burch serves as liaison between CSU and the Army, UW, UCHSC, and PVH, oversees IRB compliance, participates in biochemical analyses of cyst fluids, performs comparative biostatistical analyses of breast cyst fluids, and prepares Annual Reports, manuscripts, and Final Reports. Dr. Tom Keefe, a biostatistician in the Department of Environmental Health at Colorado State University, will assist Dr. Burch

with statistical data analyses. Ben Anderson, M.D., is a surgical oncologist at the University of Washington School of Medicine and is Director of the Breast Health Center at the University of Washington Medical Center. Dr. Anderson serves as a collaborator with respect to clinical aspects of the proposed studies, as well as oversees procurement of breast cyst fluid samples. He supervises the clinical tasks of patient identification, patient consent, breast cyst fluid aspiration, processing, and shipment at the University of Washington School of Medicine, and consults with the PI and other study investigators (see attached letter). **Christina Finlayson, M.D., will oversee the clinical tasks of patient identification, patient consent, breast cyst fluid aspiration, processing, and shipment at the University of Colorado Health Sciences Center. Winfield Craven, M.D., will oversee the same clinical tasks as Drs. Anderson and Finlayson at the Poudre Valley Hospital Harmony Imaging Center.**

15. Signature of Principal Investigator:

I have read the forgoing protocol and agree to conduct the study as outlined herein.

Robert Wells Date: September 6, 2001

Robert Wells, Ph.D.
Associate Professor
Radiological Health Sciences

ATTACHMENT B. LETTERS OF AGREEMENT AND CV'S FOR NEW SITES

4200 East Ninth Avenue
Denver, Colorado 80262

University Hospitals
School of Medicine

School of Nursing
School of Dentistry

School of Pharmacy
Graduate School

August 25, 2000

Jim Burch, M.S., Ph.D.
Assistant Professor
Department of Environmental Health
Colorado State University
Fort Collins, CO 80523

Dear Dr. Burch:

We are pleased to write a letter of support for your grant, "The Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease". After we receive IRB approval for study, we look forward to participating as an ancillary site. We expect to enroll 40 patients per year.

If you have any questions, please call me at (303) 315-8671.

Christina A. Finlayson, MD
Assistant Professor
GI, Tumor and Endocrine Surgery

CHRISTINA ANN FINLAYSON
CURRICULUM VITAE

University of Colorado Health Science Center
GI, Tumor and Endocrine Surgery
4200 E. Ninth Avenue, Box C-311
Denver, CO 80262
Telephone: 303-315-8671
Fax: 303-315-5527
e-mail: Christina.Finlayson@UCHSC.edu

Education

MEDICAL

1985-1989 University of Utah School of Medicine
Salt Lake City, Utah
M.D., 1989

GRADUATE

1982-1983 Stanford University
Palo Alto, California
A.M., Education, 1983

UNDERGRADUATE

1976-1981 Idaho State University
Pocatello, Idaho
B.S., Microbiology, 1981

1978-1979 Brigham Young University
Provo, Utah

Surgical Training

1994-1996 Fox Chase Cancer Center
Philadelphia, Pennsylvania
Fellowship, Surgical Oncology

1989-1994 University of Colorado Health Science Center
Denver, Colorado
Internship & Residency, General Surgery

Academic Appointments

1996-present University of Colorado Health Science Center
Department of Surgery
Denver, Colorado
Assistant Professor, Surgery

1996-present Veterans Administration Hospital
Denver, Colorado

1994-1996 Temple University
Department of Surgery
Philadelphia, Pennsylvania
Clinical Instructor

Hospital and Administrative Appointments
1997-present Director, University Hospital Breast Center
1998-1999 Co-director, Breast Program, University of Colorado Hospital Cancer Center

Licensure
Colorado, 1996
Pennsylvania, 1994

Board Certification
American Board of Surgery, September 1995

Honors and Awards
1998 "Top 150 Doctors in Denver", 5280 Magazine. Denver, CO
1994 Second place, Colorado Chapter American College of Surgeons resident paper competition, "Technetium 99m Sestamibi: A new scan for parathyroid adenoma"
1988 Hubbard Scholarship

Professional Organizations
Western Surgical Society, member
Association of Women Surgeons, member
Denver Academy of Surgery, member
ACOSOG, Institution Principal Investigator
Southwestern Surgical Congress, member
SWOG, member
NSABP, Institution Principal Investigator
Society of Surgical Oncology, member
American College of Surgeons, member
Association for Surgical Education, member

Resident and Medical Student Education
2000 Lecture, Pathology 6000 2nd year medical students, Multidisciplinary Management of Breast Cancer
Lecture, Pathology Residents, Multidisciplinary Management of Breast Cancer
CME Rural Outreach Program, Management of Mammographic Abnormalities, Torrington, Wyoming and Syracus, Kansas
1999 Lecture, Ob-Gyn 2nd year residents, Management of Breast Diseases

Lecture, 2nd year medical students, Surgical management of breast cancer
1998 Lecture, Ob-Gyn 2nd year residents, Management of Breast Diseases
1997-present Course director, 3rd year medical student general surgery rotation, University of Colorado Hospital
1997-present Family practice resident rotation in ambulatory/breast surgery
1996-present General surgery residency training program
1996-present Surgery core curriculum lecture series, 3rd year medical students, UCHSC

Committee Appointments
1998-present Chairman, University of Colorado Cancer Committee
ACS Cancer Liaison Physician
1998-1999 Planning Committee, Fitzsimmons Center for Advanced Medicine
1998-1999 Chairman, Operative and Invasive Procedure Committee, VAMC
1998-1999 Planning Committee, Fitzsimmons Cancer Center
1998-present Clinical Cancer Center Steering Committee
1997-present Management Committee, University of Colorado Cancer Center
1996-present Surgery Quality Improvement Committee, University Hospital
1996-present Cancer Committee, University Hospital
1996-1999 Cancer Committee, VAMC
1997 Bridge to the Future Innovations in Education Mini-Grant Program Review Committee

Community Service
2001 What women should know about breast cancer even if they don't have it, American Association of University Women, February 3, 2001.
2000 Advisory Committee, CEFP evaluation for Reach to Recovery Program, American Cancer Society.
Community Outreach Program, talk to high school biology class on medical careers, Syracus, Kansas
1999 "Ask a Doctor" Channel 4 Call in program
Regis High School Mother's Group, "Breast Cancer"
1998 University Longevity Lecture Series, "Breast Cancer"
1997 University Opinion Lecture Series, "Breast Cancer"

Research Support
2000 Susan G. Komen Foundation, "Using a social worker to increase clinical trial enrollment" $13,500.
ACOSOG Grant, $166,000
NSABP, $50,000
1999 NSABP Insitutional grant, $24,000
1997 Dean's Academic Enrichment Fund, "Medial quadrant breast cancer: can surgical staging be improved by lymphatic mapping?" $20,000.
1996 Innovations in Education Mini-Grant, "Computer Assisted Instruction with Model Simulation for Breast Disease Education", $5000.

Research Projects

1. The role of hyperinsulinemia in breast cancer, co investigator with Jim Chappel, Samia Nawaz, and Boris Draznin, 2000-present.
2. Radiation therapy primes for an augmented inflammatory cytokine response to a second stress, coinvestigator with Jyoti Arya and David Raben, 2000-present.
3. MRI as an intermediate marker for tamoxifen response, coinvestigator with John Lewin, 2000-present.
4. "A Prognostic Study of Sentinel Node and Bone Marrow Micrometastases in Women with Clinical T1 or T2 N0 M0 Breast Cancer (ACOSOG Z0010)" University of Colorado Health Sciences Center, Department of GI, Tumor and Endocrine Surgery, February 2000-Present.
5. A Randomized Trial of Axillary Node Dissection in Women with Clinical T1 or T2 N0 M0 Breast Cancer Who Have a Positive Sentinel Node" University of Colorado Health Sciences Center, Department of GI, Tumor and Endocrine Surgery, March 2000-Present.
6. Correlation between methylated p16 DNA in the blood and breast cancer tissue of patients, coinvestigator with Andrew Kraft, 2000-present.
7. A Double-Blind, Randomized, Placebo-Controlled, Multi-Center Study to Evaluate the Efficacy and Safety of Two Doses of Lexipafant for the Treatment of Acute Pancreatitis University of Colorado Health Sciences Center, Department of GI, Tumor and Endocrine Surgery, March 1997-January 1999.
8. "An 80-Patient, Phase II, Dose-Ranging, Double-Blind, Placebo-Controlled Multicenter Trial of Sterile NC67722 Suspension 150 mg/ml in Axillary Nodal Staging of Women Diagnosed with Carcinoma of the Breast" University of Colorado Health Sciences Center, Department of GI, Tumor and Endocrine Surgery, March 1997-August 1997.
9. "Medial Quadrant Breast Cancer: Can Surgical Staging Be Improved By Lymphatic Mapping" University of Colorado Health Sciences Center, Department of GI, Tumor and Endocrine Surgery, March 1997-October 1999.
10. "A Randomized, Double-Blind, Placebo-Controlled, Multi-Center Phase III Study of Intravenous Betafectin□ PGG-Glucan for the Prevention of Serious Infections in Patients Undergoing Upper GastroIntestinal Surgery Who Are at High Risk for Infection" University of Colorado Health Sciences Center, Department of GI, Tumor and Endocrine Surgery, February 1998-January 1999.
11. "Lymphography To Define Lymphatic Drainage Patterns of the Normal Mammary Gland as Compared to Drainage From a Breast Involved with Mammary Cancer" University of Colorado Health Sciences Center, Department of GI, Tumor and Endocrine Surgery, January 1999-January 2000.
12. "A Phase 2/3 Study to Evaluate the Safety and Efficacy of Recombinant Human Platelet Activating Factor Acetylhydrolase (rPAF-AH) for the Treatment of Patients with Severe Acute Pancreatitis" University of Colorado Health Sciences Center, Department of GI, Tumor and Endocrine Surgery, August 1999-May 2000.
13. "A Double-Blind, Placebo-Controlled, Minimized Phase III Study Comparing Marimastat to Placebo as Adjuvant Therapy in Patients with Resectable Pancreatic Cancer" University of Colorado Health Sciences Center, Department of GI, Tumor and Endocrine Surgery, August 1999-June 2000.

Presentations and Invited Lectures

1. DCIS: What to know, what to trust, what not to know and what to forget, Surgery Grand Rounds, University of Colorado Health Science Center, Feb. 10, 2001.
2. Neoadjuvant chemotherapy for breast cancer, Hormones in Cancer Research Seminar, Jan 23, 2001.
3. Risk modification for preventing breast cancer, Family Practice Review Course, University of Colorado Health Science Center, November 2000
4. Risk modification for preventing breast cancer, Family Practice Review Course, University of Colorado Health Science Center, June 2000.
5. Risk modification for preventing breast cancer, East Tennessee State University Department of Surgery Grand Rounds, April, 2000.
6. Risk modification for preventing breast cancer, Horizons in Surgery, University of Colorado Department of Surgery, Breckenridge, CO, March 2000.
7. Breast Cancer Management for the Gynecologist, University of Colorado Department of Ob-Gyn Winter Conference, Vail, CO, February 2000.
8. Risk modification for preventing breast cancer, Surgery Grand Rounds, St. Joseph's Hospital, December, 1999.
9. Risk modification for preventing breast cancer, Surgery Grand Rounds, University of Colorado Department of Surgery, November, 1999.
10. Breast Magnetic Resonance Imaging: Role in determining breast as a source of unknown metastatic lymphadenopathy. Invited discussant, Southwestern Surgical Congress, San Diego, California, April 18, 1999.
11. Sentinel node biopsy for staging breast cancer, Video presentation, Southwestern Surgical Congress, San Diego, California, April 17, 1999.
12. Role of Breast Magnetic Resonance Imaging in Determining Breast as a Source of Unknown Metastatic Lymphadenopathy, invited commentary at Southwestern Surgical Congress, San Diego, California, April 18, 1999. Published American Journal of Surgery 178:499, Dec. 1999.
13. Management of breast lumps, Geriatric Grand Rounds, University of Colorado Health Science Center, March, 1999.
14. Management of mammographic abnormalities, Horizons in Surgery, University of Colorado Department of Surgery, Breckinridge, CO, March, 1999.
15. Management of mammographic and palpable breast abnormalities, and Update in Breast Cancer workshop, Winter Symposium, St. Mary's Hospital, Grand Junction Colorado. January15,1999.
16. Management of mammographic abnormalities, Resident Education, University of Utah Department of Surgery, January 18, 1999.
17. Management of mammographic abnormalities, Surgery Grand Rounds, University of Colorado Department of Surgery, November, 1998.
18. Surgical considerations in the management of hereditary colon and breast cancer, practice based symposium, National Society of Genetics Counselors, Denver, CO, 1998.
19. Update in Breast Cancer, Horizons in Surgery, University of Colorado Department of Surgery, Breckinridge, CO, March, 1998.
20. Management of Breast Disease, Ob-Gyn Grand Rounds, University of Colorado Department of Ob-Gyn, 1997.

21. Update in Breast Cancer, Surgery Grand Rounds, University of Colorado Department of Surgery, October 1997.
22. Update in Breast Cancer, Surgery Grand Rounds, University of Colorado Department of Surgery, October 1996.

Publications

1. **Finlayson CA**, Eisenberg BL: Palliative Pelvic Exenteration, Patient Selection and Results. Oncology, 10(4):479-484, April, 1996.
2. **Finlayson C**, Hoffman J, Yeung R, Kessler H, Guttmann, M, Shaer A, Clair M: Intraoperative Ultrasound Does Not Improve Detection of Liver Metastases in Surgically Resectable Pancreatic Cancer. American Journal of Surgery, 175(2):99-101, Feb. 1998.
3. Bowers SP, Pearlman NW, McIntyre RC, **Finlayson CA**, Heurd S: Cost-Effective Management of Gynecomastia. American Journal of Surgery, 176: 638-641, Dec. 1998
4. Heimbach JK, Biffl AL, Mitchell EL, **Finlayson CA**, Schwartzberg BS, Myers A, Rabinovitch R and Franciose RJ: Breast Conservation Therapy in Affiliated County, University, and Private Hospitals. American Journal of Surgery 178:466-469, Dec. 1999.
5. **Finlayson CA**, MacDermott TA: Ultrasound can estimate the pathologic size of infiltrating ductal carcinoma. Archives of Surgery, 135:158-159, Feb. 2000
6. **Finlayson CA**, Arya J, Galandiuk S, Harken AH: The meaning of surgery. Surgery, 127:361-362, April, 2000.
7. Arya J, **Finlayson CA**, Shames BD, Harken AH, Anderson BO: Stimulated apoptosis as an anti-neoplastic strategy. Surgery, 127:366-369, April, 2000.
8. Norred CL and **Finlayson CA**: Hemorrhage after the preoperative use of complementary and alternative medicines. AANA Journal, 68:1-4, June, 2000.
9. Rabinovitch R, **Finlayson C**, Pan Z, Lewin J, Humphries S, Biffl W, and Franciose R: Radiographic evaluation of surgical clips is better than ultrasound for defining the lumpectomy cavity in breast boost treatment planning: A prospective clinical study. International Journal of Radiation. Oncology Biol Phys 47(2):313-317, in press 2000.

Book Chapters

1. **Finlayson** T, Pearlman N: Neck Masses. In Abernathy C and Harken A (eds):Surgical Secrets, 2nd edition. Philadelphia, Hanley & Belfus, 1991, p. 223-226.
2. **Finlayson** C, Abernathy C: The Acute Abdomen. In Parsons P and Wiener-Kronich J (eds):Critical Care Secrets. Philadelphia, Hanley & Belfus, 1992, p. 326-328.
3. **Finlayson** CA: Hodgkin's Disease and the Malignant Lymphomas. In Harken AH and Moore EE (eds): Abernathy's Surgical Secrets, 3rd edition. Philadelphia, Hanley & Belfus, 1996, p. 206-211.
4. **Finlayson** CA, Ridge JA: Tumors of the Pharynx and Larynx. In Conn RB, Borer WZ, Snyder JW (eds): Current Diagnosis 9. Philadelphia, WB Saunders, Co., 1997.
5. **Finlayson** C: The Acute Abdomen. In Parsons P and Wiener-Kronich J (eds):Critical Care Secrets. Philadelphia, Hanley & Belfus, 1998, p. 377.
6. Heimbach J and **Finlayson** CA: Management of the Palpable Breast Mass. In White M (ed): Hematology Oncology Secrets, Philadelphia, Hanley & Belfus, 1999.
7. **Finlayson** CA: Gastrointestinal Lymphoma. In Norton L, Steele G, Eiseman B (eds): Surgical Decision Making 4th edition. WB Saunders, Co., 2000.

8. **Finlayson** CA: Carcinoma of the Larynx. In Norton L, Steele G, Eiseman B (eds): Surgical Decision Making 4th edition. WB Saunders, Co., 2000.
9. **Finlayson** CA: Hodgkin's Disease and the Malignant Lymphomas. In Harken AH and Moore EE (eds): Abernathy's Surgical Secrets, 4th edition. Philadelphia, Hanley & Belfus, 2000.
10. **Finlayson** CA: Breast Masses. In Harken AH and Moore EE (eds): Abernathy's Surgical Secrets, 4th edition. Philadelphia, Hanley & Belfus, 2000.

Abstracts

1. **Finlayson** C, Hoffman J, Yeung R, Kessler H, Guttmann, M, Shaer A, Clair M: Intraoperative Ultrasound Does Not Improve Detection of Liver Metastases in Surgically Resectable Pancreatic Cancer, Pancreas Club, May, 1996.
2. Bowers SP, Pearlman NW, McIntyre RC, **Finlayson** CA, Heurd S: Cost-Effective Management of Gynecomastia, Southwest Surgical Congress, April 1998.
3. Rabinovitch R, **Finlayson** C, Pan Z, Franciose R, Humphries S, Norton L: Superiority of Radio-opaque Clips Compared to Ultrasound as Methods of Defining Lumpectomy Cavity Volumes for Breast Boost Planning, ASTRO, Oct. 1998.
4. **Finlayson** CA, MacDermott T, Sisney G: Ultrasound can estimate the pathologic size of breast tumors, 21st Annual San Antonio Breast Cancer Symposium, Dec. 1998.
5. Rabinovitch R, **Finlayson** C, Pan Z, Franciose R, Humphries S, Norton L: Superiority of Radio-opaque Clips Compared to Ultrasound as Methods of Defining Lumpectomy Cavity Volumes for Breast Boost Planning, 21st Annual San Antonio Breast Cancer Symposium, Dec. 1998.
6. Strzelczyk J, **Finlayson** C: Sentinel node biopsy: ALARA and other considerations, Radiation safety and ALARA considerations for the 21st century joint meeting of Health Physics Society, American Academy of Health Physics, International ALARA Symposium, National Registry of Radiation Protection Technologists, and Nuclear Suppliers Association, accepted for podium presentation February 6, 2001.
7. Marks JL, **Finlayson** CA, Singh M, Seligman PA: Clinical and Pathological Analysis of Locally Advanced Breast Cancer Before and After Neoadjuvant Chemotherapy. Presented at NACT, Carmel, CA, February 2001.
8. Gibans L, Rabinovitch RA, and **Finlayson** CA: Two portal tangential breast irradiation includes the bed of the sentinel lymph node in women receiving breast-conservation treatment for early-stage breast cancer. Accepted for oral presentation, American Radium Society, April, 2001.
9. **Finlayson** CA, MacDermott T, Arya J: Can specific preoperative counseling increase the likelihood a woman will choose post-mastectomy breast reconstruction? Accepted for oral presentation, Southwest Surgical Congress, April 2001.
10. Gonzalez RJ, McIntyre RC, Stiegmann G, **Finlayson** CA, MacDermott T, Pearlman N: Local recurrence after total mesorectal excision in a general surgery teaching service. Accepted for presentation at Southwest Surgical Congress, April 2001.
11. Singh M, Marks JL, Nawaz S, **Finlayson** CA, Seligman PA: Vascular Endothelial Growth Factor (VEGF) expression before and after neoadjuvant chemotherapy in locally advanced breast carcinoma, accepted for presentation.

12. Singh M, Marks JL, Nawaz S, **Finlayson** CA, Seligman PA: Mib-1 expression before and after neoadjuvant chemotherapy in locally advanced breast carcinoma, accepted for presentation.

Miscellaneous

2000 University of Colorado Cancer Center Annual Report
1999 University of Colorado Cancer Center Annual Report
1997 Esophagus Patient Care Evaluation, University Hospital
Esophagus Patient Care Evaluation, VAMC

POUDRE VALLEY HOSPITAL
1024 South Lemay Avenue
Fort Collins, Colorado 80524-3998
(970) 495-7000
www.pvhs.org

September 27, 2000

James Burch, M.S., Ph.D
Assistant Professor
Department of Environmental Health
Colorado State University
Fort Collins, CO 80523

Dear Dr. Burch:

I am pleased to have the opportunity to collaborate with you on your project: "The Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease". After Institutional Review Board approval, the Breast Diagnostic Center anticipates providing patient data and breast cyst fluid for biochemical analysis. I will also be available for consultation regarding data analysis and manuscript preparation. We expect to enroll between 40 and 60 patients per year.

If you have any questions, please contact me at 970-495-8600.

Sincerely,

Winfield M. Craven, M.D.
Co-Director
Breast Diagnostic Center
Poudre Valley Health Systems

WMC/lm

WINFIELD M. CRAVEN, M.D.

PII Redacted

[redacted]

POST GRADUATE TRAINING:

Fellowship in Angiography and
Interventional Radiology,
University of Colorado,
July 1989 to July 1990.

Chief Resident in Radiology,
University of Colorado,
July 1988 to July 1989.

Resident in Diagnostic Radiology,
University of Colorado,
July 1985 to July 1989.

Resident in General Surgery,
University of Washington (Seattle),
July 1982 to July 1984.

ADDITIONAL TRAINING:

Course in Radiology/Pathology,
Armed Forces Institute of Pathology,
Washington, DC.,
January 1988-February 1988.

TEACHING:

Clinical Faculty Member,
Department of Medical Imaging,
Oregon Institute of Technology,
August 1990 to June 1992.

Instructor in Radiology and Angiography,
University of Colorado,
July 1989 to July 1990.

PUBLICATIONS:

"Percutaneous Removal of a Gianturco
Coil from the Pulmonary Artery with
Use of Flexible Intravascular
Forceps", JVIR 1991; 2:105-106.

"Planned Delayed Nephrectomy After
Ethanol Embolization of Renal Carcinoma"
J. of Urology 1991; 146: 704-708.

BOARD CERTIFICATION:

American Board of Radiology,
Certificate of Added Qualifications,
Vascular/Interventional Radiology,
November 1996.
American Board of Radiology,
June 1989.
National Board of Medical Examiners,
July 1983.

AWARDS AND HONORS:

Outstanding Resident Award,
June 1989.
Rufus Choate Scholar,
January 1975 to June 1978.
Illinois State Scholar, 1974-1975.

MEDICAL EDUCATION:

University of Cincinnati,
College of Medicine,
Cincinnati, Ohio 1978 to1982,
Doctor of Medicine.

UNDERGRADUATE EDUCATION:

Dartmouth College,
Hanover, New Hampshire 1974 to1978,
Major: Anthropology, high distinction,
Bachelor of Arts with honors.

PROFESSIONAL ORGANIZATIONS:

Society of Cardiovascular and
Interventional Radiology.
Radiological Society of North America.
American College of Radiology.
Colorado Radiological Society.
Colorado Medical Society.
Larimer County Medical Society.

PROFESSIONAL EMPLOYMENT:

Medical Director
Harmony Imaging Center,
Fort Collins, CO 11/00 - present.

Co-Director
Breast Diagnostic Center,
Poudre Valley Health System,
Fort Collins, CO 6/93 - present.

Radiologist
Poudre Valley Health System,
Fort Collins, CO 6/92 - present.

Radiologist
Merle West Medical Center,
Klamath Falls, OR 8/90 - 6/92.

LICENSURE: Colorado, Nebraska.

ATTACHMENT C. OLD CONSENT FORMS

Oct. 1997 version

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE
COLORADO STATE UNIVERSITY

CONSENT FORM

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

Investigators:

Benjamin O. Anderson, MD, Assistant Professor, Dept. Surgery, Univ. Wash. School of Med (543-3680)
Julie R. Gralow, MD, Assistant Professor, Medicine/Oncology. Univ. Wash School of Med (598-4100)
James B. Burch, MS, Ph.D., Res. Assoc., Dept. of Environmental Health, Colo. State Univ., (970) 491-6178
Robert Wells, Ph.D., Assoc. Prof., Dept. of Radiological Health Sciences, Colo. State Univ., (970-491-1087)
Sharon Bennett, BA, Research Study Coordinator, University of Washington Medical Center (598-4100)

University of Washington Medical Center Medical Oncology 24-hour emergency phone number: (206) 548-6180 (ask the Operator to page the Oncology Fellow on call).

INVESTIGATORS STATEMENT

Purpose and Benefits: Melatonin, which is a substance found naturally in the body, has been shown to slow the growth of cancer cells. How melatonin works on cancer cells is not known. This study will look at the relationship between melatonin and cystic breast disease (CBD) to better understand its effect on breast cancer. It will also study other proteins found naturally in the body, called growth hormones and growth factors, which are found in breast fluid and how they effect melatonin. You have been told by your doctor that you have CBD. In order to treat your cysts, your doctor will need to remove the fluid inside these cysts with a needle, this is also known as an aspiration. You have consented to this aspiration and are being asked to give part of this breast cyst fluid (BCF) for this study.

You will not receive any direct benefit from participating in this study, however, the results of this study may provide researchers with a better understanding of CBD and its relation to breast cancer.

Procedures: If you agree to be in this study, we will ask you to fill out a questionnaire to provide us with information regarding your breast cancer risk factors. Some of these risk factors include information such as whether or not you have had breast aspirations before, whether or not there is a history of breast cancer in your family, your history of menstruation, abortion, pregnancy, and hormone use. The questionnaire is voluntary, which means you do not have to fill it out. Should you agree to fill out the questionnaire, it will only take about 5-10 minutes. You do not have to answer all the questions.

The information that you give us from the questionnaire will be entered into the University of Washington Breast Cancer Epidemiologic Register and Neoplastic Index (BERNI). Your identity will be kept confidential, which means that researchers who use the database to look up information will not be able to identify you by your name. You will only be identified by a number. This data will be used by researchers doing breast cancer studies who need information about breast cancer risk factors from women with different backgrounds. Information entered into the database will stay in the database for an indefinite length of time for researchers to use.

After your doctor collects your BCF, part of the sample you donate for research (which will be no more than approximately 10 milliliters or equal to about 1 tablespoon) will be stored in the University of Washington Breast Cancer Tumor Bank for an indefinite length of time. The other part of your BCF sample will be shipped to Colorado State University where it will be tested for melatonin levels and related growth factors and hormones. Any BCF that is not used at Colorado State University will be destroyed after approximately 3 years. The samples that will be sent to CSU will be linked to information stored about you in the database. However, this link will be coded such that the researchers at CSU will not know your identity unless you give your permission as described below.

To follow your long-term health status, we would like to review the Washington State Cancer Registry, or another cancer registry in case you move from Washington State, in the future. In order to do this, we will [redacted] and Social Security number. Only the investigators will have access to this information and to the cancer registry data. You will not be contacted by any investigators at CSU regarding this study or any future studies. Providing us with this information is voluntary.

Subject Initials _____ **Witness Initials** _____

Other Information: Your participation in this study is voluntary. You can withdraw from this study at any time. You can withdraw your fluid sample from the University of Washington Breast Cancer Tumor Bank at any time, if it has not already been used. You will not receive payment for being in this study. You will not be charged for study procedures. Your costs for treatment of your cysts are yours and your insurance agency's responsibility. Giving the study team your date of birth and Social Security number is optional:

_____ I give my permission for the study team to use my name, date of birth, and Social Security number to access and review the Washington State or another cancer registry.

_____ I do not agree to the use of my name, date of birth, or Social Security number to access a cancer registry.

We will keep all data from this study indefinitely. Information you provide for the database will be coded so that only the study team will know your identity. The study team will keep the link between your identity and the data in locked files, indefinitely. Representatives from the sponsor of this study, the U.S Army Medical Research and Materiel Command, are eligible to review research records as a part of their responsibility to protect human subjects in research. If we publish the results of this study, we will not use your name. You will not be contacted by Colorado State University.

Investigator's Signature

_____
Physician/Investigator Name (Print or Type)

_____ _____
Signature of Physician/Investigator Date

Subject's Statement and Signature: The study described above has been explained to me. I understand that there is a possibility that the cyst fluid sample I am providing for this study may also be used in other research and could potentially have some commercial use. I am 18 years of age or older and voluntarily agree to participate in this study. I have had an opportunity to ask questions. I understand that if I have questions or concerns about this research or about my rights as a subject that I can contact Sharon Bennett (206 598-4100) or one of the other investigators listed above. I will receive a signed copy of this consent form.

_____
Subject Name (Print or Type)

_____ _____
Signature of Subject Date

_____ _____
Social Security Number (Optional) Date of Birth: MM /DD/YY (Optional)

_____
Permanent Address: Street

_____ _____
State Zip Code

Witness' Signature:

_____
Witness Name (Print or Type)

_____ _____
Signature of Witness Date

Copies to: Subject
Investigator's File

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE
COLORADO STATE UNIVERSITY

Sample Donation Form

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

I voluntarily and freely donate my breast cyst fluid to the University of Washington School of Medicine and Colorado State University and give up all right, title, and interest in this fluid.

Investigator's Signature:

_____
Physician/Investigator Name (Print or Type)

_____ _____
Signature of Physician/Investigator Date

Subject's Signature:

_____
Subject Name (Print or Type)

_____ _____
Signature of Subject Date

_____ Subject
Permanent Address: Street

_____ _____
State Zip Code

Witness' Signature:

_____
Witness Name (Print or Type)

_____ _____
Signature of Witness Date

Apr. 7, 2000 version

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE
COLORADO STATE UNIVERSITY

CONSENT FORM UW

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

Investigators:

Benjamin O. Anderson, MD	Associate Professor	UW Department of Surgery	206-543-3680
Julie R. Gralow, MD	Assistant Professor	UW Medicine/Oncology	206-598-4100
James B. Burch, MS, PhD	Dept. of Environ Health	Colorado State Univ.	970-491-6148
Robert Wells, PhD	Associate Professor	CSU Dept. of Radiology	978-491-1087
Beth Aaron, RN	Research Coordinator	UW Department of Surgery	206-543-9322

University of Washington Medical Center Medical Oncology 24-hour emergency phone number: 206-598-6180 Ask that the Oncology Fellow "on-call" be paged.

INVESTIGATORS' STATEMENT

Purpose and Benefits

Melatonin, which is a substance found naturally in the body, has been shown to slow the growth of cancer cells. How melatonin works on cancer cells is not known. This study will look at the relationship between melatonin and cystic breast disease (CBD) to better understand its effect on breast cancer. It will also study other proteins found naturally in the body, called growth hormones and growth factors, which are found in breast fluid and how they effect melatonin. Your doctor has told you that you have CBD. In order to treat your cysts, your doctor will need to remove the fluid inside these cysts with a needle; this is also known as an aspiration. You have consented to this aspiration and are being asked to give part of this breast cyst fluid (BCF) for this study.

You will not receive any direct benefit from participating in this study, however, the results of this study may provide researchers with a better understanding of CBD and its relation to breast cancer.

Procedures

If you agree to be in this study, we will ask you to fill out a questionnaire to provide us with information regarding your breast cancer risks factors. Some of these risk factors include information such as whether or not you have had breast aspirations before, whether or not there is a history of breast cancer in your family, your history of menstruation, abortion, pregnancy and hormone use. The questionnaire is voluntary which means you do not have to fill it out. Should you agree to fill out the questionnaire, it will only take about 5-10 minutes. ~~You may skip any question you do not want to answer.~~ The information that you give us from the questionnaire will be entered into ~~a computer database called the University of Washington Breast Cancer Epidemiologic Register and Neoplastic Index (BERNI).~~ Your identity will be kept confidential which means researchers who use the database to look up information will not be able to identify you by your name. You will only be identified by a number. This data will be used by researchers doing breast cancer studies and who need information about breast cancer

_____ Witness's Initials

APPROVED

_____ Subject's Initials

03/27/00

APR 0 7 2000

UW Human Subjects
Review Committee

risk factors of women who come from many different backgrounds. The information that is entered into the database will stay in the database for an indefinite length of time for researchers to use.

After your doctor collects your BCF, part of the sample you donate for research (which will be no more than approximately 1 tablespoon) will be stored in the University of Washington Breast Cancer Tumor Bank for an indefinite length of time. The other part of your BCF will be shipped to Colorado State University (CSU) where it will be tested for melatonin levels and related growth factors and hormones. Any BCF that is not used at Colorado State University will be destroyed after approximately 3 years. The samples that will be sent to CSU will be linked to information stored about you in the database. However, this link will be coded such that the researchers at CSU will not know your identity unless you give permission as described below.

To follow your health status, the investigators at CSU would like to review the Washington State Cancer Registry or another cancer registry in case you move from Washington State, in the future. In order to do this, we will need your social security number (SSN). Only the investigators will have access to your SSN and the information from the cancer registry. You will not be contacted by any investigators at CSU in regards to this study or any other future studies. Giving us your SSN is voluntary.

Other Information

Your participation in this study is completely voluntary. You can withdraw from this study at any time. You can withdraw your fluid sample from the University of Washington Breast Cancer Tumor Bank at any time, if it has not already been used. You will not receive payment for being in this study. You will not be charged for any study procedures. Your costs for treatment of your cysts are yours and your insurance agency's responsibility. Giving the study team your Social Security Number and date of birth is optional.

In order to access and review the Washington State Cancer Registry, I agree to give the study team my: **(please check all of the lines that you agree with)**

_____ Social Security Number _____ Date of Birth

_____ I do not agree to give the study team my SSN or date of birth.

We will keep all data for this study indefinitely. Information you provide for the database will be coded so that only the study team will know your identity and the data will be in locked files indefinitely. If we publish the results of this study, we will not use your name. The sponsor of this study is the US Army Medical Research and Material Command. The sponsor will also have access to your study records as part of their responsibility to protect human subjects in research. You will not be contacted by Colorado State University.

_____ Witness's Initials

APPROVED

_____ Subject's Initials

03/27/00

APR 0 7 2000

UW Human Subjects Review Committee

Investigator's Signature

Name of Physician/Investigator (printed or typed) Date

Signature of Physician/Investigator

Subject's Statement and Signature

The study described above has been explained to me. I understand that there is a possibility that the cyst fluid sample I am providing for this study may also be used in other research and could potentially have some commercial use in the future. I voluntarily give up all right, title, and interest to any future developments. I am 18 years of age or older and I voluntarily consent to participate in this study. I have had an opportunity to ask questions. I understand that future questions I may have about the research or about my rights as a research subject will be answered by contacting Beth Aaron RN (206-543-9233) or one of the investigators listed above. I will receive a signed copy of this consent form.

Subject Name (printed /typed) Date

Subject Signature

Subject's permanent mailing address, city, state, zip code

Social Security Number (**OPTIONAL**) Date of birth mm/dd/yy (**OPTIONAL**)

Witness Name (printed/typed) Date

Witness Signature

Copies to: Subject
Investigator's File

_____ Witness's Initials _____ Subject's Initials

03/27/00

APPROVED

APR 0 7 2000

UW Human Subjects Review Committee

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE
COLORADO STATE UNIVERSTIY

SAMPLE DONATION FORM

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

I voluntarily and freely donate any and all breast cyst fluid samples to the University of Washington and Colorado State University. I understand that there is a possibility that the breast cyst fluid sample(s) that I am providing under this study may also be used for other research studies and could potentially have some commercial applicability in the future. I hereby relinquish all rights, title, and interest in said items.

Signature of Subject _____ Date _____

Printed name of Subject _____

Permanent Address of the Subject _____

Signature of Witness _____ Date _____

Printed Name of Witness _____

3/24/2000

APPROVED

APR 07 2000

UW Human Subjects Review Committee

ATTACHMENT D. REVISED CONSENT FORMS

Consent Form Approval

Allan Prochazka, MD/Steve Bartlett, R.Ph, Co-Chairs, COMIRB
Christopher Kuni, MD/Ken Easterday, R.Ph, Co-Chairs, COMIRB
Adam Rosenberg, MD/David Lawellin, Ph.D., Co-Chairs, COMIRB

Date: Valid Through:_____

Colorado Multiple Institutional Review Board

Project Title: Role of Melatonin In the Prevention of Breast Cancer in Patients with Cystic Breast Disease

COMIRB#: 00-596

Principal Investigator: Christina A. Finlayson, MD
SUBJECT CONSENT FORM
DATE: August 29, 2001 Version 3

Project Description: You are being asked to take part in a research study because you have Cystic Breast Disease. Melatonin, which is a substance found naturally in the body, has been shown to slow the growth of cancer cells. How melatonin works on cancer cells in not known. This study will look at the relationship between melatonin and cystic breast disease (CBD) to better understand its effect on breast cancer. It will also study other proteins found naturally in the body, called growth hormones and growth factors, which are found in breast fluid and how they affect melatonin. In order to treat your cysts, your doctor will need to remove fluid inside these cysts with a needle; this is also known as an aspiration. You have consented to this aspiration and are being asked to give part of this breast cyst fluid (BCF) for this study. Your participation is completely voluntary.

Procedures: If you agree to participate in this study, your BCF sample will be frozen and shipped to Colorado State University, where it will be tested for melatonin levels and related growth factors and hormones. This information will be combined with questionnaire information in the analysis. Any BCF that is not used up by the tests by Colorado State University will be stored at Colorado State University indefinitely. These samples may be analyzed for other substances related to melatonin or breast cancer. If in the future you decide you wish to withdraw from the study, you may contact one of the study investigators. Any remaining BCF will then be removed from storage and discarded, and the link between your name and the database will be destroyed. We will ask you to fill out a questionnaire to provide us with information regarding your breast cancer risk factors. Risk factors include information such as whether or not you

have had breast aspirations before, whether or not there is a history of breast cancer in your family, your history of menstruation, abortion, pregnancy, and hormone use. Completing the questionnaire is voluntary. If you do not want to answer a question, you can skip it. It should take about 5-10 minutes to fill out the questionnaire. Information from the questionnaire will be entered into a computerized database at Colorado State University. You will only be identified by a number and the list linking your name and identification number will be kept in locked storage in the custody of a study investigator. Information in the database will stay there for an indefinite length of time.

To follow your long-term health status, we would like to review the Colorado State Cancer Registry, or another cancer registry in case you move from Colorado in the future. In order to do this, we request permission to use your name, date of birth, and Social Security number to access the registry. After we determine your case status from the registry, the link between your identity and the data will be destroyed. You will not be contacted by CSU investigators regarding this study or future studies. Just like your questionnaire, the personal information you provide will be kept confidential. Only the investigators will have access to this information and to the cancer registry.

Sample Size: Approximately 150 patients will be enrolled nationally and up to 125 patients through the University of Colorado Health Sciences Center in Denver.

Discomforts and Risks

Your doctor has recommended that you undergo breast aspiration. You would have this procedure regardless of whether you choose to participate in the CSU study. The principal risk from participating in the CSU study is a loss of confidentiality. To minimize this risk, you will be assigned a code number and your data will be linked to the code. Your name or other personal identifiers will not be entered into the study database. Consent forms and the list linking the code number to your name will be kept in locked storage in the custody of a study investigator. The risks associated with the breast aspiration procedure are summarized below.

> **Breast Aspiration:** Although benign, some simple cysts can be painful or they can be large enough to be bothersome. Some cysts contain debris or areas of solid tissue and these are referred to "complex" cysts. Complex cysts have some increased chance, although small, of being cancerous. Therefore, ultrasound guided cyst drainage may be recommended by your doctor or by the radiologist to relieve the cyst pressure or to evaluate a complex cyst further.

During ultrasound guided cyst aspiration, the skin over the cyst is cleaned and numbed with local anesthetic. The radiologist identifies the cyst using ultrasound. A thin needle is directed into the cyst and the cyst is drained.

Complex cysts can disguise themselves by looking like a solid mass. Cyst aspiration may be attempted to determine if the lesion is a complex cyst. If the cyst drains completely, no other procedure is needed. If the cyst does not drain, (thus, it is solid), biopsy, either surgical excisional biopsy or ultrasound guided needle core biopsy should be performed.

When the needle is inserted into the breast mass you may feel pain. You may have bleeding associated with this procedure. It is possible to develop an infection or a pnemothorax.

There may also be risks that are currently unforeseeable.

Benefits: You will receive no health benefit from participating in this study and there are risks as mentioned in the risk section.

Source of Funding: The U.S. Army Medical Research and Material Command under the Breast Cancer Research Program.

Cost to Subject: There is no cost for participating in this study. There will be no charge for procedures or drugs required by the study. You will not be paid for your participation in the study.

Study Withdrawal: You may choose not to enter the study or withdraw from the study at any time and your doctor will continue to take care of you without the loss of benefits to which you are entitled. Your doctor may also choose to withdraw you from the study at any time if s/he feels that it would be harmful to your health for you to continue or the side effects are too severe. You can withdraw your fluid sample from this study at any time, if it has not already been used up by the tests at CSU. If in the future you decide you wish to withdraw from the study, you may contact one of the study investigators. Any remaining BCF will then be removed from storage and discarded, and the link between your name and the database will be destroyed. Significant new findings that relate to your participation in this study will be discussed with you.

Invitation for Questions: You will receive a copy of this consent form. Please ask questions about this research or consent either now or in the future. You may direct your questions to Dr. Christina Finlayson at (303) 315-8671. If you have questions regarding your rights as a research subject, please call the Colorado Multiple Institutional Review Board (COMIRB) office at (303) 724-1055.

Confidentiality: Your physician/investigator and Colorado State University will treat your identity with professional standards of confidentiality. However, information from this study will be submitted to the sponsor and if appropriate to the U.S. Food and Drug Administration. It may also be submitted to governmental health agencies in other countries that have the authority to regulate the study. Medical records which identify you and the consent form signed by you will be inspected and/or copied by:

- The sponsor
- An agent for the sponsor

And may be inspected or copied by:

- The FDA
- Department of Health and Human Services (DHHS) agencies
- Governmental agencies in other countries which have authority to regulate the study
- COMIRB
- The Western Institutional Review Board

Injury and Compensation: If you are hurt by this research, we will provide medical care if you want it, but you will have to pay for the care that is needed. You will not be paid for any other loss as result of the injury, such as loss of wages, pain and suffering. Further information can be obtained by calling Dr. Christina Finlayson at (303) 315-8671.

Authorization: I have read this paper about the study or it was read to me. I understand the possible risks and benefits of this study. I know that being in this study is voluntary. I choose to be in this study: I know I can stop being in the study and I will still get the usual medical care. I will get a copy of this consent form. (initial all the previous pages of the consent form)

____I give my permission for the study team to use my name, date of birth, and Social Security number to access and review the Colorado State or another cancer registry.

____I do not agree to the use of my name, date of birth, or Social Security number to access a cancer registry.

_____
Subject Name (Print or Type)

_____ _____
Signature of Subject Date

_____ _____
Social Security Number (Optional) Date of Birth: MM/DD/YY (Optional)

_____
Permanent Address: Street

_____ _____ _____
City State Zip

_____ _____
Witness Signature Date

_____ _____
Consent form explained by Date

_____ _____
Investigator Signature Date

SAMPLE DONATION FORM

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

I voluntarily and freely donate any and all breast cyst fluid samples to the University of Colorado Health Sciences Center and Colorado State University. I understand that there is a possibility that the breast cyst fluid sample(s) that I am providing under this study may also be used for other research studies and could potentially have some commercial applicability in the future. There are no plans to provide financial compensation to me should this occur.

_____ _____

Signature of Subject Date

_____

Printed name of Subject

_____

Permanent Address of the Subject

_____ _____

Signature of Witness Date

_____

Printed Name of Witness

POUDRE VALLEY HOSPITAL
COLORADO STATE UNIVERSITY

CONSENT FORM

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

Investigators:

Winfield Craven, MD, Fort Collins Radiologic Associates (970) 495-8600
James B. Burch, MS, Ph.D., Asst. Prof., Dept. of Environmental Health, Colo. State Univ. (970) 491-6178
Robert Wells, Ph.D., Assoc. Prof., Dept. of Radiological Health Sciences, Colo. State Univ. (970) 491-1087
Kim Woods-McCormick, Research Coordinator, Poudre Valley Hospital (Phone: 495-7333)

Poudre Valley Hospital 24-hour phone number: (970) 495-7000

INVESTIGATORS STATEMENT

Purpose and Benefits: Melatonin, is a substance found naturally in your body that slows the growth of cancer cells. How melatonin works on cancer cells is not known. This study will look at the relationship between melatonin and cystic breast disease (CBD) to better understand their relation with breast cancer. It will also study other hormones and growth factors that are found in breast fluid and how they effect melatonin. You have been told by your doctor that you have CBD. In order to treat your cysts, your doctor will need to remove the fluid inside these cysts with a needle. You have consented to this procedure and are being asked to give part of this breast cyst fluid (BCF) for research. You will not receive any direct benefit from participating in this study. However, the results of this study may provide researchers with a better understanding of CBD and its relation to breast cancer.

Procedures: If you agree to be in this study, your BCF sample will be given to Colorado State University where it will be tested for melatonin and related growth factors and hormones. This information will be combined with questionnaire information in our analysis. **If your doctor has your sample tested for the presence of unusual cells, we will also include that information in our analysis.** Any BCF that is not used will be **stored at Colorado State University indefinitely. These samples may be analyzed in the future for other agents related to melatonin or breast cancer.** We will ask you to fill out a questionnaire to provide us with information regarding your breast cancer risk factors. Risk factors include information such as whether or not you have had cyst fluid removed before, your history of menstruation, pregnancy, and hormone use, and whether or not there is a history of breast cancer in your family. Completing the questionnaire is voluntary. If you do not want to answer a question, you can skip it. It should take about 5-10 minutes to fill out the questionnaire. Information from the questionnaire will be entered into a computerized database at Colorado State University. You will only be identified by a number and the list linking your name and identification number will be kept confidential. Information in the database will stay there for an indefinite length of time.

To follow your long-term health status, we would like to review the Colorado State Cancer Registry, or another cancer registry in case you move from Colorado in the future. In order to do this, we request permission to use your name, date of birth, and Social Security number to access the registry. This information will only be used to access a registry. You will not be contacted by CSU investigators regarding this study or future studies. Just like your questionnaire, the personal information you provide will be kept confidential. Only the investigators will have access to this information and to the cancer registry data.

Other Information: Your participation in this study is voluntary. You can withdraw from this study at any time. You can withdraw your fluid sample from this study at any time, if it has not already been used up by the tests. If in the future you decide you wish to withdraw from the study, you may contact one of the study investigators. Any remaining BCF will then be removed from storage and discarded. and the link between your name and the database will be destroyed. You will not receive payment for being in this study. You will not be charged for study procedures. Your costs for the treatment of your cysts are yours and your insurance agency's responsibility.

_____ I give my permission for the study team to use my name, date of birth, and Social Security number to access and review the Colorado State or another cancer registry.

_____ I do not agree to the use of my name, date of birth, or Social Security number to access a cancer registry.

Subject Initials _____ Witness Initials _____

We will keep information from this study indefinitely. Information you provide for the database will be coded so that only the study team will know your identity. The study team will keep the link between your identity and the data in locked files. **After we determine your case status from the registry, the link between your identity and the data will be destroyed.** Representatives from the sponsor of this study, the U.S Army Medical Research and Materiel Command, are eligible to review research records as a part of their responsibility to protect human subjects in research. If we publish the results of this study, we will not use your name. You will not be contacted by Colorado State University.

Investigator's Signature

_____
Physician/Investigator Name (Print or Type)

_____ _____
Signature of Physician/Investigator Date

Subject's Statement and Signature: The study described above has been explained to me. I understand that there is a possibility that the cyst fluid sample I am providing for this study may also be used in other research and could potentially have some commercial use. There are no plans to provide financial compensation to me should this occur. I am 18 years of age or older and voluntarily agree to participate in this study. I have had an opportunity to ask questions. If in the future I decide I wish to withdraw from the study, I may contact one of the study investigators. Any remaining BCF will then be removed from storage and discarded, and the link between my name and the database will be destroyed. I understand that if I have questions or concerns about this research or about my rights as a subject that I can contact Dr. Craven (495-8600) or one of the other investigators listed above. (You will receive a signed copy of this consent form).

_____
Subject Name (Print or Type)

_____ _____
Signature of Subject Date

_____ _____
Social Security Number Date of Birth: MM /DD/YY

_____
Permanent Address: Street

_____ _____
State Zip Code

Witness' Signature;

_____
Witness Name (Print or Type)

_____ _____
Signature of Witness Date

Copies to: Subject
Investigator's File

POUDRE VALLEY HOSPITAL
COLORADO STATE UNIVERSITY

Sample Donation Form

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

I voluntarily and freely donate my breast cyst fluid to the Poudre Valley Hospital and Colorado State University. I understand that there is a possibility that the breast cyst fluid sample(s) that I am providing under this study may also be used for other research studies and could potentially have some commercial applicability in the future. There are no plans to provide financial compensation to me should this occur.

Investigator's Signature:

_____
Physician/Investigator Name (Print or Type)

_____ _____
Signature of Physician/Investigator Date

Subject's Signature:

_____
Subject Name (Print or Type)

_____ _____
Signature of Subject Date

_____
Subject Permanent Address: Street

_____ _____
State Zip Code

Witness' Signature:

_____
Witness Name (Print or Type)

_____ _____
Signature of Witness Date

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE
COLORADO STATE UNIVERSITY

CONSENT FORM

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

Investigators:			
Benjamin O. Anderson, MD	Associate Professor	UW Department of Surgery	206-543-3680
Julie R. Gralow, MD	Assistant Professor	UW Medicine/Oncology	206-598-4100
James B. Burch, MS, PhD	Assistant Professor	CSU Environ. Health Dept.	970-491-6148
Robert Wells, PhD	Associate Professor	CSU Dept. of Radiology	970-491-1087
Participating Physicians:			
Connie Lehman MD	Assistant Professor	UW Dept. of Radiology	206-288-2046
Katherine Dee MD	Assistant Professor	UW Dept. of Radiology	206-288-7200
Diane Georgian-Smith MD	Assistant Professor	UW Dept. of Radiology	206-543-3320
Anne Freitas MD	Associate Professor	UW Dept. of Radiology	206-543-3320
Hilarie Gutierrez MD	Acting Instructor	UW Dept. of Radiology	206-543-3320
Eva Khan ARNP	Clinician	Women's Health Care Center	206-598-5500
Research Coordinators:	**Office** 206-543-9322		
Beth Aaron, RN	Research Coordinator	UW Department of Surgery	206-559-8782
Mari Nosal, MSW	Study Coordinator	UW Department of Surgery	206-989-5580

University of Washington Medical Center Medical 24-hour emergency phone number: 206-598-6190 Ask that Dr. Anderson be paged.

INVESTIGATORS' STATEMENT

We are asking you to be in a research study. The purpose of this consent form is to give you the information you will need to help you decide whether or not to be in the study. Please read the form carefully. You may ask questions about the purpose of the research, what we would ask you to do, the possible risks and benefits, your rights as a volunteer, and anything else about the research or this form that is not clear. When all your questions have been answered, you can decide if you want to be in the study or not. This process is called 'informed consent.'

Purpose and Benefits

Melatonin, which is a substance found naturally in the body, has been shown to slow the growth of cancer cells. How melatonin works on cancer cells is not known. This study will look at the relationship between melatonin and cystic breast disease (CBD) to better understand its effect on breast cancer. It will also study other proteins found naturally in the body, called growth hormones and growth factors, which are found in breast fluid and how they affect melatonin. Your doctor has told you that you have CBD. In order to treat your cysts, your doctor will need to remove the fluid inside these cysts with a needle; this is known as an aspiration. You have consented to this aspiration and are being asked to give part of this breast cyst fluid (BCF) for this study.

You will not receive any direct benefit from participating in this study; however, the results of this study may provide researchers with a better understanding of CBD and its relation to breast cancer.

_____ Witness's Initials _____ Subject's Initials

Procedures

If you agree to be in this study, we will ask you to fill out a questionnaire to provide us with information regarding your breast cancer risks factors. Some of the questions asked are sensitive, such as whether or not you have had breast aspirations before, whether or not there is a history of breast cancer in your family, your history of menstruation, abortion, pregnancy and hormone use. The questionnaire is voluntary which means you do not have to fill it out. Should you agree to fill out the questionnaire, it will only take about 5-10 minutes. You may skip any question you do not want to answer. The information that you give us from the questionnaire will be entered into a computer database called the University of Washington Breast Cancer Epidemiologic Register and Neoplastic Index (BERNI). This data will be used by researchers doing breast cancer studies who need information about breast cancer risk factors. The information that is entered into the BERNI database will stay in the database for an indefinite length of time for researchers to use.

After your doctor collects your BCF, part of the sample you donate for research (which will be no more than approximately 1 tablespoon) will be stored in the University of Washington Breast Cancer Tumor Bank for an indefinite length of time. The other part of your BCF will be shipped to Colorado State University (CSU) where it will be tested for melatonin levels and related growth factors and hormones. Any BCF that is not used up by the tests will be stored at Colorado State University indefinitely. These samples may be analyzed in the future for other agents related to melatonin or breast cancer. The samples sent to CSU will be linked to information stored about you in the database. However, this link will be coded such that the researchers at CSU will not know your identity unless you give permission as described below. The link between you and your sample will be kept indefinitely. If in the future you decide you wish to withdraw from the CSU study, you may contact one of the CSU investigators. Any remaining BCF at CSU will then be removed from storage and discarded, and the link between your name and the database will be destroyed.

To follow your health status, the investigators at CSU would like to review the Washington State Cancer Registry or another cancer registry in case you move from Washington State, in the future. In order to do this, they will need your social security number (SSN). Only the investigators will have access to your SSN and the information from the cancer registry. You will not be contacted by any investigators at CSU with regard to this study or any other future studies. Giving your SSN is voluntary.

Risks, Stresses or Discomforts

Some people feel that providing information for research is an invasion of privacy. Some women may feel that the questions asked are sensitive.

Other Information

Your participation in this study is completely voluntary. You can withdraw from this study at any time. You can withdraw your fluid sample from the University of Washington Breast Cancer Tumor Bank at any time, if it has not already been used up by the tests. Future research using your cyst fluid may lead to the development of commercial products. You will not share in any profits that this work may produce. You will not receive payment for being in this study. You will not be charged for any study procedures. Your costs for treatment of your cysts are yours and your insurance agency's responsibility. Giving the study team your Social Security Number and date of birth is optional:

In order to access and review the Washington State Cancer Registry, I agree to give the study team my: **(please check all of the lines that you agree with)**

_____ Social Security Number _____ Date of Birth

_____ I do not agree to give the study team my SSN or date of birth.

_____ Witness's Initials _____ Subject's Initials

We will keep all data for this study indefinitely. Information you provide for the database will be coded so that only the study team will know your identity. The written data will be in locked files; the computer database will be secured by a password only the researchers will have. After we determine your case status from the registry, the link between your identity and the data will be kept indefinitely. It will be kept in a computer file that is only available using a password. If we publish the results of this study, we will not use your name. The sponsor of this study is the US Army Medical Research and Material Command. The sponsor will also have access to your study records as part of their responsibility to protect human subjects in research. You will not be contacted by Colorado State University.

Investigator's Signature

_____ _____

Name of Physician/Investigator (printed or typed) Date

_____

Signature of Physician/Investigator

Subject's Statement and Signature

The study described above has been explained to me.
I understand that there is a possibility that the cyst fluid sample I am providing for this study may also be used in other research and could potentially have some commercial use in the future. There are no plans to provide financial compensation to me should this occur. I am 18 years of age or older and I voluntarily consent to participate in this study. I have had an opportunity to ask questions and I voluntarily consent to participate in this study. I understand that future questions I may have about the research can be answered by contacting Beth Aaron RN (206-543-9322), or Mari Nosal (206-989-5580 pager) or one of the investigators listed above. If I have questions about my rights as a subject, I may call the University of Washington Human Subjects Division at (206) 543-0098. I give the investigator permission to review my medical records as described above. I will receive a copy of this consent form.

_____ _____

Subject Name (printed /typed) Date

_____

Subject Signature

_____

Subject's permanent mailing address, city, state, zip code

_____ _____

Social Security Number (**OPTIONAL**) Date of birth mm/dd/yy (**OPTIONAL**)

_____ _____

Witness Name (printed/typed) Date

_____

Witness Signature

Copies to: Subject
Investigator's File

_____ Witness's Initials _____ Subject's Initials

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE
COLORADO STATE UNIVERSTIY

SAMPLE DONATION FORM

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

I voluntarily and freely donate any and all breast cyst fluid samples to the University of Washington and Colorado State University. I understand that there is a possibility that the breast cyst fluid sample(s) that I am providing under this study may also be used for other research studies and could potentially have some commercial applicability in the future. There are no plans to provide financial compensation to me should this occur.

_____ _____

Signature of Subject Date

_____

Printed name of Subject

_____

Permanent Address of the Subject

_____ _____

Signature of Witness Date

_____

Printed Name of Witness

_____ Witness's Initials _____ Subject's Initials

ATTACHMENT E. UNIVERSITY OF WASHINGTON IRB APPROVAL FOR BERNI DATABASE

Institutional Review Board
Fred Hutchinson Cancer Research Center
IRB Committee A (01) ☒
IRB Committee B (02XS) ☐
Assurance #: M1008

CONTINUATION REVIEW REPORT

Institutional Review Office
Mailstop: D5-100
Telephone: (206) 667-5784
Date Received: IRO RECD JAN 09
Agenda Date: 2-14-01

TITLE OF PROTOCOL:

BREAST CANCER DATABASE AND BIOLOGIC RESOURCE BANK

73

PRINCIPAL INVESTIGATOR: Ben Anderson, M.D.
MAILSTOP: C3-168
DIVISION of Clinical Research

IR FILE #: 4472

I. CURRENT STATUS OF PROTOCOL * (*Check one*):

- ☑ Accrual Continuing
- ☐ Accrual complete with treatment intervention and/or participant interviews/surveys continuing
- ☐ No Subjects enrolled and no new risk(s) identified **
- ☐ Accrual complete, data analysis only, no further research intervention **
- ☐ Cooperative Review **
- ☐ Category 3, Standard Treatment Protocol – Closure Requested **
- ☐ Accrual complete, no further treatment intervention or follow-up required – closure requested. Please submit a Progress Report only. Include a final summary under "findings to date". (No protocol or consent required).
- ☐ Study never activated, closure requested – (Submit this page only with PI Initial/date)

PI initial _____ date _____

- ☐ Other, Please describe: _____

* *Please see Continuation Review Report Request Letter for Copy Instructions*
** *Qualifies for Expedited Review*

II. Does this file include ancillary studies in addition to the original IRB-approved study proposal? ☐yes ☑no
If yes, please complete an annual progress report for the original study and each ancillary study. Be sure to attach the relevant protocol, questionnaire(s), consent form(s), etc., to each progress report.

III. Is Scientific Review Committee approval required for this study? ☐yes ☑no
If yes, please provide the most recent approval date _____.

IV. FUNDING UPDATE
Has this activity been previously reported to the funding agency? ☑yes ☐no
Has the funding source changed? ☐yes ☑no
If yes, please provide a supplemental sheet explaining the nature of the change.
Is this research funded by a federal funding source? ☐yes ☑no
If yes, please specify funding source: _____ Identification #: _____ Project #: _____

V. PERFORMANCE SITES
Is this a multi-site trial where FHCRC serves as the Coordinating Center for this research activity? ☐yes ☑no
If Yes, Is FHCRC the Applicant Organization? ☐yes ☐no If yes, please contact the IRO Specialist at ext. 5900 about IRB documentation requirements for each performance site.

FINAL APPROVAL: See attached materials. Type of Review: Full ☐ Expedited ☑

JOAN CLARK, M.D., IRB CHAIR, COMM. A

Signature _____ Date Jan 15, 2001

Dates Of Approval 12-20-00 TO 12-19-01

VALID ONLY AS LONG AS APPROVED PROCEDURES ARE FOLLOWED

cc: Helen Devitt, C3-168

"irb\iro\forms.iro\applicat\annual1.doc " (Revised 3/1/00)

University of Washington Correspondence

INTERDEPARTMENTAL

Department of Medicine
Division of Oncology
Box 356043

January 8, 2001

TO: Institutional Review Board
Fred Hutchinson Cancer Research Center

FROM: Tove Thompson
University of Washington

RE: IR File 4472

Enclosed please find three copies of the Continuation Review Report for Dr. Ben Anderson's project titled, "Breast Cancer Database and Biologic Resource Bank." The primary review for this project is by the University of Washington Human Subjects Committee. Copies of the reviewed and approved annual renewal are attached to the first page of the Continuation Review Report as required for projects that under go cooperative review. If you have any questions concerning the report, please feel free to call me.

Telephone: *(206) 598-4935*
Fax: *(206) 598-4509*

HUMAN SUBJECTS STATUS REPORT – PLEASE RESPOND PROMPTLY

BOX FOR COMMITTEE USE ONLY
MASTER ☐ COMM. ☐ INVEST. ☐
96-3456-A 06
APPLICATION NO.

Julie Gralow, M.D.; Assistant Professor
Department of Medicine, Division of Oncology
Box 356043
598-7722, 598-4509, pink@u.washington.edu

RECEIVED
Human Subjects Division
NOV 13 2000
UW

96-3456-A: 05 Breast Cancer Tumor Bank

Human subjects approval for this activity expires on **December 22, 2000**. Please complete this form according to the instructions below and send it to the Human Subjects Division, Box 355752, at least **six weeks before the expiration date**. This Status Report form and other Human Subjects Division forms are available on our web site, http://depts.washington.edu/hsd. Please note: You may not recruit new human subjects or continue your activity with previously enrolled subjects unless you have active human subjects approval.

☐ **DO NOT RENEW**: Send **one** typed, completed and signed original Status Report form. Please note that you should maintain approval until the data analysis for this activity is complete.

X **RENEW**: Send **one** typed, completed and signed original and **eight** copies of the Status Report plus **nine** copies of each consent and assent form you are currently using (**two** copies for Committee E applications).

☐ No changes proposed

X Changes proposed (Please send nine copies of a memorandum describing the changes and nine copies of the revised consent form and any other revised study material.)

Status Report Checklist

X Nine copies of Status Report, signed by the principal investigator*
X Nine copies of the current consent and assent forms*
X Nine copies of memorandum describing any changes*
X Nine copies of revised consent or other revised materials*
X One copy of each new proposal for funding
X Status Report typed, not handwritten
☐ Radiation Safety Committee (543-0463) renewal requested, if appropriate
*three for Committee E applications

I acknowledge that this Status Report represents an accurate and complete description of my research.

Julie E Gralow MD — 11/9/00
Typed name and original inked signature of principal investigator — Date
Julie Gralow

BOX FOR COMMITTEE USE ONLY

Abad Quinn BK — 12/20/00 — Approve X — Disapprove ☐
Human Subjects Review Committee Signature — Date

Subject to the following conditions: Approval contingent on revised consent form as discussed on 1/3/01

Period of approval is one year, from 12/20/00 through 12/19/01

VALID ONLY AS LONG AS APPROVED PROCEDURES ARE FOLLOWED

A. Activity Status

X New subject enrollment still in progress
☐ Enrollment closed but subjects are still undergoing study procedures
☐ Enrollment closed, subjects have completed study procedures, but are still in follow-up
☐ Subject involvement completed, need approval for data analysis only
☐ Enrollment closed, study completed
☐ No enrollment, study never begun

B. Subject Numbers

	Normals (Controls)	Patients (Cases)
1. No. subjects you are approved to enroll	NA	NA
2. No. of subjects enrolled since initial approval	NA	1,328
3. No. of new subjects since last approval	NA	94
4. No. of subjects actively enrolled in the study	NA	NA
5. No. of additional subjects needed to complete study	NA	NA

C. Summaries

1. **Provide an abstract summarizing the purpose of this research activity, the procedures subjects will undergo, and a description of the subject populations.**

The purposes of this project are to develop:

1. A comprehensive longitudinal clinical database of breast cancer patients seen in the Surgery Breast Clinic or the Breast Cancer Specialty Center at the University of Washington Medical Center (BERNI = Breast Cancer Epidemiologic Register and Neoplastic Index).
2. A breast cancer tumor bank with tissue from breast cancer patients who have biopsies or resections at the University of Washington Medical Center.
3. A breast cancer patient serum and lymphocyte bank with blood products collected from breast cancer patients seen in the Surgery Breast Clinic or the Breast Cancer Specialty Center at the University of Washington Medical Center.

All breast cancer patients seen in the Breast Surgery Clinic or the Breast Cancer Specialty Center at the University of Washington Medical Center are approached about this project. Patients in the Breast Surgery Clinic are asked whether they are willing to allow some of their tumor tissue to be collected for the breast cancer tumor bank. If the patient consents, extra tissue from the biopsy or surgical specimen is collected, processed, cataloged and stored in a freezer housed at the Fred Hutchinson Cancer Research Center. Patients seen either in the Breast Surgery Center or at the Breast Cancer Specialty Center are approached concerning:

1. Inclusion of data about them in the longitudinal clinical database.
2. Willingness to provide a blood sample (approximately 50 mL) for the serum and lymphocyte bank.

The population of potential subjects includes all patients evaluated for breast cancer in either the Breast Surgery Clinic or the Breast Cancer Specialty Center at the University of Washington Medical Center.

2. **Provide a summary of your progress to date. If you have not yet enrolled subjects, please explain why. Send one copy of each manuscript based on the data from this study, written since the last approval.**

No manuscripts have been written. The following table describes the progress to date:

	To date	Since 12/22/1999
Entries into database (BERNI)	1328	94
Tissue samples collected	88	3
Serum samples collected	134	0
Lymphocyte samples collected	11	0

3. **Summarize any changes you have made during the last period of approval.**

No changes have been made since the last approval period.

4. **Describe changes in the risks or benefits to subjects over the last period of approval.**

There has been no change in the risks or the benefits to subjects over the last period of approval.

5. **If you propose changes in this activity for the next period of approval, attach nine copies of a memorandum describing the changes. Also attach nine copies of the revised consent or assent forms and any other revised study materials.**

D. **Adverse Events:** Provide this information about subjects enrolled under this HSRC approval for the period since your last report. If there were none, enter 0.

1. **No. of adverse events:** 0 **Explain how you handled each adverse event.**

Were any of these adverse events unexpected or more serious than expected? ☐ Yes ☐ No

If yes, did you send us an Adverse Event report? ☐ Yes ☐ No

If no, please download the form from our web site, complete it and send it to the Human Subjects Division immediately with this Status Report.

2. **No. of complaints:** 0 **Explain how you handled each one.**

3. **No. of subject withdrawals:** 0 **For each, explain why the subject chose to withdraw or why you withdrew the subject from the study.**

4. No. of protocol violations: 0 Explain how you handled each one.

E. Funding: Please review and update the grant and contract information on the following page(s).

Is there new funding proposed for this activity? X Yes ☐ No If yes, send us one complete copy of the proposal and explain in the space below if there are any differences between this new proposal and what is approved in this application.

There are no differences between the new proposal and what is approved in this application. The new proposal, if funded, will provide financial support for the work currently being done for this project. At the present time there is no on going funding for this project.

Please include all funding, current and pending, for this Human Subjects Application. For Center or Program grants, list the Principal Investigator and the Title for each separate project or core. If you wish to add a new funding proposal, also submit one entire copy of the new grant.

Funding Type:
☐ Research Grant ☐ Fellowship ☐ Training Grant ☐ Contract ☐ Other, specify:

Funding Agency:

Principal Investigator (on proposal):

Agency Number (if known):

Proposal Title:

Status: ☐ New ☐ Competing ☐ Non-Competing Start Date: End Date: Funded? ☐ Yes ☐ No

Submitted through GCS? ☐ Yes ☐ No If NO, explain

Funding Type:
☐ Research Grant ☐ Fellowship ☐ Training Grant ☐ Contract ☐ Other, specify:

Funding Agency:

Principal Investigator (on proposal):

Agency Number (if known):

Proposal Title:

Status: ☐ New ☐ Competing ☐ Non-Competing Start Date: End Date: Funded? ☐ Yes ☐ No

Submitted through GCS? ☐ Yes ☐ No If NO, explain

ATTACHMENT F. SURGICAL/PROCEDURAL CONSENT FORMS

I hereby authorize Dr. _____, and such assistants as may be designated, to perform:

_____
(NAME OF TREATMENT / PROCEDURE)

and any other related procedures or forms of treatment that they deem necessary for the welfare of:

_____
(NAME OF PATIENT)

I acknowledge that the University of Washington Medical Centers are teaching hospitals and that teachers, trainees and students may observe or participate in the care provided.

I consent to the administration of anesthesia and/or such drugs as may be necessary. I understand that all anesthetics involve risks of complication, serious injury, or rarely death from both known and unknown causes.

I consent to the administration of blood and/or blood products if deemed medically necessary. I understand that all blood and blood products involve risks of allergic reaction, fever, hives, and in rare circumstances infectious diseases such as hepatitis and HIV/AIDS. I understand that precautions are taken by the blood bank in screening donors and in matching blood for transfusion to minimize those risks.

OR, I REFUSE CONSENT FOR BLOOD AND BLOOD PRODUCTS. (DOCUMENT ON FORM UH 0399) | PATIENT INITIALS |

I consent to the examination, use and distribution for educational, scientific and research purposes by the University of Washington medical staff of all biological materials such as body fluids, tissues and organs removed during the course of the above treatment/procedure with privilege of ultimate use and disposal resting with said medical staff. I hereby acknowledge that all such biological materials may be used for the development of one or more research, diagnostic, or therapeutic products. I authorize such potential use and distribution at the discretion of the University of Washington medical staff.

I understand that each person reacts differently to treatments/procedures, therefore, the expected results of said treatment cannot be guaranteed. The physicians, surgeons, or dentists of the University of Washington has discussed to my satisfaction the following:

A. The nature and character of the proposed treatment/procedure.
B. The anticipated results of the proposed treatment/procedure.
C. The recognized alternative forms of treatment/procedure.
D. The recognized serious possible risks and complications of the treatment/procedure and of the recognized alternative forms of the treatment/procedure, including non-treatment.
E. The anticipated date and time of the proposed treatment/procedure.

Additional Comments: _____

_____ | PATIENT INITIALS |

My physician has offered to answer all inquiries concerning the proposed treatment/procedure. I understand that I am free to decline consent to the proposed treatment/procedure at any time.

HEALTH CARE PROVIDER OBTAINING CONSENT (PRINT NAME & INITIAL)			SIGNATURE OF PERSON GIVING CONSENT
DATE SIGNED	TIME	☐ A.M. ☐ P.M.	RELATIONSHIP TO PATIENT (IF APPLICABLE)

☐ PLEASE CHECK IF THIS IS A TELEPHONE MONITORED CONSENT.
THIS CONSENT WILL BE PERMANENTLY FILED IN THE PATIENT'S MEDICAL RECORD.

MRC.

NAME

003

UNIVERSITY OF WASHINGTON MEDICAL CENTERS
HARBORVIEW MEDICAL CENTER - UW MEDICAL CENTER
UNIVERSITY OF WASHINGTON PHYSICIANS
SEATTLE, WASHINGTON

SPECIAL CONSENT TO TREATMENT (DIAGNOSTIC & SURGICAL PROCEDURES, ANESTHESIA, MEDICAL TREATMENT & OTHER PROCEDURES)

UH 0173 REV DEC 98

UNIVERSITY HOSPITAL
4200 E. Ninth Avenue
Denver, CO 80262

CONSENT TO MEDICAL PROCEDURE (SURGERY, DIAGNOSTIC, THERAPEUTIC, ANESTHESIA)

Not to be used for experimental procedures

HOSPITAL #
PATIENT NAME

Date:_____

I. Surgery, Diagnostic, Therapeutic

I authorize Dr. _____ and/or any assistant working with this physician to conduct the following procedure described to me in plain language:_____

1. I have been advised that the following benefits might be reasonably expected from undergoing the recommended procedure:_____

2. I have been advised that the following risks and discomforts might reasonably be expected from undergoing the recommended procedure:_____

3. I have been advised that there are other risks, such as severe loss of blood, infection, heart failure, etc. to the performance of any surgical procedure. I have been advised that the reasonably anticipated result if I refuse to undergo the recommended procedure is as follows:_____

4. Alternative measures, including the decision not to undergo the procedure, have been explained to me in plain language and I have been advised that these, and their reasonably expected benefits, risks, and results are as follows

II. Blood

I understand that I may need a transfusion of blood or blood products. While the blood has been screened for hepatitis, HIV, and other abnormal antibodies, there is a remote risk of contracting these and other infectious disease from transfusions. Alternatives to transfusion, including the risks of not receiving this therapy, have been explained to me.

_____ I consent to transfusion therapy. _____ I refuse transfusion therapy.

III. Specimen Disposition

I authorize this health care facility to examine, photograph or preserve for scientific research or teaching purposes or to otherwise dispose of the tissue, limbs, organs or foreign objects resulting from the procedures authorized above

I understand that if I wish to retrieve embryo, fetus, or other pregnancy tissue for private funeral services, I must indicate such wishes on this consent form. If no preference is indicated, the pregnancy tissue will be disposed of by the Hospital. If applicable, initial your preference below:

_____ Please dispose of embryo, fetus, or pregnancy tissue.

_____ I wish to retrieve the embryo, fetus, or other pregnancy tissue for private funeral services. I will contact Decedent Affairs within 24 hours of the procedure or Hospital will dispose of pregnancy tissue.

I have been advised that the practice of medicine is not an exact science, and acknowledge that no guarantees have been made to me concerning the results of the procedure. I have been advised that, at any time prior to the performance of the operation or procedure, I may withdraw my consent and not have the procedure performed. I have been advised that I may delete any provisions in the form about which I do not agree or consent, and that I may add any provisions in the form which I want included in my consent. I have been given the opportunity to ask questions concerning this procedure and such questions have been answered to my satisfaction. All applicable blanks in this form were filled in prior to my signing it.

PHYSICIAN DECLARATION:
I believe the patient is an appropriate candidate to undergo the planned procedure. I have explained the risks and alternatives of the operation/procedure to the patient and have answered all the patient's questions and to the best of my knowledge, I feel the patient has been adequately informed and has consented.

_____
Signature of Patient or Legal Guardian

_____ _____
Signature of Informing Physician Relationship to Patient

_____ _____
Date Time Print Name of Patient

IF PATIENT SPEAKS A LANGUAGE OTHER THAN ENGLISH OR IS COMMUNICATIVELY DISABLED:

I have translated the information and advice presented orally to this patient by the person obtaining this consent form in _____ language and explained its contents to him/her. To the best of my knowledge and belief he/she understood this translation.

_____ _____ _____ _____
Interpreter Date Time Phone #

IV. Request for and Consent to Anesthesia Service

My anesthetic care will be provided by or under the supervision of a physician anesthesiologist. Anesthetic techniques recommended for my planned procedure are: _____

_____

_____. Anesthetics have rare complications such as pain during operation, allergic reactions, cardiac arrest, nerve damage, brain damage, paralysis, or other organ damage or death. Common side effects of general anesthesia include sore or scratchy throat, nausea and vomiting, muscle or joint pains or headaches. If undergoing regional anesthesia such as epidural or spinal blocks, I understand the risks of such procedures include pain at the needle site, pain during the operation, nerve and/or spinal damage or failure of the regional anesthesia, in which case, another type of anesthesia, usually general anesthesia, is substituted. I have been given the opportunity to ask questions concerning the recommended anesthesia and such questions have been answered to my satisfaction. I consent to the recommended anesthetic procedures except: _____

_____

_____
Signature of Patient or Legal Guardian

_____ _____
Signature of Informing Anesthesiologist Relationship to Patient

_____ _____
Date Time Print Name of Patient

Breast Diagnostic Center
at Harmony Imaging Center, LLC

2127 East Harmony Road • Fort Collins, Colorado 80528 • (970) 207-4700 • Fax (970) 207-4755

SURGERY OR OTHER PROCEDURE: I, _____ permit Dr. _____ and any other doctors or assistants needed to assist in performing the surgery or procedure my doctor has recommended. The surgery or procedure my doctor has recommended is _____
stereotactic or ultrasound-guided breast biopsy or ultrasound-guided cyst aspiration

I also allow the use of any anesthetics that may be necessary.

THIS SURGERY OR PROCEDURE HAS BEEN RECOMMENDED BECAUSE:
Breast mass or calcification

MY OTHER CHOICES ARE: **Surgical biopsy, follow-up mammography**

I UNDERSTAND THAT:

- Any surgery or procedure and the use of anesthesia have some risks. These risks can be serious and in rare cases result in death.
- Treatment results are not guaranteed and may not cure the condition.
- The risks listed below are the more common risks but are not all the possible risks associated with this operation or procedure.
- As is deemed necessary by the surgeon of record, medical product representatives may be present in the Operating Room solely to observe the use of that supplier's surgical and/or medical equipment.

RISKS: The most common risks are bleeding, infection, nerve injury, blood clots, heart attack, allergic reactions and pneumonia. Other risks of this particular operation or procedure include: _____

- **Possible infection**
- **Bleeding**
- **Inadequate sampling**

SURGERY PATIENTS

If during my surgery the doctor finds an unsuspected medical need, I permit him/her to provide the necessary treatment(s). My doctor has fully explained the surgical procedure in words I understand. I have read and fully understand this consent form, and all of my questions have been answered. **Do not sign unless you have read and thoroughly understand this form.**

Patient/
Responsible Party _____ Date _____ Time _____

Witness _____ Date _____ Time _____

Doctor _____ Date _____ Time _____

Patient Identification

(continued on next page)

CONSENT FOR SURGERY OR OTHER PROCEDURE

ATTACHMENT G. RECRUITMENT POSTER

If you are having a Breast Cyst drained, you can participate in a Research Project.

The UW Department of Surgery is participating in a research study to learn about the relationship of Melatonin, and other growth factors found in breast cyst fluid, and breast disease.

- **You can donate your breast cyst fluid to this study.**
- **There is no cost to you for donating your cyst fluid.**
- **If you are interested, please review this consent form.**
- **Let your practitioner know you would like to participate.**
- **If you have questions, please call Mari @ 543-9322 or page 559-8782.**

MODIFICATION FORM

RECEIVED
Human Subjects Division

JAN 05 2001

86

University of Washington
Human Subjects Division, Box 355752
3935 University Way NE, Seattle, WA 98105-6613

UW

This box is for Human Subjects Review Committee Use Only		
HSRC signature: Jeri C Fais	Date approved: FEB 5 2001	Master ☐ Committee ☐ Reviewer ☐ Investigator ☐
Contingencies:		

Instructions: Complete, sign and date this form. Submit **four** copies of this form plus **four** copies of revised materials. Modifications may not be implemented until they have received approval. The approval of this modification does not change the original period of approval of your Human Subjects Review Committee application. For funding modifications, please submit HSRC form *"Adding a New Funding Source"* available at http://depts.washington.edu/hsd or by calling our office. If you have questions, please call us at (206) 543-0098.

Principal Investigator: Benjamin O. Anderson Current HSRC approval no.: 99-9845-E 01

Dept./Div.: Surgery Box: 356410 Phone: 543-9322 Fax: 543-8136 Email: banderso@u...

Title of application: Role of Melatonin in Prevention of Breast Cancer in Patients with Gross Cystic Breast Disease

☐ **1. PURPOSE:** Describe the proposed changes below. Submit 4 copies of new consent/assent form(s), if necessary.

No changes

☐ **2. PROCEDURES:** Briefly summarize proposed changes below. Assess impact on risk level. If changes require addition or deletion of an investigational drug, device, or radiological procedure, provide appropriate documentation from the sponsor or Radiation Safety Committee. If change is a protocol amendment, submit 4 copies of the amendment and 1 copy of the protocol. If necessary, submit 4 copies of any revised or new instruments and/or consent/assent form(s).

No changes

☒ **3. POPULATION & RECRUITMENT:** Briefly describe proposed changes in subject population or recruitment below. Include the following for each new population: inclusion criteria, exclusion criteria, approach and recruitment methods. If new or revised recruitment materials, please submit 4 copies.

This is a change in recruitment methods only. The study population is not changed. We would like to post the attached flyer, with a supply of consent forms, in the interventional radiology/ultrasound waiting rooms at the Women's Health Care Center at the Roosevelt Clinic and UWMC Breast Health Radiology area. The clinicians have expressed reticence to participate because of the time it takes them to introduce the study to potential subjects, obtain informed consent, have the subject complete the study-related questionnaire and then do the actual aspiration. Usually this procedure is scheduled for a 15 minute time slot. We feel that if the potential subject had the opportunity to review the informed consent form while waiting, that there would be less pressure on the schedule for both clinicians and the patients. The pre-review of the consent form will introduce the purpose of the study and answer many questions. The questionnaire can be taken with the subject to be completed at a later time and returned in the stamped envelope we will provide. This will also potentially provide advance notice for the research coordinator to travel over to Roosevelt clinic to enroll the patient and answer questions in lieu of the physician doing so.

☐ **4. INVESTIGATORS:** Provide information requested below for each new investigator. Submit 4 copies of consent and/or assent form(s) revised to show changes.

Name and Title	University Position	Dept./Div.	Phone	Box

☐ **5. SITE:** Provide a letter of cooperation from each non-UW site.

☐ **6. CONSENT FORM:** Submit 4 copies of each revised consent/assent form. Highlight changes on one copy.

☐ **7. Other:**

Signature of P.I.: _____ **Date:** 1/4/01

ATTACHMENT H. SUMMARY OF THE YEAR 1 ANNUAL REPORT

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

March 20, 2001

Research Data Management

SUBJECT: Review of Annual Report Dated September 2000, Award Number DAMD17-99-1-9143

Robert L. Wells, Ph.D.
Department of Radiological
Health Sciences
Colorado State University
408 University Services Center
Fort Collins, Colorado 80523

Dear Doctor Wells:

Subject report has been reviewed and is acceptable as written. A copy of your report has been forwarded to the Defense Technical Information Center's Technical Reports database.

To assist you in preparing future reports under the subject award, we have enclosed the reviewer's comments. We ask that you review these comments and incorporate any recommended changes into future reports.

Point of contact for this action is Ms. Judy Pawlus at 301-619-7322 or by email at judy.pawlus@det.amedd.army.mil.

Sincerely,

Judy Pawlus
Technical Editor

Enclosure

ANNUAL REPORT REVIEW
USAMRMC FY98 BREAST CANCER RESEARCH PROGRAM

Grant/Contract/MIPR No.:	**DAMD17-99-9143**
Principal Investigator:	Robert L. Wells, Ph.D. James B. Burch, M.S., Ph.D. Maxine Hennesey, M.S.
Institution:	Colorado State University Fort Collins, Colorado 80523-2002
Report Title:	Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease
Report Type:	Annual (First)
Award Mechanism:	Idea
Date of Report:	September 2000
Reporting Period:	1 September 1999 - 31 August 2000

SUMMARY REVIEW: Melatonin and breast cyst fluids (BCFs) both exhibit antiproliferative effects on breast cancer cells, which are potentially mediated by growth factors. The purpose of this study is to identify melatonin in BCF and determine whether it is responsible for BCF's proliferative characteristics. The principal investigator (PI) intends to create a clinical BCF sample bank among patients with gross cystic breast disease in order to evaluate the connection between this disease, melatonin, and related growth agents in BCF, and breast cancer risk. BCF samples will be used to clarify the role of melatonin and related growth agents such as epidermal growth (EGF), transforming growth factor-beta (TGF-beta), and dehydroepiandrosterone-sulfate (DHEA-S) with the oncostatic effects of BCFs in the MCF-7 human breast cancer model. Thus far, the PI has established informed consent procedures and a BCF sample bank. Patient enrollment and BCF sample collection are ongoing. Cell culture experiments have verified that physiological melatonin levels hinder MCF-7 cell growth. Baseline melatonin, sodium, potassium, and TGF-beta measurements in BCF have been completed.

The data acquired thus far will permit progression to the next phase of this study, which includes the treatment of MCF-7 breast cancer cells with human BCF to determine how BCF composition

relates to and affects breast cancer cell proliferation. Results from cell culture experiments using BCF samples and corresponding biochemical assays will enhance the understanding of breast cancer biology and potentially explain the mechanism whereby BCF and melatonin hinder breast cancer cell growth. The data from this study may be important in analyzing the potential prognostic and therapeutic applications of BCF constituents including melatonin in the development and treatment of breast cancer.

FORMAT/EDITORIAL ISSUES: This report conforms to USAMRMC reporting requirements.

CONTRACTUAL ISSUES: Information is provided in this annual report that supports the following:

Task 1	Months 1-30	In progress
Task 2	Months 1-30	In progress
Task 3	No timeline	In progress
Task 4	Months 3-36	Not yet initiated

This report is in general compliance with the goals outlined in the Statement of Work (SOW).

TECHNICAL ISSUES: No major technical issues were noted during the review of this report.

SPECIFIC DISCREPANCIES AND RECOMMENDATIONS: This reviewer recommends accepting this annual report.

KEY RESEARCH ACCOMPLISHMENTS: The PI has indicated unlimited distribution for the following key research accomplishments:

- Approval of informed consent procedures for research in human subjects was obtained from the Colorado State University, the University of Washington, and USAMRMC institutional review boards.
- BCF sample collection and handling procedures and laboratory safety protocols have been established.
- Patient enrollment has been initiated and a BCF sample bank has been established.
- Collaborative agreements for patient recruitment and BCF sample collection have been established at two additional medical centers.
- Baseline biochemical assay procedures have been established for melatonin, sodium, potassium, and TGF-beta.
- Cell culture conditions and a treatment protocol that induces melatonin-mediated growth inhibition in MCF-7 human breast cancer cells has been established.

- An alternative cell counting procedure (CyQuant) that minimizes the required BCF volume necessary for cell proliferation assays and optimizes the data yield for each BCF sample has been developed and validated.

REPORTABLE OUTCOMES:

Products

Informatics

- A human breast cyst fluid sample bank has been established as a biological resource for performing studies on the relationship between breast cyst fluid composition, gross cystic breast disease, and breast cancer risk in women.

REVISED REPORT RECOMMENDED: **YES**_____ **NO** √

REVISED SOW RECOMMENDED: **YES**_____ **NO** √

ATTACHMENT I. HSRRB APPROVAL AT THE CURRENT STUDY SITE (UW)

DEPARTMENT OF THE ARMY
OFFICE OF THE SURGEON GENERAL
5109 LEESBURG PIKE
FALLS CHURCH VA 22041-3258

REPLY TO ATTENTION OF
MCMR-RCQ (70-1n)

27 October 1999

MEMORANDUM FOR Director, U.S. Army Medical Research Acquisition Activity, ATTN: MCMR-AAA-B (Ms. Marken), Fort Detrick, MD 21702-5014

SUBJECT: Protocol Entitled, "Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease," Submitted by, Robert Wells, Ph.D., Colorado State University. Proposal Log No. BC981197, HSP Log No. A-8953

1. Revisions received on 21 and 22 October 1999 to the subject protocol have been reviewed and found to comply with all applicable human subject protection regulations.

2. The study is approved for implementation at Colorado State University and at the University of Washington School of Medicine. Please review the responsibilities of the Principal Investigator as outlined in HSP Clause 13.01.

3. Please enter the Use of Human Subjects Clause and the Prohibition of Use of Anatomical Substances Clause into the contract.

4. Because the study poses no greater than minimal risks to the subjects, the Volunteer Registry Data Sheet is not required.

5. Point of contact for this action is Sonya Lewis at DSN 343-7486, facsimile DSN 343-7803, or e-mail Sonya.Lewis@det.amedd.army.mil.

Julie K. Zadinsky

JULIE K. ZADINSKY
COL, AN
Acting Chair, Human Subjects
Research Review Board

CF:
MCMR-SGS
MCMR-PLF (Dr. Mishra)

APPENDIX B

Laboratory Protocols for Biochemical Assays

Melatonin Assay

Obtain sample to be used, thaw sample, and spin down for 15 minutes at 2000 rpm (Beckman tabletop centrifuge), to remove any particulate matter. An extraction procedure should be performed for each sample prior to running the ELISA.

Melatonin Extraction Procedure:

1. Use laboratory tape to label each extraction column with the proper sample identification.
2. Place extraction columns into 15ml conical polypropylene centrifuge tubes (Do not put caps back on after inserting extraction columns).
3. Add 1 ml methanol to the column and centrifuge for 1 minute at 200 x g (1200 rpm). Repeat.
4. Add 1 ml of deionized water to the column and centrifuge for 1 minute at 200 x g. Repeat.
5. Empty centrifuge tube at this point to avoid sample contamination.
6. Load 200 µl of sample into the extraction column marked to receive that sample and centrifuge for 1 minute at 200 x g.
7. Add 1 ml of 10% methanol of column and centrifuge for 1 minute at 500 x g (1800 rpm). Repeat.
8. Add 1 ml of hexane to each column and centrifuge for 2 minutes at 500 x g. (Note: Perform this step in a fume hood to avoid breathing in too much hexane).
9. Place each extraction column into a CLEAN centrifuge tube and label tube with sample identification.
10. Add 1 ml of methanol to each column and centrifuge for 1 minute at 200 x g.
11. Evaporate the methanol to dryness under a stream of particle free nitrogen.
12. Reconstitute the sample with 250µl of reconstitution buffer.
13. Vortex thoroughly, then place onto an orbital shaker and equilibrate the sample for 30 minutes with slow to moderate shaking.
14. Transfer the sample to an appropriately labeled eppendorf tube and place in a -20 freezer until ready to assay.

ELISA Method for Melatonin Detection:

1. Allow all reagents to warm to room temperature prior to use.
2. Secure 8 well strips into one 96 well plate holder. Make sure you have enough strips to run a standard curve (each

concentration in duplicate), and each sample in duplicate.
3. Wash the coated strips twice with wash buffer. After the second wash, aspirate wells to ensure removal of all wash buffer.
4. Carefully pipette 100µl in duplicate of each of the following: blanking reagent, zero calibrator, standards, high control, low control, extracted standards.
5. To each well add 50µl melatonin biotin conjugate. Cover with plate sealer and shake for 60 seconds at 900 rpm.
6. Incubate for 3 hours at 2-8°C.
7. Aspirate wells and wash 4 times using 400µl wash buffer each time. After final wash be sure to aspirate wells to ensure removal of all wash buffer.
8. To all wells, add 100µl enzyme label.
9. Cover plate with new plate sealer, and keeping plate at room temperature, shake at 900 rpm for 60 minutes.
10. After 60 minutes, aspirate wells and again wash 4 times with 400µl wash buffer each time.
11. Aspirate thoroughly after the last wash, and then add 100µl TMB substrate to all wells.
12. Cover wells with new plate sealer and place plate on shaker again. Keeping the plate at room temperature AND protected from light, shake at 800-1000 rpm for 30 minutes.
13. After 30 minutes add 100µl STOP solution to all wells and be sure to remove ALL air bubbles that may have formed. Read on plate reader set to 450nm within 30 minutes.

Melatonin Data Reduction:

1. To analyze data use a curve fit program capable of fitting the standards to a 4-parameter curve. Using a semi-log scale, plot absorbance on the y-axis and melatonin concentration along the x-axis.
2. Ensure that the high control and low control values fall within the acceptable range provided by ALPCO.

Electrolyte Assays

Samples are analyzed for sodium and potassium content by flame ionization atomic absorption spectroscopy. Commercial sodium standard solutions (RICCA Chemical, Arlington, TX) are diluted with 1% nitric acid in double de-ionized water to concentrations of 0.1, 0.50, 1.0, and 2.0 µg/ml and a standard curve is prepared at an absorbance of 589 nm (sodium lamp). Potassium standards are prepared in similar fashion from a commercial stock standard solution (RICCA Chemical, Arlington, TX) and diluted with 1% nitric acid in double de-ionized water to

concentrations of 0.1 0.5 1.5 2.0 and 3.0 µg/ml and a standard curve is prepared at an absorbance of 766.5 nm (potassium lamp). Data are integrated and recorded using Varian Spectra 300/400 software. Cyst fluid samples are diluted in 1% nitric acid-double de-ionized water with final dilution factors of 1,000X for potassium and sodium. Analytic blanks were prepared form 1% nitric acid in double de-ionized water.

Transforming Growth Factor-Beta 1 (TGF-B1) Assay

For analysis of TGF-B1, an ELISA kit purchased from ALPCO (Windham, NH) is used. This particular ELISA is based on the sandwich principle. In short, samples and standards that have been acidified and then neutralized are added to antibody coated microtiter wells, allowed a specified amount of time to bind to the antibody in the wells, and then excess is washed off. To what is left bound, consecutive incubations with a monoclonal mouse anti-TGF-B1 antibody, a biotinilated anti-mouse IgG antibody and the Streptavidin-POD Enzyme complex are performed. The result is an immuno enzyme sandwich. Once this sandwich is formed, a substrate solution is added and allowed to develop a stable color for a definite amount of time and then the intensity of the color is measured with a microtiter plate reader set to read at 450 nm.

TGF-B1 Sample Preparation:

1. Centrifuge both cyst fluid and supernatant samples for 15 minutes at 2000rpm.
2. Dilute cyst fluid samples 1:5 with assay buffer. Do not dilute supernatant samples.
3. Label clean 1.5 ml eppendorf centrifuge tubes and pipette 200µl diluted (or undiluted) samples into appropriately labeled tubes and do the same with the standards (standards should not be diluted again after initial serial dilutions).
4. Add 20µl 1N HCl to each eppendorf tube and vortex.
5. Allow samples and standards to incubate at room temperature for 15 minutes.
6. After 15 minutes, add 20µl 1N NaOH and vortex again.
7. Check pH to ensure all samples and standards are between 7 and 8. Samples and standards are now ready to assay.

ELISA Method for TGF-B1 Detection:

1. Secure the correct number of 8 well strips to run all standards and samples in duplicate.

2. Pipette 100µl prepared standards and samples into the appropriate wells.
3. Cover the plate with a plate sealer and incubate overnight at 2-8°C.
4. Aspirate wells and wash 3 times with wash buffer, aspirating after the final wash.
5. Pipette 100µl of Specific Antibody into the wells.
6. Cover plate with a plate sealer and incubate at room temperature for 2 hours.
7. Aspirate wells and wash 3 times with wash buffer, aspirating after the final wash.
8. Pipette 100µl Anti Mouse Biotin into each well.
9. Cover plate with a plate sealer and incubate at room temperature for 45 minutes.
10. Aspirate wells and wash 3 times with wash buffer, aspirating after the final wash.
11. Pipette 100µl Enzyme Complex into each well.
12. Cover plate with a plate sealer and incubate at room temperature for 45 minutes.
13. Aspirate wells and wash 3 times with wash buffer, aspirating after the final wash.
14. Pipette 100µl Substrate solution (TMB) into each well, cover with plate sealer and incubate at room temperature for 15 minutes.
15. Pipette 50µl STOP solution into each well and shake plate for 10-15 seconds on a plate shaker to ensure complete mixing.
16. Determine absorbance using a microtiter plate reader set to read at 450nm.

TGF-B1 Data Reduction:

1. Using a data analysis program capable to plotting a 4-parameter curve to determine the equation and slope of the standard curve.
2. Use the average absorbance of each sample to determine the corresponding TGF-B1 value by simple interpolation from the standard curve, remembering to multiply by the dilution factor if necessary.

Transforming Growth Factor - Beta 2 (TGF-B2) Assay

To determine TGF-B2 in breast cyst fluids, an ELISA kit was purchased from R&D Systems (Minneapolis, MN). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TGF-B2 has been pre-coated onto a microplate. Standards and samples are pipetted into the

wells and any TGF-B2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-B2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TGF-B2 bound in the initial step. The color development is stopped and the intensity of the color is measured.

TGF-B2 Standard Preparation:

1. Reconstitute TGF-B2 standard with2 ml of Calibrator Diluent RD5I.
2. Allow the standard to sit for a minimum of 15 minutes prior to use.

TGF-B2 Sample Preparation:

Use polypropylene tubes. Do not activate the kit standards as they already contain active TGF-B2.

1. To 125µL sample add 25µL 1N HCl. Mix well.
2. Incubate 10 minutes at room temperature.
3. Add 25µL 1.2 N NaOH/0.5 M HEPES. Mix well.
4. Add 800µL Calibrator Diluent RD5I. Mix well and assay within 2 hours.

Note: Sample results must be multiplied by the dilution factor, 7.8. If samples generate values higher than the highest standard, further dilute the samples after activation with the Calibrator Diluent and repeat the assay.

ELISA Method for TGF-B2 Detection:

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents, working standards and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 100µL of Assay Diluent RD1-17 to each well.
4. Add 100µL of Standard or activated sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at

room temperature.

5. Aspirate each well and wash with 400µl wash buffer, repeating the process twice for a total of three washes. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating.
6. Add 200µL of TGF-B2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Protect from light.
9. Add 50µL of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

TGF-B2 Data Reduction:

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation. To determine the TGF-B2 concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding TGF-B2 concentration. Because the samples have been diluted in the activation step prior to assay, the measured concentrations must be multiplied by the dilution factor, 7.8.

Dehydroepiandrosterone Sulfate (DHEA-S) Assay

To determine the amount of DHEA-S in breast cyst fluid, an ELISA kit was purchased form ALPCO (Windham, NH). This particular kit is based on the competition principle and the microplate separation. An unknown amount of DHEA-S present in the sample and a fixed amount of DHEA-S conjugated with horse-radish

peroxidase compete for the binding sites of a polyclonal DHEA-S antiserum coasted onto the wells. After one hour incubation the microtiterplate is washed to stop the competition reaction. Having added the substrate solution the concentration of DHEA-S is inversely proportional to the optical density measured.

DHEA-S Sample Preparation:

1. Dilute breast cyst fluid samples 1:10 with zero standard.

ELISA Method for DHEA-S Detection:

1. Determine the correct number of coated, 8-well strips that will be needed to run samples and standards in duplicate.
2. Pipette 25µl of DHEA-S standards and samples into wells.
3. Pipette 200µl of Enzyme-Conjugate into each well.
4. Cover plate with a plate sealer and shake at 800 rpm for 5 min.
5. Incubate at room temperature for 60 minutes.
6. After 60 minutes, aspirate wells and wash 3 times, using 400µl of wash buffer each time. Aspirate after final wash.
7. Pipette 100µl of Substrate solution to each well at timed intervals.
8. Incubate for 15 minutes at room temperature.
9. Stop the enzymatic reaction by adding 50µl of STOP solution to each well at the same timed intervals as before and determine the absorbance of each well at 450nm.

DHEA-S Data Reduction:

1. Using a data analysis program capable to plotting a 4-parameter curve to determine the equation and slope of the standard curve.
2. Use the average absorbance of each sample to determine the corresponding TGF-B1 value by simple interpolation from the standard curve, remembering to multiply by the dilution factor if necessary.

Epidermal Growth Factor (EGF) Assay

To determine the quantity of EGF in human breast cyst fluid, an ELISA kit was purchased from ALPCO (Windham, NH). The ALPCO ELISA is a sandwich enzyme immunoassay which measures the "free" forms of the cytokine EGF. The coated microtiter wells bind the human EGF in the sample. Simultaneously, EGF specific rabbit anti-human polyclonal antibodies detect EGF in the sample. With the addition of goat anti-rabbit conjugated alkaline

phosphatase, and followed by the addition of the color reagent solution, the amount of cytokine is detected.

EGF Standard Preparation:

1. EGF Standard is reconstituted with Assay Buffer to make a solution that has a concentration of 10,000 pg/ml.
2. After stock is made, dilutions are made in the appropriate amount of Assay Buffer in order to obtain the following concentrations: 1000, 250, 62.5, 15.6, and 0.0 pg/ml.

EGF Assay Procedure:

1. Obtain enough pre-coated wells to perform a standard curve and run the samples in duplicate.
2. Pipette 100µl of Standards into duplicate wells.
3. Pipette 50µl of samples into wells and then add 50µl of Assay Diluent to bring the volume up to 100µl.
4. Pipette 25µl of diluted Rabbit Anti-Human EGF Polyclonal Antibody into each well.
5. Seal plate with plate sealer and incubate at room temperature for 3 hours.
6. After 3 hours, wash plate 4 times with Wash Buffer, using 250µl for each wash. Then pipette 250µl wash buffer into the wells and allow the buffer to remain in the wells for 10 minutes. Aspirate each well to remove any excess fluid.
7. Prepare color reagent, by mixing equal amounts of color reagent A and color reagent B. Set aside.
8. Pipette 50µl of diluted Goat Anti-Rabbit Conjugated Alkaline Phosphatase into each well. Seal plate again, and incubate at room temperature for 45 minutes.
9. Wash plate 5 times, using 250µl of wash buffer in each well for each wash, and, again, allowing the 5^{th} wash to remain in the wells for 10 minutes and removing the buffer thoroughly with aspiration.
10. Pipette 200µl of the Color Reagent (prepared in step 7) into each well, cover with plate sealer and incubate at room temperature for 12 minutes.
11. Check absorbance after 12 minutes of the #1 Standard (900 pg/ml), if O.D. for that standard is 1.6, dispense 50µl of STOP solution to all the wells and read plate in a microtiter plate reader set to read at 492nm.
12. If absorbance has not yet reached 1.6 after 12 minutes, continue reading plate in 3-5 minute intervals until an O.D. reading of 1.6 is reached.

APPENDIX C

Protocol for Treatment of MCF-7 Cells with Breast Cyst Fluids

General Maintenance of MCF-7 Cell Cultures:

MCF-7-W cells are routinely maintained and passaged on a weekly basis. Passage number, dates of passaging, and general cell characteristics are maintained in a notebook. The cells are maintained in a 37□C water jacketed, humidified incubator with 5% CO_2. Media requirements for general cell maintenance are as follows:

RPMI 1640	425 ml
Pen/Strep	5 ml
L-glutamine	5 ml
MEM Amino Acids	5 ml
Sodium Pyruvate	5 ml
Insulin	5 ml
Fetal Bovine Serum	50 ml

Culturing Frozen MCF-7 cells:

1. Warm RPMI 1640 media to 37□C.
2. Remove 1 cryovial of MCF-7 cells from liquid nitrogen (record in Master Log Book) and thaw in 37□C water bath.
3. Working under a laminar flow hood - Label a T-75 flask with the date, your initials and the information printed on the cryovial, and place 10 ml of warmed media into flask.
4. Saturate the outside of the cryovial with ethanol to ensure decontamination, then using a 1 ml pipette, remove the cells from the cryovial and carefully place the cells into the T-75 flask.
5. Rinse the pipette by pipetting up and down in the media.
6. Discard pipette and cap the T-75 flask.
7. Lay the T-75 flask down and rock gently, until all the cells and media coat the bottom of the T-75 flask.
8. Look through microscope to ensure that cells are present, then place the T-75 into a 37□C incubator, supplemented with 5% CO_2.
9. After 24 hours, remove old media and dead cells with an aspirator and replace with 10 ml of fresh, warmed RPMI 1640 media.
10. Change media every 48 hours and monitor cells until they reach 80-90% confluency, at which time they will need to be split.

Splitting the MCF-7 cells:

1. Warm RPMI media to 37□C.
2. Aspirate media off of cells in the flask.
3. For a T-75 flask - place 2.0 ml of Phosphate Buffered

Saline (PBS) into the flask. Rock flask back and forth 5-10 times then aspirate off the trypsin.

4. Place 2-3 mls of fresh trypsin onto the cells and return cells to the incubator for 3 to 5 minutes.
5. After 3 minutes, check to see if the cells have loosened from the bottom of the flask by rapping the flask on the heel of your hand and then looking through the microscope to be sure that all of the cells have detached.
6. If the more than 30% of the cells are still adhered to the bottom of the flask, return the flask to the incubator for an additional 2-3 minutes.
7. When the cells are detached, immediately add 5 ml of warmed media to the flask and mix thoroughly making sure that the entire flask is rinsed with media (this will inactivate the trypsin).
8. Label a new T-75 flask with the date, the new passage number for the cells and your initials and add 9 ml of warm media to the flask.
9. Transfer 1 ml of cells from the flask containing the split cells to the new flask.
10. Make sure that the cells and media coat the bottom of the new flask and place the flask back into the incubator.
11. Report passaging in notebook.

Counting Cells:

1. After cells have been trypsinized, transfer the cells to a small, clean, sterile beaker and break up large cell clumps using a syringe with a 22 gauge needle attached.
2. NOTE: when syringing cells, make sure to draw cells up gently and expel them gently to avoid shearing the cells.
3. Pass cells through the needle 3 or 4 times.
4. Make a 1:10 dilution of cells for counting. To do this: Remove 100µl of cells and mix with 900µl of media.
5. To set up hemacytometer: Remove hemacytometer from the 70% alcohol container, rinse with distilled water, and gently dry with a kimwipe to avoid scratching the counting surface. Do the same for the coverslip. Carefully place the coverslip on the hemacytometer over the metal counting chamber. Using a 20µl pipette, carefully pipette cells onto the metal counting chamber taking care not to let the media overflow into the wells on either side of the counting chamber. Focus scope on counting chamber and count areas marked A, B, C and D. Calculate the number of cells per ml:

(Number of cells counted)/4*1x10^4*(dilution factor)=cells/ml

Cells/ml * total ml = number of cells total

To count cells using Particle Data Counter:

1. Turn on the Particle Data Counter.
2. Press _ key until you get to the SELECT command
3. Press 1
4. Make sure status = 1
5. Normalize the machine: Move metal bar (located on top right of machine), to the Vacuum position, press the SET button followed by the NORM button. After normalizing, move metal bar back to Count position.
6. Now you are ready to count the samples. First, press the SET button and make sure that the cursor next to sample ID disappears.
7. Place a sample that has been diluted 1:10 with PBS/formal in solution onto the counting platform and make sure that the orifice on the glass tube is submerged.
8. Move metal bar to vacuum position and allow the mercury to go down past the second electrode.
9. Press the START button and then move metal bar back to Count.
10. Repeat with same sample to get the number of cells in 1 ml of fluid.
11. Calculate the number of cells per ml:
    [(value from count#1)+(value from count#2)] * 10 = cells/ml

Protocol for Exposure to Breast Cyst Fluid:

In brief, the method used for determining growth inhibition is as follows. The MCF-7 cells were plated in 6-well plates at a

density of $1.5x10^5$ cells/well. After plating, the cells were allowed to regain adherence to the bottom of the wells for 24 hours. After 24 hours all media was removed and was replaced with media that contained no FBS (SFM), media that contained FBS (RM) or SFM media that contained 10% cyst fluid. There were no media changes performed. After 120 hours of exposure, the cells were trypsinized from the bottom of the wells and counted with a hemacytometer using trypan blue exclusion. A more detailed protocol is a follows:

0 HOURS:

1. Aspirate media from cells.
2. Rinse flask with 1.5 ml of trypsin and aspirate off.
3. Replace with 2 ml of trypsin and place in incubator for 3-5 minutes.
4. Loosen cells by rapping flask against the palm of your hand.
5. Add 5 ml of media to inactivate the trypsin.
6. Transfer cells to a clean, sterile beaker and using a syringe with a 22 gauge needle, carefully and gently draw cells into syringe and expel gently - repeat 3 or 4 times.
7. Count cells using the Data Particle Counter.
8. Determine dilution that accommodates 60,000 cells/ml.

   $\frac{(\#\text{ ml needed for test})(60{,}000)}{\text{for dilution (cells/ml that you have)}}$ = number of ml needed

9. Plate 2.5 ml of cells (150,000 cells total) into each well of a 6-well plate and place into 37□C incubator overnight.

24 HOURS:

1. Aspirate media from the 6-well plates.
2. Rinse each well with 1 ml of PBS and aspirate.
3. Replace with either regular growth media, serum free growth media or serum free growth media supplemented with 10% cyst fluid.
4. Return plates to 37□C incubator for 120 hours

120 HOURS:

1. Count cells using particle data counter.
2. Record results in computer data base.

APPENDIX D

Publication:

Disruption of Mitochondrial Respiration by Melatonin in MCF-7 Cells

(Tox Appl Pharmacol 171, 149-156. 2001.)

Toxicology and Applied Pharmacology 171, 149–156 (2001)
doi:10.1006/taap.2000.9115, available online at http://www.idealibrary.com on IDEAL®

Disruption of Mitochondrial Respiration by Melatonin in MCF-7 Cells

Aaron E. Scott,*[,1] Gregory N. Cosma,* Anthony A. Frank,† Robert L. Wells,‡ and Henry S. Gardner, Jr.§

*Department of Environmental Health, †Department of Pathology, and ‡Department of Radiological Health Sciences, Colorado State University, Fort Collins, Colorado; and §U.S. Army Center for Environmental Health Research, Ft. Detrick, Maryland

Received August 10, 2000; accepted November 1, 2000; published online February 9, 2001

Disruption of Mitochondrial Respiration by Melatonin in MCF-7 Cells. Scott, A. E., Cosma, G. N., Frank, A. A., Wells, R. L., and Gardner, H. S. (2001). *Toxicol. Appl. Pharmacol.* 171, 149–156.

Clinical and laboratory studies have provided evidence of oncostatic activity by the pineal neurohormone melatonin. However, these studies have not elucidated its mechanism of action. The following series of MCF-7 breast tumor cell studies conducted in the absence of exogenous steroid hormones provide evidence for a novel mechanism of oncostatic activity by this endogenous hormone. We observed a 40–60% loss of MCF-7 cells after 20-h treatment with 100 nM melatonin, which confirmed and extended previous reports of its oncostatic potency. Interestingly, there were no observed changes in tritiated thymidine uptake, suggesting a lack of effect on cell cycle/nascent DNA synthesis. Further evidence of a cytocidal effect came from morphologic observations of acute cell death and autophagocytosis accompanied by degenerative changes in mitochondria. Studies of mitochondrial function via standard polarography revealed a significant increase in oxygen consumption in melatonin-treated MCF-7 cells. Enzyme-substrate studies of electron transport chain (complex IV) activity in detergent permeabilized cells demonstrated a concomitant 53% increase ($p < 0.01$) in cytochrome *c* oxidase activity. Additional studies of succinate dehydrogenase activity (complex II) as determined by reduction of (3-4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide demonstrated a significant increase ($p < 0.05$) in melatonin-treated cells and further confirmed the accelerated ET activity. Finally, there was a 64% decrease ($p < 0.05$) in cellular ATP levels in melatonin-treated cells. The G-protein-coupled melatonin receptor antagonist luzindole abrogated the cytotoxic and mitochondrial effects. These studies suggest a receptor-modulated pathway of cytotoxicity in melatonin-treated MCF-7 tumor cells with apparent uncoupling of oxidative phosphorylation. © 2001 Academic Press

Key Words: melatonin; MCF-7 cells; *in vitro;* mitochondria; morphology; electron transport.

Melatonin is a neurohormone, synthesized and secreted by the pineal gland in humans and other vertebrate species (for review, Brezezinski, 1997; Vanacek, 1998), and appears to play a number of homeostatic physiologic roles in the immunology and neuroendocrinology of most mammals (for review, Cos and Sanchez-Barcelo, 2000a; Brezezinski, 1997; Vanacek, 1998). In addition to its interactions with these physiologic systems, melatonin also displays several other biological properties, including a direct quenching effect on oxygen free radicals, which we have previously described (Zang *et al.*, 1998). More importantly, melatonin treatment substantially decreases tumor development in animal models of breast cancer (Bojkova *et al.*, 2000; Teplitzky *et al.*, 1999) and has been suggested as a naturally occurring oncostatic agent in humans (Cos and Sanchez-Barcelo, 2000b; Panzer and Viljoen, 1997). Tissue and serum levels of melatonin are significantly and inversely related to nuclear grade of human breast malignancy (Maestroni and Conti, 1996), and excretion of melatonin metabolites has been shown to be decreased in breast cancer patients and to be negatively correlated with tumor size (Bartsch and Bartsch, 1999). Clinical studies have also provided generally supportive evidence of oncostatic activity by melatonin, both in cohort studies of melatonin levels in breast cancer/noncancer patients, as well as in studies of breast cancer survival in patients enrolled in melatonin supplementation regimens (Viljoen, 1997; Lissoni *et al.*, 1999; Bartsch and Bartsch, 1999; Neri *et al.*, 1998).

Several laboratory studies have demonstrated an oncostatic activity by melatonin against breast cancer cell proliferation *in vitro* after 4 to 6 days in culture (Hill and Blask, 1988; Hill *et al.*, 1992; Lemus-Wilson *et al.*, 1995; Cos and Sanchez-Barcelo, 1994). These reports indicate growth inhibition by melatonin on the estrogen-responsive human breast carcinoma cell line MCF-7, at nanomolar concentrations that approach circulating physiologic levels of the hormone (Molis *et al.*, 1994; Blask and Hill, 1986; Hill *et al.*, 1992; Cos and Blask, 1994).

Despite these confirmatory reports of oncostatic activity by melatonin, the mechanism of action by which this endogenous agent inhibits tumor cell proliferation remains unresolved (for recent review see Viljoen, 1997; Cos and Sanchez-Barcelo, 2000a). For example, a number of diverse morphologic changes have been described in MCF-7 cells following treatments with melatonin, but no distinct or insightful pattern of subcellular degenerative changes has emerged from these prior investigations (Hill and Blask, 1988; Crespo *et al.*, 1997). In

[1] To whom correspondence should be addressed at P.O. Box 460783, Aurora, CO 80046-0783. Fax: (303) 680-7188; E-mail: Aaron.Scott@uchsc.edu.

0041-008X/01 $35.00

addition, studies have focused on changes in cell cycle kinetics following MCF-7 cell treatments with the hormone, including slight delays in the G_1–S transition and accumulation of G_1 phase cells; however, there has been no elucidation of the underlying mechanism of cell cycle arrest or block (Cos *et al.*, 1996a, b, 1991). Interestingly, an "estrogen-rescue" on melatonin-induced cell cycle effects has been described wherein the cell cycle arrest was reversed by the addition of estradiol (Cos *et al.*, 1991).

Several other biochemical changes have been demonstrated in melatonin-treated MCF-7 cells, including changes in the secretion of estrogen-regulated growth factors (e.g., c-*myc*, TGFα, c-*fos*, TGF-β) (Molis *et al.*, 1997) and downregulation of the estrogen receptor (Molis *et al.*, 1993, 1994). Additionally, inhibition of MCF-7 cell growth has been correlated with estrogen responsiveness as well as level of estrogen receptor mRNA and appears to be transduced through the membrane-associated G-protein coupled mt1 melatonin receptor (Ram *et al.*, 2000).

Perhaps what is more unclear are the previously described effects of melatonin on the MCF-7 cell line grown with and without estrogen in the medium. Separate studies with varying concentrations of estrogen in culture medium have provided evidence of cell cycle arrest (Cos *et al.*, 1996a, b), proliferation (Cos and Sanchez-Barcelo, 1995; Blask *et al.*, 1997; Blask and Hill, 1986), induction of the estrogen early responsive gene c-*myc* (Molis *et al.*, 1997); and morphologic aberrations (Hill and Blask, 1988; Crespo *et al.*, 1997). In light of these reports suggestive of interactions between melatonin and estrogen signaling pathways, the studies described herein were performed in estrogen-free culture conditions, so that analysis of melatonin effects on MCF-7 cell growth and respiration could be explored in a direct fashion.

Because melatonin has been described as an oncostatic agent in rodents, humans, and *in vitro*, yet is essentially nontoxic to human patients (Waldhauser *et al.*, 1990), we performed the following set of studies to explore the toxic mechanism of this hormone in the MCF-7 cancer cell model. We have proposed the hypothesis that melatonin is cytotoxic to MCF-7 breast cancer cells through a mechanism disrupting oxidative respiration in the absence of estrogen. In addition, since melatonin has been identified as a ligand for a G-coupled receptor (Ram *et al.*, 2000) as well as being a potent antioxidant, we investigated whether the melatonin receptor antagonist luzindole would block the mitochondrial and cytotoxic effects that we observed in the MCF-7 cells.

MATERIALS AND METHODS

Cell culture. MCF-7 human breast adenocarcinoma cells were obtained from American Type Culture Collection at passage 154. Cell cultures were maintained in a humidified incubator at 5% CO_2 and 37°C. All reagents and supplies were purchased from Sigma (St. Louis, MO) unless otherwise specified. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS; 10% v/v), MEM nonessential amino acids (1% v/v), penicillin–streptomycin (1% v/v), hydrocortisone (200 nM), sodium pyruvate (1 mM), and insulin (0.003 unit/ml). Prior to experiments, cells were grown in phenol-red-free medium supplemented with charcoal–dextran-treated FBS to minimize exogenous hormone levels ($\leq$0.114 pM). Melatonin concentration in the treated serum was below the detection limit of 1.9 pmol/liter as measured by radioimmunoassay (Rollag and Niswender, 1976). Cells were maintained in the estrogen-depleted medium for 3 to 4 days and were in mid- to late log phase growth at the time of experimental treatments. Cells were enumerated by hemocytometer count using trypan blue dye viability assay or by Coulter cell counter.

Tritiated thymidine uptake. MCF-7 cells were seeded at a density of 8×10^5 cells per 60 mm culture dish and, after 16 h of exposure to melatonin, vehicle, or estradiol, were pulsed with [^{3}H]thymidine (0.65 μCi/ml) for 30 min. After rinsing with phosphate-buffered saline, ice-cold trichloroacetic acid was added, and followed with ice-cold 70% ethanol. Cells were lysed with Toplayer II (18.15 g NaOH, 29.3 g NaCl, 3.81 g tetra-sodium EDTA, 5.23 g Brij-58, in 1 liter of double-deionized water). A scintillation cocktail was prepared with 75 μl HCl, 300 μl H20, and 10 ml of Ready Safe, and cell lysate radioactivity was analyzed via liquid scintillation using decays per minute (DPM) from the tritium window. DPMs were standardized to mean cell counts from cultures grown in parallel.

Electron microscopy. Cells were prepared by routine glutaraldehyde fixation, osmium tetraoxide postfixation, sequential dehydration, infiltration, and plastic imbedding prior to staining and examination.

Polarography/oxygen consumption. MCF-7 cells were harvested and resuspended in medium to provide a concentration of 1×10^6 cell per analysis. Samples were incubated for 30 min at 37°C and 5% CO_2 immediately prior to polarographic measurements. Oxygen levels were calibrated from 100 to 0% oxygen saturation by the addition of sodium sulfite to air-saturated deionized water. Data measurements were taken over a linear range of oxygen consumption ($R^2 > 0.99$) for 20 min.

Cytochrome c oxidase assays. The cytochrome *c* oxidase microtiter plate assay was performed with minimal modification using the method of Chrzanowska–Lightowlers (Turnbull *et al.*, 1993). Briefly, MCF-7 cell membranes were permeabilized with 0.25% saponin prior to adding substrate mix containing cytochrome *c* and 3,3′-diaminobenzidine-tetrachloride (DAB). Enzyme assays were standardized to cell number in 96-well microtiter plates with approximately 1.5×10^6 cells required per assay well for optimal detection of enzyme activity. Kinetic readings were linear for at least 15 min in all samples. Background absorption was measured in wells containing medium but no cells (assay blank) and subtracted from cell samples. The addition of 3 mM potassium cyanide to control wells inhibited 100% of enzyme activity, thus verifying that the reaction was catalyzed by cytochrome *c* oxidase. Data are reported as mean velocity of enzyme activity (linear slope of DAB oxidation = Δabsorbance/minute) per 10^6 cells.

In addition, we used the AlamarBlue dye assay (AccuMed International, Inc., Westlake, OH) to detect changes in electron transport complex IV activity. AlamarBlue dye is reported by the manufacturer to be a final electron acceptor between the reduction of oxygen and cytochrome *c* oxidase (cyt.a_3). The dye is reduced by the removal of oxygen and its replacement by hydrogen, with the amount of reduced dye (emitting at 590 nm) being proportional to the activity of cyt.a3. Data are reported as fluorescent units of reduced dye per cell per 4-h incubation.

Analysis of succinate dehydrogenase activity. Mitochondrial complex II activity was measured by the MTT (3-4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) assay with minor modification of the original Mosmann method (Mosmann, 1983). Briefly, 5×10^4 MCF-7 cells were seeded in 48-well microtiter plates. After 3 days of growth, the cells were refed and then treated 2 days later with fresh medium containing treatments. MTT was dissolved in PBS at and was added after 17 h of cell treatment and incubated for 3 additional hours. DMSO was added, the plate was placed on a shaker at low speed for 5 min, the solubilized dye solution was transferred to a 96-well plate, and absorbance was read at 550 nm. Cells from parallel treatments were enumerated by hemocytometer and trypan blue dye exclusion to standardize MTT results by cell number.

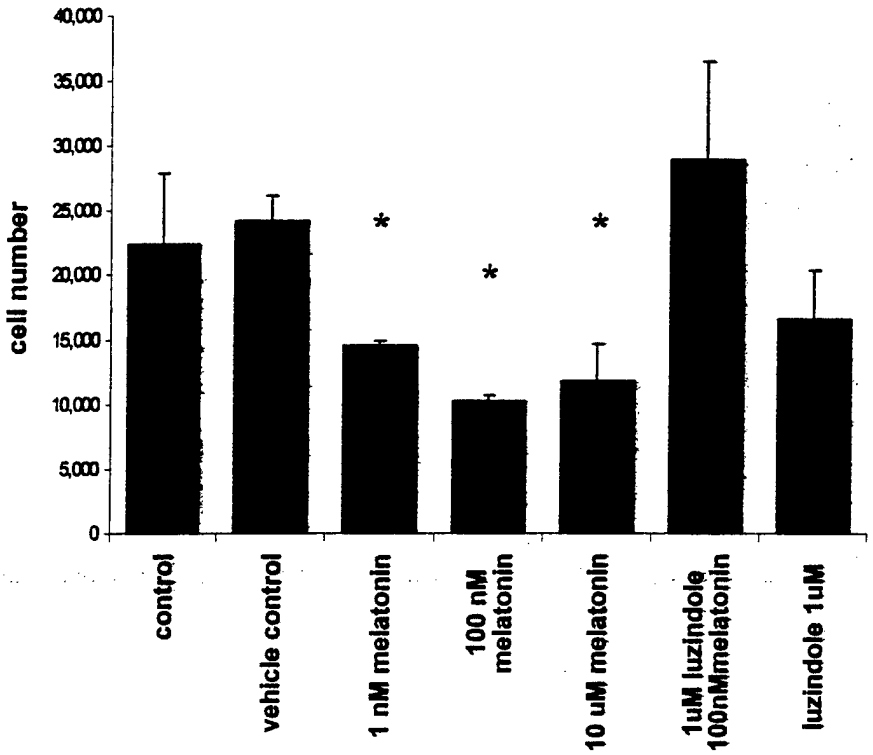

FIG. 1. MCF-7 cells treated for 18 h with vehicle control, melatonin, and luzindole. Cells were enumerated by trypan blue dye exclusion assay and hemocytometer count. Data are representative of at least three independent experiments and demonstrate a 40 to 60% decrease in cell number following melatonin treatment. *Statistically significant differences from control ($p < 0.05$).

Luciferin–luciferase chemiluminescent ATP assay. Quantification of cellular ATP by chemiluminescent measurement was performed using luciferase–luciferin in glycine buffer. Cell samples were extracted with 0.1% Triton X and diluted fivefold in assay buffer (100 mg BSA and 10 ml each of 200 mM Tris HCL, 20 mM EDTA, and 100 mM $MgSO_4$, pH 7.8). Results were standardized to cell protein using the BCA protein assay and reported as moles of ATP per mg protein.

Statistical analysis. Where applicable, results were analyzed by one-way analysis of variance using Minitab software. Comparison of sample means to control means was determined by Dunnett's multiple means test or Student's *t* tests with the assumption of equality of variance. Two-tailed critical values were used in all experiments with level of significance as indicated. Statistical analysis of oxygen consumption data was done with Minitab software by comparison of

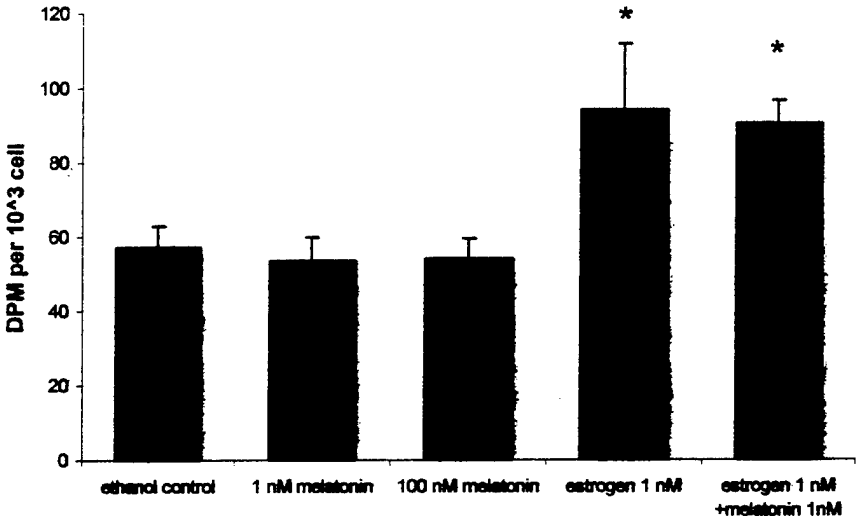

FIG. 2. Tritiated thymidine uptake by MCF-7 cells 16 h following melatonin and 17β-estradiol treatment. No difference in DNA synthesis was evident after either 1 or 100 nM melatonin treatment, although the addition of estrogen significantly increased tritiated thymidine uptake with or without melatonin. All treatments were performed in triplicate ($*p < 0.05$).

FIG. 3. Microscopic changes in MCF-7 cells exposed to 100 nM melatonin 18 h previously. (A) Photomicrograph of vehicle-exposed control cells with typical MCF-7 cell morphology (hematoxylin and eosin, bar represents 10 μm). (B) Photomicrograph of melatonin-exposed cells. Note a markedly degenerate cell characterized by cell swelling, pallor, and dissolution of the cell membrane (arrow) and a morphologically normal cell containing a prominent phagolysosome (arrowhead; hematoxylin and eosin, bar represents 10 μm). (C) Electron micrograph of melatonin-exposed cells. Note the prominent membrane-bound phagolysosome with electron-dense whorls (remnants of digested cell membrane; "P"). Disorganized bands of filaments (arrowhead) and the edge of the nucleus (arrow) are also visible (bar represents 500 nm).

oxygen consumption slopes with the context of multiple linear regression (see for example, Kleinbaum *et al.*, 1998). All experiments were conducted in triplicate or quadruplicate fashion.

RESULTS

Our initial proliferation studies of melatonin-treated MCF-7 cells confirmed and extended previous reports of its oncostatic activity in breast tumor cells. In our studies, we performed 17- to 20-h melatonin treatments, after which time the cells were enumerated by directly counting the cells (via Coulter counting and/or hemocytometer). As shown in Fig. 1, we observed a 40 to 60% dose-responsive loss of cells following these short-term treatments with melatonin, at concentrations as low as 1 nM. However, in cultures treated simultaneously with 100 nM melatonin and 1 μM luzindole, cell numbers were not different from control. Considering the abbreviated time course of these effects, the results suggested a cytolethal mechanism of oncostatic action, rather than an inhibition of cell proliferation. Further evidence for this cytolethality came from our tritiated thymidine uptake/nascent DNA synthesis studies. As depicted

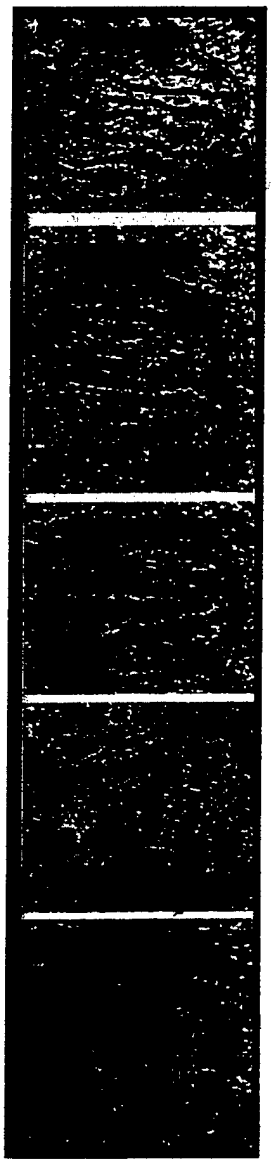

FIG. 4. Electron micrographs of mitochondria from a single MCF-7 cell exposed to 100 nM melatonin 18 h previously. Mitochondrial morphology varies from normal (A), to swollen cristae of varying severity (B and C), to dissolution of cristae (D), to dissolution of outer mitochondrial membrane (E). Bars represent 500 nm.

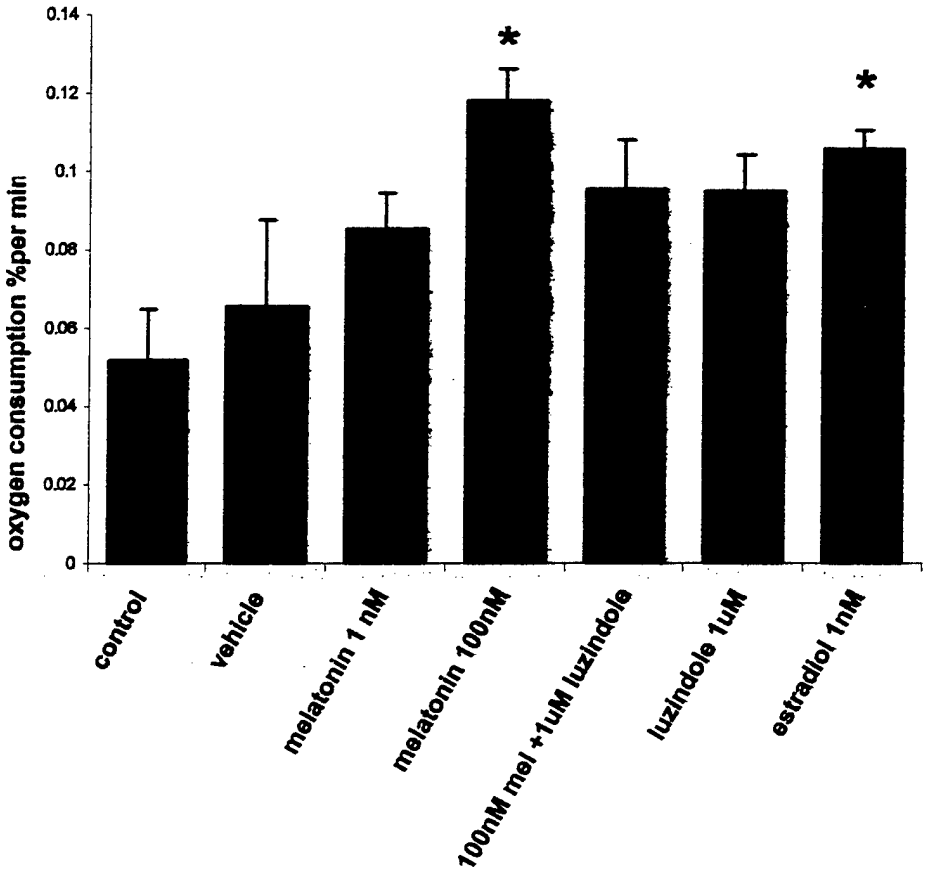

FIG. 5. Oxygen uptake by MCF-7 cells 18 h after treatments with vehicle (0.00005% ethanol), melatonin, luzindole, and estrogen. Bars represent mean slope of oxygen consumption curves with error bars representing one standard deviation of each set mean. The slope of both 1 nM estradiol- and 100 nM melatonin-treated groups were significantly higher than vehicle control ($*p < 0.05$). The significant increase due to 100 nM melatonin was negated by concurrent incubation with luzindole.

in Fig. 2, following the same melatonin treatments described above, we found no significant inhibition of tritiated thymidine uptake in treated vs control MCF-7 cell cultures. On the other hand, cells treated with 1 nM 17 beta-estradiol demonstrated a significant increase in tritiated thymidine uptake, thus confirming a positive proliferative response to estrogen in the MCF-7 cells. Furthermore, flow cytometric analyses of melatonin-treated MCF-7 cells failed to show measurable change in cell cycle parameters (data not shown). Together, these results suggested a lack of inhibitory effect by melatonin on the cell cycle/DNA synthesis and provided further evidence for cell death as a cause of reduced cell numbers.

Acute cytotoxicity in melatonin-treated MCF-7 cells was observed by light and electron microscopy. Figure 3B displays one of the pale staining "ghost"-like bodies commonly observed in the melatonin-treated cells. In addition to these obviously degenerating cells, a number of inclusion bodies were present within the cytoplasm of otherwise morphologically normal cells. A 15,000× transmission electron micrograph (Fig. 3C) of an inclusion body similar to the one shown in Fig. 3B identified it as a phagocytized cell in advanced stages of degeneration. These morphologic observations suggested that melatonin treatment of the MCF-7 carcinoma cells resulted in acute cytotoxicity accompanied by autophagocytosis.

Further evaluation of ultrastructure by electron microscopy demonstrated morphologic alterations of mitochondria in the melatonin-treated cells. The electron micrograph depicted in Fig. 4 shows mitochondria from an MCF-7 cell 18 h after melatonin treatment and demonstrates effects ranging from normal ultrastructure to swollen cristae, degenerate cristae, and dissolution of the outer mitochondrial membrane.

Because of the acute toxicity that melatonin appeared to inflict in the MCF-7 cells, as well as morphologic evidence for specific organellar degradation, mitochondrial activity/respiration in melatonin-treated breast tumor cells was determined. Using standard polarography, oxygen consumption in MCF-7 cells treated with melatonin was measured in a similar fashion to the previously described proliferation studies. As shown in Fig. 5, 100 nM melatonin treatment resulted in an increase (185% of control respiration, $p < 0.05$) in oxygen consumption in MCF-7 cells by 17 h. No significant change in oxygen consumption occurred when luzindole was added concurrently with the melatonin.

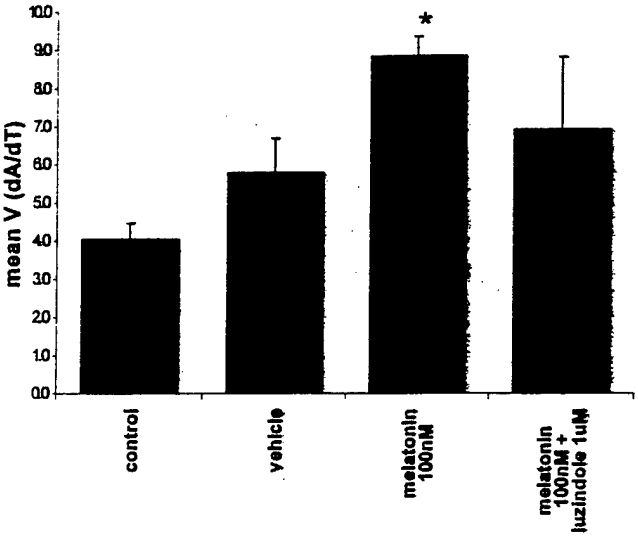

FIG. 6. Cytochrome *c* oxidase activity by microtiter plate method (colorimetric measurement of oxidized DAB) in MCF-7 cells 18 h after treatment with vehicle, melatonin, luzindole, or 17β-estradiol. The enzyme reaction rate (mean V/min) was standardized to cell number. Treatment with 100 nM melatonin was significantly different from control ($*p < 0.01$). The addition of 1 μM luzindole to the 100 nM melatonin treatment group negated the increased cytochrome *c* oxidase activity seen with melatonin alone.

Changes in mitochondrial respiration via modulation of electron transport enzyme activities was determined by directly performing enzyme–substrate assays in detergent permeabilized MCF-7 cells. These studies demonstrated a 53% increase in cytochrome *c* oxidase activity in melatonin-treated MCF-7 cells (Fig. 6). We further investigated this apparent change in complex II electron transport chain activity by employing the use of a mitochondrial vital dye, AlamarBlue, which undergoes a detectable change in fluorescence following reduction by complex IV. Eighteen hours following melatonin treatment, a dose-responsive 70 to 125% increase in AlamarBlue dye reduction had occurred (Fig. 7). Once again, the addition of 1 μM luzindole to the 100 nM melatonin-treated cells negated these effects.

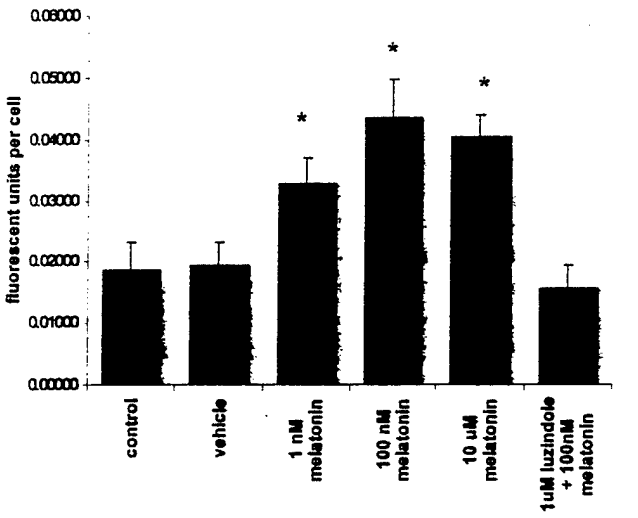

FIG. 7. Cytochrome *c* oxidase activity (reduction of AlamarBlue) in MCF-7 cells 18 h after treatment. Data are reported as fluorescent units of AlamarBlue dye reduction during a 4-h incubation period per million cells. Fluorescence after 1 nM, 100 nM, and 10 μM melatonin treatment was significantly higher than control fluorescence ($*p < 0.05$). The addition of 1 μM luzindole to 100 nM melatonin returned the fluorescence units (activity of the enzyme) to control values.

TABLE 1
Activity of Succinate Dehydrogenase by MTT Assay

Treatment of cells	Average OD of MTT
Ethanol control	22.39
1 nM melatonin	25.551**
100 nM melatonin	25.697**
100 nM melatonin + luzindole	22.926
1 μM luzindole	22.322

Note. Optical density of the microtiter plate assay was standardized to cell number and results are represented as absorbance per million cells. OD following 1 and 100 nM melatonin treatments were significantly higher than vehicle control OD ($p < 0.05$) but were not significant when cells were incubated with luzindole and melatonin. **Significantly different from control ($p < 0.05$).

To confirm that the increase in electron transport activity was not restricted to complex IV, succinate dehydrogenase activity (complex II) was evaluated. Results from the MTT assay (Table 1) demonstrated a 15% ($p < 0.05$) increase in formazon dye reduction in the MCF-7 cells following 19 h of treatment with melatonin, suggesting increased activity of complex II of the electron transport chain. Complex II activity in cells treated simultaneously with 1 μM luzindole and 100 nM melatonin was not significantly different from control values.

Since melatonin altered mitochondrial respiration in the MCF-7 cells, studies to determine whether the hormone directly altered respiration in isolated rat liver mitochondria were performed. These studies were unable to demonstrate melatonin-induced state 3 or state 4 changes in oxygen consumption or activity of complex I, II, or IV in treated mitochondria and submitochondrial particles (data not shown).

TABLE 2
ATP Concentration in MCF-7 Cells

Treatment of cells	ATP nmol/mg protein
Ethanol control	11.187
100 nM melatonin	6.180

Note. Crude data (relative light units) were converted to nmol of ATP from a standard curve and reported as nmol of ATP per mg protein. Melatonin treated cells had significantly lower total ATP than vehicle controls ($p < 0.05$ by Student's *t* test).

The increase in oxygen consumption and electron transport activity in melatonin-treated cells suggested that melatonin may act to uncouple oxidative phosphorylation from electron transport in MCF-7 breast tumor cells. Measurement of cellular ATP levels by chemiluminescence following 100 mM melatonin treatment demonstrated a statistically significant decrease in ATP content (Table 2). Thus, melatonin appears to act as an uncoupler of oxidative phosphorylation in MCF-7 cells and may exert toxicity to the breast tumor cells via this mechanism of mitochondrial disruption.

DISCUSSION

Acute toxicity as well as perturbation of mitochondrial respiration was clearly apparent in MCF-7 cells cultured under estrogen-free conditions following 16- to 20-hour melatonin treatments. Although as many as 60% of the cells were lost in conjunction with autophagocytosis and morphologic alterations of mitochondrial ultrastructure, we were unable to demonstrate changes characteristic of apoptosis by electron microscopy or with the comet assay (data not shown). More significantly, the cytotoxicity resulted from melatonin concentrations that were within human physiological ranges. Because these concentrations were at levels previously reported to inhibit MCF-7 cancer cell growth, we explored potential mechanisms of cytotoxicity in the MCF-7 cancer cell model.

In addition to cell death following exposure to melatonin, our data demonstrated increased electron transport activity along with reduced ATP levels in MCF-7 cells culture in the absence of estrogen and suggested that oxidative phosphorylation was uncoupled. In contrast, parallel experiments conducted on MCF-7 cells grown in estrogen-supplemented medium failed to show melatonin-induced cytotoxicity or changes in mitochondrial respiration. The absence of melatonin-induced toxicity by estrogen-containing medium may result from estrogen upregulation of electron transport subunit proteins, which undergo as much as a 16-fold increase in some estrogen-dependent cell types (Van Itallie and Dannies, 1988). In this manner, cells could overcome the uncoupling effect of melatonin and provide sufficient mitochondrial ATP for continued cell growth. Conversely, in the absence of estrogen, limited synthesis of cytochrome *c* oxidase subunit proteins could impede electron flow and thereby increase cell susceptibility to melatonin cytotoxicity by the uncoupling of oxidative phosphorylation. Thus, cells such as the MCF-7 carcinoma cells that rapidly increase metabolism in response to estrogen would be eliminated by melatonin in physiologic periods characterized by the absence of estrogen.

Interestingly, the cytotoxicity and increased respiratory activity was not observed when the melatonin G-protein-coupled receptor antagonist luzindole was present. In each set of experiments (Figs. 1, 5, 6, 7, and Table 1) and in the morphologic studies (luzindole-treated plates not shown), luzindole negated the melatonin-induced effects. Supportive of a receptor-mediated pathway for melatonin toxicity, a recent MCF-7 cell study (Ram *et al.*, 2000) demonstrated correlation of cell growth inhibition with expression of the mt1 melatonin receptor. Furthermore, our experiments with isolated rat liver mitochondria and submitochondrial particles were unable to demonstrate direct enhancement of respiration by melatonin (data not shown). Together, these data suggest an indirect and receptor-mediated pathway of respiratory modulation by melatonin in the MCF-7 cells.

Previously described effects of melatonin in rodent mammary cancer models (Bojkova *et al.*, 2000; Teplitzky *et al.*, 1999), inhibition of *in vitro* breast cancer cell proliferation (Hill and Blask, 1988), and the data presented in this report (Hill *et al.*, 1992) clearly implicate melatonin as an agent toxic to the growth of breast tumor cells. However, the hormone has minimal or no toxicity reported in clinical application. For example, melatonin was essentially nontoxic in human studies even at megadoses of 6 g per night (Waldhauser *et al.*, 1990). Thus, our data suggest a potential target for receptor-mediated toxicity in MCF-7 cancer cells and further describe a mechanism through which the hormone melatonin may block aberrant breast cell growth during periods of low estrogen concentration. Future studies using *in vivo* models and human subjects will be required to further address the clinical relevance of these data with respect to the physiological role of melatonin in breast cancer prevention.

ACKNOWLEDGMENTS

We thank the Colorado State University College of Veterinary Medicine and Biomedical Sciences and the U.S. Army Center for Environmental Health Research for their support. The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as official Department of the Army position, policy, or decision, unless so designated by other official documentation.

REFERENCES

Bartsch, C., and Bartsch, H. (1999). Melatonin in cancer patients and in tumor-bearing animals. *Adv. Exp. Med. Biol.* **467,** 247–264.

Blask, D., and Hill, S. (1986). Effects of melatonin on cancer: Studies on MCF-7 human breast cancer cells in culture. *J. Neural Transm.* **21**(suppl.), 443–449.

Blask, D., Wilson, S. T., and Zalatan, F. (1997). Physiologic melatonin inhibition of human breast cancer cell growth *in vitro:* Evidence for a glutathione mediated pathway. *Cancer Res.* **57,** 1909–1914.

Bojkova, B., Kubatka, P., Mocikova, K., Mnichova, M., Ahlersova, E., and Ahlers, I. (2000). Effects of retinyl acetate and melatonin on *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in rats. A preliminary report. *Folia Biol.* **46,** 73–76.

Brezezinski, A. (1997). Melatonin in humans. *N. Engl. J. Med.* **336,** 186–195.

Cos, S., and Blask, D. (1994). Melatonin modulates growth factor activity in MCF-7 human breast cancer cells. *J. Pineal Res.* **17,** 25–32.

Cos, S., Blask, D. E., Lemus-Wilson, A., and Hill, A. B. (1991). Effects of melatonin on the cell cycle kinetics and "estrogen-rescue" of MCF-7 human breast cancer cells in culture. *J. Pineal Res.* **10,** 36–42.

Cos, S., Fernandez, F., and Sanchez-Barcelo, E. J. (1996a). Melatonin inhibits

DNA synthesis in MCF-7 human breast cancer cells in vitro. *Life Sci.* **58,** 2447–2453.

Cos, S., Recio, J., and Sanchez-Barcelo, E. J. (1996b). Modulation of the length of the cell cycle time of MCF-7 human breast cancer cells by melatonin. *Life Sci.* **58,** 811–816.

Cos, S., and Sanchez-Barcelo, E. J. (1994). Differences between pulsatile or continuous exposure to melatonin on MCF-7 human breast cancer cell proliferation. *Cancer Lett.* **85,** 105–109.

Cos, S., and Sanchez-Barcelo, E. J. (1995). Melatonin inhibition of MCF-7 human breast cancer cells growth: Influence of cell proliferation rate. *Cancer Lett.* **93,** 207–212.

Cos, S., and Sanchez-Barcelo, E. J. (2000a). Melatonin and mammary pathological growth. *Front. Neuroendocrinol.* **21,** 133–170.

Cos, S., and Sanchez-Barcelo, E. J. (2000b). Melatonin, experimental basis for a possible application in breast cancer prevention and treatment. *Histol. Histopathol.* **15,** 637–647.

Crespo, D., Fernandez-Viadero, R., Verduga, R., Ovejero, V., and Cos, S. (1997). Interaction between melatonin and estradiol on morphological and morphometric features of MCF-7 breast cancer cells. *J. Pineal Res.* **16,** 215–222.

Hill, S., and Blask, D. (1988). Effects of the pineal hormone melatonin on the proliferation and morphological characteristics of human breast cancer cells (MCF-7) in culture. *Cancer Res.* **46,** 6121–6126.

Hill, S., Spriggs, L. L., Simon, M., Muraoka, H., and Blask, D. (1992). The growth inhibitory action of melatonin on human breast cancer cells is linked to the estrogen response system. *Cancer Lett.* **64,** 249–256.

Kleinbaum, D. G., Kupper, L. L., Muller, K. E., and Nizam, A. (1998). *Applied Regression Analysis and Multivariate Methods.* Duxbury Press, Pacific Grove, CA.

Lemus-Wilson, A. M., Kelly, P. A., and Blask, D. (1995). Melatonin blocks the stimulatory effects of prolactin on human breast cancer cell growth. *Br. J. Cancer* **72,** 1435–1440.

Lissoni, P., Tancini, G., Paolorossi, F., Mandala, M., Ardizzoia, A., Malugani, F., Giani, L., and Barni, S. (1999). Chemoneuroendocrine therapy of metastatic breast cancer with persistent thrombocytopenia with weekly low-dose epirubicin plus melatonin: A phase II study. *J. Pineal Res.* **26,** 169–173.

Maestroni, G. J., and Conti, A. (1996). Melatonin in human breast cancer tissue: Association with nuclear grade and estrogen receptor status. *Lab. Invest.* **75,** 557–561.

Molis, T. M., Spriggs, L. L., and Hill, S. M. (1994). Modulation of estrogen receptor mRNA expression by melatonin in MCF-7 human breast cancer cells. *Mol. Endocrinol.* **8,** 1681–1690.

Molis, T., Spriggs, L. L., Jupiter, Y., and Hill, S. (1997). Melatonin modulation of estrogen regulated proteins, growth factors, and proto-oncogenes in human breast cancer. *J. Pineal Res.* **18,** 93–103.

Molis, T. M., Walters, M. R., and Hill, S. M. (1993). Melatonin modulation of estrogen receptor expression in MCF-7 human breast cancer cells. *Int. J. Oncol.* **3,** 687–694.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65,** 55–63.

Neri, B., de, L. V., Gemelli, M. T., di Loro, F., Mottola, A., Ponchietti, R., Raugei, A., and Cini, G. (1998). Melatonin as biological response modifier in cancer patients. *Anticancer Res.* **18,** 1329–1332.

Panzer, A., and Viljoen, M. (1997). The validity of melatonin as an oncostatic agent. *J. Pineal Res.* **22,** 184–202.

Ram, P. T., Yuan, L., Dai, J., Kiefer, T., Klotz, D. M., Spriggs, L. L., and Hill, S. M. (2000). Differential responsiveness of MCF-7 human breast cancer cell line stocks to the pineal hormone, melatonin. *J. Pineal Res.* **28,** 210–218.

Rollag, M. D., and Niswender, G. D. (1976). Radioimmunoassay of serum concentrations of melatonin in sheep exposed to different lighting regimens. *Endocrinology* **98,** 482–489.

Teplitzky, S. R., Blask, D. E., Cheng, Q., Myers, L., and Hill, S. M. (1999). Melatonin and 9-*cis*-retinoic acid in the chemoprevention of NMU-induced rat mammary carcinoma. *Adv. Exp. Med. Biol.* **460,** 363–367.

Turnbull, D. M., Chrzanowski-Lighttowlers, Z. M. A., and Lightowlers, R. N. (1993). A microtiter plate assay for cytochrome *c* oxidase in permeabilized whole cells. *Anal. Biochem.* **214,** 45–49.

Vanacek, J. (1998). Cellular mechanisms of melatonin action. *Physiol. Rev.* **78,** 687–721.

Van Itallie, C. M., and Dannies, P. S. (1988). Estrogen induces accumulation of the mitochondrial ribonucleic acid for subunit II of cytochrome oxidase in pituitary tumor cells. *Mol. Endocrinol.* **2,** 332–337.

Viljoen, P. A. (1997). The validity of melatonin as an oncostatic agent. *J. Pineal Res.* **22,** 184–202.

Waldhauser, F., Saletu, B., and Trinchard-Lugan, I. (1990). Sleep laboratory investigations on hypnotic properties of melatonin. *Psychopharmacology* **100,** 222–226.

Zang, L. Y., Cosma, G., Garnder, H., and Vallyathan, V. (1998). Scavenging of reactive oxygen species by melatonin. *Biochim. Biophys. Acta* **1425,** 469–477.