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Award Number: W81XWH-04-1-0525

TITLE: Proteinated Subnano Particles of Elemental Selenium for the Treatment of Breast Cancer

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REPORT DATE: September 2005

20060223 005

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;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-09-2005		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 Sep 04 – 31 Aug 05	
4. TITLE AND SUBTITLE Proteinated Subnano Particles of Elemental Selenium for the Treatment of Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0525	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Fritz Sieber, Ph.D. E-Mail: fsieber@mcw.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Medical College of Wisconsin Milwaukee, WI 53226				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. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The purpose of this award is to test in preclinical models the hypothesis that cytotoxic conjugates of elemental selenium and proteins are safe and effective for the systemic therapy of invasive breast cancer. The grant has three specific aims, 1) to evaluate the safety and efficacy of systemically administered Se(0)-protein conjugates in athymic nude mice bearing xenografts of human breast cancer cells, 2) to assess the functional integrity of conjugatetreated normal human hematopoietic stem cells, and 3) to determine by use of the combination index method how Se(0)-protein conjugates interact with standard chemotherapeutic agents that are commonly used in the treatment of invasive breast cancer. We report here on the successful custom synthesis and subsequent physical, chemical, and biological characterization of the selenomercocyanine dye MC54, the essential pro-drug for the generation of Se(0)-protein conjugates, as well as an extension of the in vitro evaluation of Se(0)-protein conjugates to additional human breast cancer cell lines.					
15. SUBJECT TERMS Treatment - systemic therapies - discovery and development					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 9	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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Introduction

Early detection, adjuvant hormone therapy, and adjuvant chemotherapy have improved survival rates in breast cancer. However, for patients with advanced disease, the prognosis remains poor with 5-year survival rates as low as 23% for patients with distant metastases. In the 1990s, large numbers of breast cancer patients were treated with high-dose chemotherapy and autologous stem cell transplants. The expectation was that the dose escalation afforded by the autologous stem cell transplants would have a major impact on survival. However, controlled trials have failed to show a significant advantage of high-dose chemotherapy over standard therapy. It thus appears that currently available forms of chemotherapy - even when used at high doses - are unable to reliably eradicate the disease in patients with high-risk breast cancer.

Most of the currently used anti-cancer drugs were developed based on their good performance in leukemia/lymphoma-based screening systems. Not surprisingly, they tend to perform best when used in the treatment of leukemias and lymphomas. Major breakthroughs in the treatment of breast cancer most likely require new agents whose mechanism of action is different from that of typical anti-leukemia/lymphoma drugs.

Grant W81XWH-04-1-0525 proposes to assess the safety and efficacy of a novel class of cytotoxic agents whose mechanism of action is fundamentally different from that of established anti-cancer drugs. The novel cytotoxic agents consist of high-affinity conjugates of extremely small (subnano) particles of elemental selenium (Se[0]) and (lipo)proteins. The (lipo)protein component acts as a Trojan horse that delivers the cytotoxic entity (selenium in oxidation state zero) to breast cancer cells as part of a physiological process. It exploits the fact that breast cancer cells have an increased requirement for serum albumin (and possibly also lipoproteins) and, therefore, an increased capacity to bind and internalize albumin.

Breast cancer cells internalize Se(0)-protein conjugates by an endocytotic process. Once inside their target cells, Se(0)-protein conjugates act as powerful air oxidation catalysts that rapidly deplete cells of glutathione and induce a loss of mitochondrial potential, a loss of plasma membrane asymmetry, and the activation of several caspases. The cytotoxic action of Se(0)-protein conjugates is not cell-cycle specific and appears to be only minimally affected by drug resistance mechanisms. Se(0)-protein conjugates potentiate - often synergistically - the cytotoxic effects of ionizing radiation and several standard chemotherapeutic agents.

Incorporating Se(0)-protein conjugates into the treatment of invasive breast cancer may prove particularly rewarding because breast cancer tissue is known to accumulate exceptionally large quantities of serum albumin. Typically, about one fifth of the cytosolic protein content of breast cancer cells consists of serum albumin. Despite low blood flow in breast cancer tissue, albumin clearance is very high in breast cancer tissue, and albumin extraction is 3-20 times higher than in any normal tissue. The inverse correlation between albumin content and estrogen-receptor expression suggests that Se(0)-protein conjugates may prove particularly useful in the treatment of estrogen-receptor negative breast cancer.

Grant W81XWH-04-1-0525 is designed to test the hypothesis that proteinated subnano particles of elemental selenium can be developed into safe and effective agents for the systemic treatment of invasive breast cancer if used either as single-modality

agents or in combination with certain other drugs. The grant has 3 specific aims. 1) It will evaluate the safety and efficacy of systemically administered Se(0)-protein conjugates in athymic nude mice bearing xenografts of MCF7 or MDA-MB-435 human breast cancer cells. 2) It will assess the *functional* integrity of conjugate-treated normal human hematopoietic stem cells. 3) It will determine by use of the combination index method how Se(0)-protein conjugates interact with standard chemotherapeutic agents that are commonly used in the treatment of invasive breast cancer. The objective is to identify drug combinations and dose schedules that are synergistic or at least additive with regard to the depletion of breast cancer cells but well tolerated by normal cells.

Body

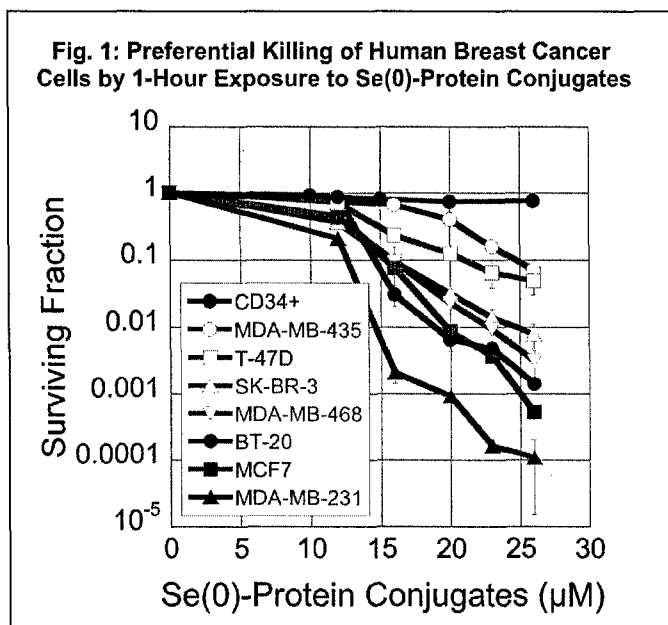
Work on this grant did not get underway until recently because we did not have access to adequate laboratory space for most of the past two years. A brief description of the circumstances that led to the delay follows below.

In August 2003, I had to move my laboratory from the 5th floor of the MACC Fund Research Center to the 4th floor of the Human Research Center (HRC). This move caused three major problems. 1) An initial lack of basic utilities (electrical outlets, water faucets) in the newly assigned laboratory space forced us to suspend all tissue culture work until January 2004. 2) The newly assigned laboratory space was only a few feet away from a walk-in incubator that was used for the production of yeast. This caused an unprecedented number of massive yeast infections in our cell cultures. 3) The HRC is plagued by severe intermittent building vibrations caused by a faulty design of the air handling equipment. The vibrations made it impossible for us to perform *in vitro* clonal assays because emerging colonies were invariably broken up into small fragments or, in extreme cases, dispersed into single cells. This defeated the very purpose of clonal assays. Since *in vitro* clonal assays are an essential component of every specific aim of grant W81XWH-04-1-0525, the building vibrations in the HRC made it virtually impossible for us to do any research relevant to grant W81XWH-04-1-0525. Like the three previous occupants of this laboratory space, we tried to isolate incubators with vibration dampening materials but were not successful. Since the institution had no plans to correct the vibration problem at the source, I accepted an offer to move my laboratory to the Clement Zablocki VA Medical Center (VAMC).

Since the move to the VAMC, we are again able to perform *in vitro* clonal assays. We also got rid of the recurrent yeast infections. Unfortunately, a full year (September 2004 to September 2005) elapsed from the time our major equipment had to be moved out of the HRC and the time the laboratory at the VA became operational. A number of factors contributed to this unexpected delay.

Since major renovations got underway in the HRC in September 2004, we had to move our major equipment out of the HRC and into storage long before essential renovations at the VAMC were completed. The renovations at the VA included the installation of additional electrical circuits (especially circuits with amperage ratings sufficient to power major equipment such as biosafety cabinets and incubators), the installation of water purifiers and a dishwasher suitable for the cleaning of tissue culture grade glassware, and the installation of tables wide enough to accommodate our

spectrophotometers. Additional delays were incurred when we could not bring our two 6-ft biosafety cabinets into the laboratory because the hallway at the VAMC was too narrow, when a third (smaller) biosafety cabinet could not be installed because the ceiling was too low, and a newly purchased (more compact) replacement biosafety cabinet was damaged in transport. As a result, tissue culture experiments could not resume until mid July 2005. Furthermore, the largest of the three laboratories at the VAMC remained essentially unusable until September 15 because a defect in the air conditioning system maintained room temperatures at 93 °F to 96 °F throughout spring and summer.



As long as my laboratory was not operational, I left the postdoctoral position unfilled because I did not want to let a year's worth of postdoctoral support go to waste. Hiring a postdoctoral fellow as an extra pair of hands to assist with the move would have made little sense because the delays were beyond our control and not caused by a lack of manpower in my laboratory. The Medical College of Wisconsin recognizes that the lack of adequate laboratory space prevented us from performing work relevant to grant W81XWH-04-1-0525

during most of this past year. Therefore, no salaries and fringe benefits were charged to grant W81XWH-04-1-0525 between September 1, 2004 and June 30, 2005. As a result, we still have most of the funds necessary to perform the work proposed in the original application, and we would like to receive permission to roll over the unused funds into the next budget year.

While I had no functioning laboratory, I tried to maintain some level of productivity by collaborating with other investigators/organizations. As part of such a collaboration, we supplied the Developmental Therapeutics Program of the National Cancer Institute with cytotoxic Se(0)-protein conjugates for testing in the 60-tumor cell line assay. Results were obtained on 54 cell lines. Fifty-three of the 54 cell lines were inhibited by Se(0)-protein conjugates, among them all 7 breast cancer cell lines included in the panel (MCF7, NCI/ADR-RES, MDA-MB-231, HS 578T, MDA-MB-435, BT-549, and T-47D). BT-549 cells were the most sensitive cells whereas HS 578T were the least sensitive cells. To the extent that there was overlap between our panel of breast cancer cell lines (Fig. 1) and the NCI's panel, both panels showed similar rank orders of sensitivity despite the fact that assay procedures used by the NCI and our laboratory were very different. Although this comparison was not part of the original proposal, the results obviously benefit grant W81XWH-04-1-0525 by confirming the efficacy of Se(0)-protein

conjugates against a broad range of human breast cancer cell lines. The results also argue that our positive findings were not assay-specific.

MDA-MB-435 cells have been widely used in breast cancer research. However, the cell line has recently come under suspicion of being a melanoma rather than a breast cancer cell line. It is worth noting in this context that the relatively low sensitivity of MDA-MB-435 cells to Se(0)-protein conjugates is consistent with the response to Se(0)-protein conjugates of melanoma cell lines.

We originally proposed to use MDA-MB-435 cells for about 50% of our experiments. Unless it can be shown in the near future that the melanoma traits in MDA-MB-435 cells are limited to a subline and do not exist in the stock distributed by the American Type Culture Collection, we will substitute another cell line. For this purpose, we have screened additional lines, and as Fig. 1 indicates, there are several lines that appear to be suitable candidates.

In accordance with Task 1a of the original Statement of Work, we have had an initial amount of selenomerocyanine dye MC54 synthesized by Aldrich (Milwaukee). We have completed the physical (mass spectroscopy, absorbance spectroscopy, fluorescence emission spectroscopy), chemical (elemental analysis), and biological characterization of the dye, and the dye appears to be in every respect equivalent to our authentic reference material. The biological characterization included both the capacity to generate fluorescent photoproduct-albumin conjugates and the capacity to generate cytotoxic Se(0)-protein conjugates. Based on the results of the first custom synthesis, we are confident that future syntheses of larger quantities by the same contractor will also be satisfactory. Pilot experiments with high potency preparations of Se(0)-protein conjugates suggest that we may want to consider substituting a less hydrophobic analogue of MC54 (e.g. a benzoxazole or a benzothiazole analogue) to improve solubility in water. Switching to a benzo-derivative should not pose any problems as the synthesis for these analogues has been worked out and is considered less difficult than the synthesis of the naphthiazole dye.

Key Research Accomplishments

- The in vitro preclinical evaluation of cytotoxic Se(0)-protein conjugates has been extended to additional breast cancer cell lines including the 7 human breast cancer cell lines that are part the NCI's 60-tumor cell line panel. All breast cancer cell lines tested sensitive to Se(0)-protein conjugates.
- Our contractor (Aldrich) has successfully synthesized an initial quantity of the selenomerocyanine dye MC54, the essential pro-drug for the generation of cytotoxic Se(0)-protein conjugates. The physical, chemical, and biological characterizations of the dye have indicated that the newly synthesized material is in every respect equivalent to our authentic reference material.

Reportable Outcomes

Poster Presentation

Era of Hope Department of Defense Breast Cancer Research Program Meeting, Philadelphia, June 8-11, 2005, p 298.

Abstract

Sampson RW, Miyagi K, Ostrowski MD, Günther WHHG, Krieg M and Sieber F: Elemental selenium for the treatment of breast cancer. Proceedings, Era of Hope Department of Defense Breast Cancer Research Program Meeting, Philadelphia, June 8-11, 2005, p 298.

Conclusions

The custom synthesis of the pro-drug (selenomerocyanine dye MC54) was successful. An extension of the in vitro preclinical evaluation of Se(0)-protein conjugates to additional breast cancer cell lines including the breast cancer cell lines that are part of the NCI's 60-tumor cell line panel confirmed that a broad range of human breast cancer cell lines are susceptible to cytotoxic Se(0)-protein conjugates. There was considerable variability with regard to the sensitivity of individual breast cancer cell lines. So far, no patterns have emerged between sensitivity to Se(0)-protein conjugates and any of the known characteristics of the cell lines.

References

None

Appendix

Sampson RW, Miyagi K, Ostrowski MD, Günther WHHG, Krieg M and Sieber F: Elemental selenium for the treatment of breast cancer. Proceedings, Era of Hope Department of Defense Breast Cancer Research Program Meeting, Philadelphia, June 8-11, 2005, p 298.

ELEMENTAL SELENIUM FOR THE TREATMENT OF BREAST CANCER

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Many cancers, including breast cancer, are known to have an increased capacity to bind and internalize serum albumin and/or lipoproteins, especially low-density lipoprotein. We recently discovered that extremely small particles of elemental selenium (Se(0)) form conjugates with albumin and lipoproteins that are highly cytotoxic if internalized by target cells. The purpose of this study was to determine in preclinical models if Se(0)-protein conjugates could be used to preferentially kill breast cancer cells. Se(0)-protein conjugates were prepared by photobleaching a photosensitizing selenomercocyanine dye in the presence of serum. Single cell suspensions of human or murine breast cancer cell lines or normal human or murine bone marrow cells were incubated in graded concentrations of the photobleached dye (Se content: $\leq 26 \mu\text{M}$) for 1 hour at 37 °C. Cells were subsequently washed free of excess Se(0)-protein conjugates, and the surviving fraction of tumor cells was determined by in vitro clonal assay. The survival of CD34-positive normal hematopoietic stem and progenitor cells was assessed by flow cytometry after staining with an appropriate fluorescent antibody. All 10 human and 3 murine breast cancer cell lines have tested sensitive to Se(0)-protein conjugates. Depletions of in vitro clonogenic cells range from 75% to $\geq 99.9\%$ at the highest drug dose tested. By contrast, identically treated human and murine bone marrow cells show no significant loss of CD34-positive cells. On average, breast cancer cells thus appear to be less sensitive to Se(0)-protein conjugates than leukemia and lymphoma cells. However, tumor cell depletions are still large enough to be of potential therapeutic interest. The cytotoxic mechanism of Se(0)-protein conjugates is not yet completely understood. Currently available data indicate that conjugates are internalized by an endocytotic process, that albumin and lipoproteins act as delivery vehicles, and that elemental selenium is the toxic entity. Conjugate accumulation and intracellular glutathione (GSH) content appear to be important determinants of sensitivity. Uptake of cytotoxic conjugates is associated with a rapid depletion of intracellular GSH, a loss of plasma membrane asymmetry and mitochondrial potential, and the activation of caspases. Se(0)-protein conjugates potentiate the cytotoxic action of ionizing radiation and several established anti-cancer drugs. Their cytotoxic activity is either not affected or only minimally affected by most multi-drug resistance mechanisms. Our discovery of cytotoxic Se(0)-protein conjugates challenges the long-held notion that selenium in oxidation state zero is biologically inert. Drugs modeled after our cytotoxic Se(0)-protein conjugates may prove useful for the treatment of metastatic breast cancer.

The U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0525 supported this work.