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| 13. ABSTRACT (Maximum 200 Words) The research effort is directed at evaluation of the alpha-emitting radionuclide, Bi-213 targeted to a metastatic breast cancer model (subrenal capsules) in an antibody-targeted protocol called "pretargeting". In the studies, biotinylated anti-breast cancer antibody, huBrE-3 was to be used as a pretargeting agent. However, the antibody has not been provided to us (as previously promised). A substantial amount of work has been conducted to find an alternative antibody for use in the studies. The antibodies, L6 and NR-LU-10 are being evaluated as alternatives. Subrenal capsules of MCF-7 cells have been prepared, with a successful growth rate of 75%. The carrier for Bi-213 in the pretargeting protocols can be either modified streptavidin or a biotin-chelate derivative. Three biotin derivatives containing the CHX (DTPA) chelate and one containing the DOTA chelate were synthesized for the study. The biotin-DOTA derivative labeled too slowly for use with Bi-213. Streptavidin has been conjugated with the CHX-A'' chelate and succinylated to obtain low kidney concentrations. Biodistribution of the Bi-213 labeled succinylated CHX-A''-streptavidin vs the same material radioiodinated demonstrated that the Bi-213 label was very stable in vivo. | | | |
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FOREWORD

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A. INTRODUCTION

This report describes the research efforts conducted during the first year of funding (August 15, 1998 thru August 14, 1999) of grant number DAMD17-98-1-8258. The research project is directed at developing a new approach to the therapy of metastatic breast cancer. In the new approach, metastatic prostate cancer an alpha-emitting radionuclide, ^{213}Bi , will be targeted to breast cancer cells through an antibody-based targeting system which has been termed "pretargeting". Three objectives were to be addressed in 14 tasks in the first year of study. We were unable to complete the objectives as outlined (Statement of Work) as the monoclonal antibody, BrE-3, that was promised to us was not obtained. A fair amount of effort has been spent to develop a working antibody/tumor xenograft model that was not previously anticipated. More on this problem is provided later. Although not completed, significant progress has been made toward the Year 1 tasks outlined in the Statement of Work" of the proposed studies. A description of the progress made / results obtained is provided in the following section. Areas where new information indicates changes from the proposal are pointed out.

B. RESEARCH RESULTS

The research results are outlined below in sections based on the **Objectives** outlined. The **Tasks** that each subheading addressed are included after the objective.

Objective 1: Evaluate stability and tissue distribution of ^{213}Bi labeled biotin chelates

Task 1: Prepare and fully characterize biotin-sarcosine-CHX-B and biotin-sarcosine-DOTA

We successfully synthesized and characterized four biotin derivatives containing chelates for ^{213}Bi . The first two biotin derivatives that we targeted, biotin-sarcosine-CHX-B and biotin-sarcosine-DOTA have been synthesized and fully characterized. The synthesis of these two compounds is outlined in Scheme 1. Biotin-sarcosine-CHX-B, **9**, was prepared from biotin as we previously published [1], and the biotin-sarcosine-DOTA, **11**, was prepared in a similar manner. As part of the study, Dr. Martin Brechbiel (NIH) provided the nitrobenzyl-CHX-B and nitrobenzyl-DOTA to make these compounds. Those compounds were easily reduced to the aminobenzyl derivatives, **8** and **10**, by catalytic hydrogenation for application to the biotin derivatives

We also studied the stable and radioactive bismuth labeling of biotin derivatives **9** and **11** as depicted in Figure 1. As previously reported [1], the CHX-B derivative, **9**, labeled very quickly (almost instantaneous). Contrary to this, labeling of the biotin-DOTA derivative, **11**, was found

to be slow, but it went to completion at room temperature (see chromatograms in Figure 2). The radiolabeling experiments with ^{213}Bi did not provide good yields, so we have abandoned the DOTA chelation group in our studies. Animal studies have shown the CHX DTPA to be quite stable in vivo [2,3](see also biodistribution of Bi-labeled streptavidin).

Scheme 1: Synthetic pathways to prepare biotin-sarcosine-CHX and biotin-sarcosine-DOTA

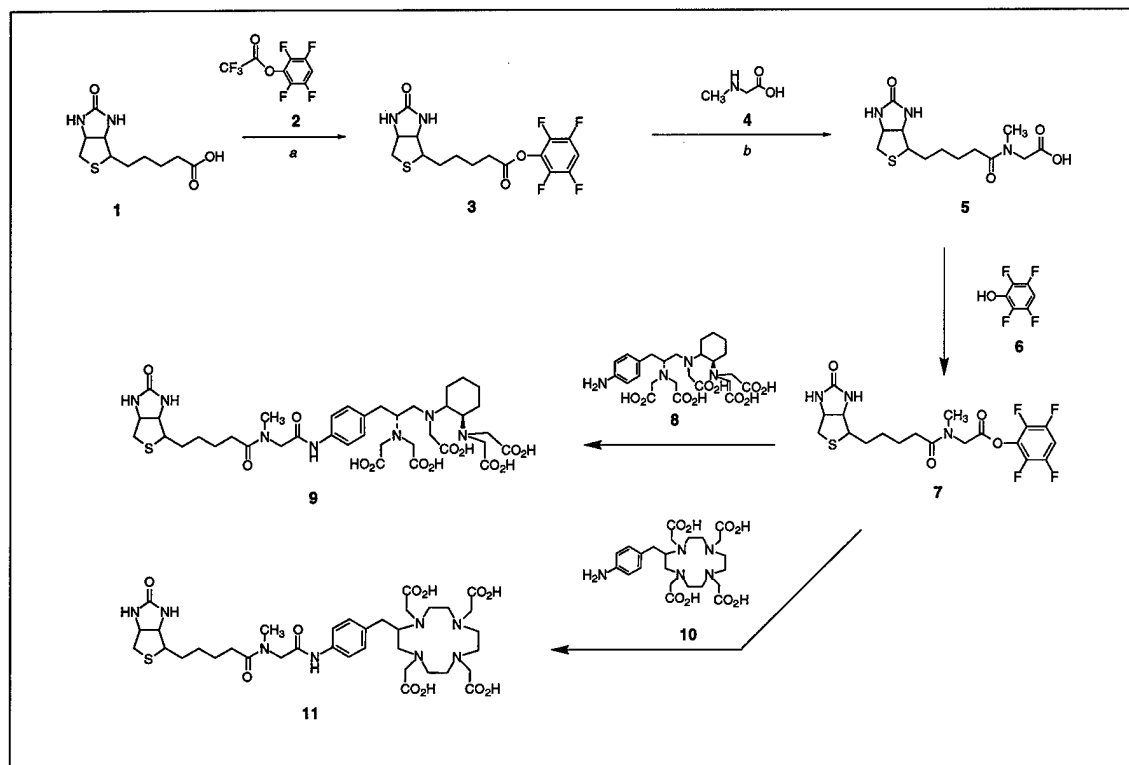


Figure 1: Schematic representation of bismuth labeling of biotin derivatives

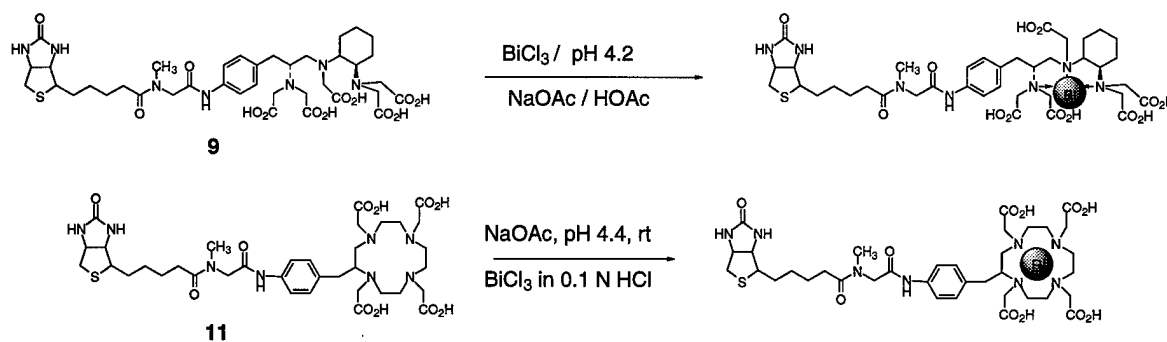
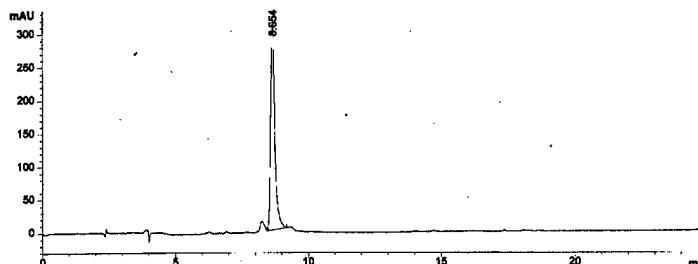
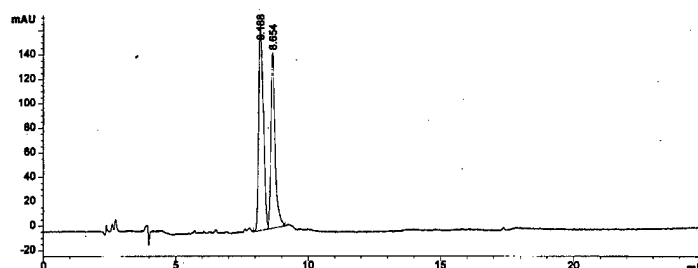
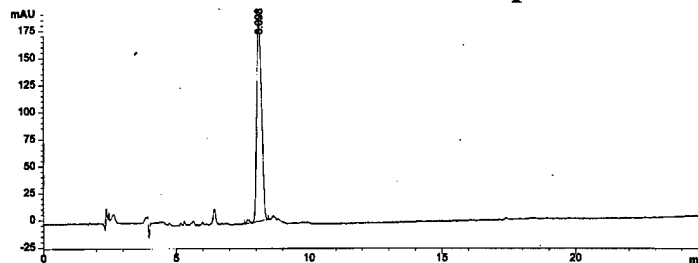
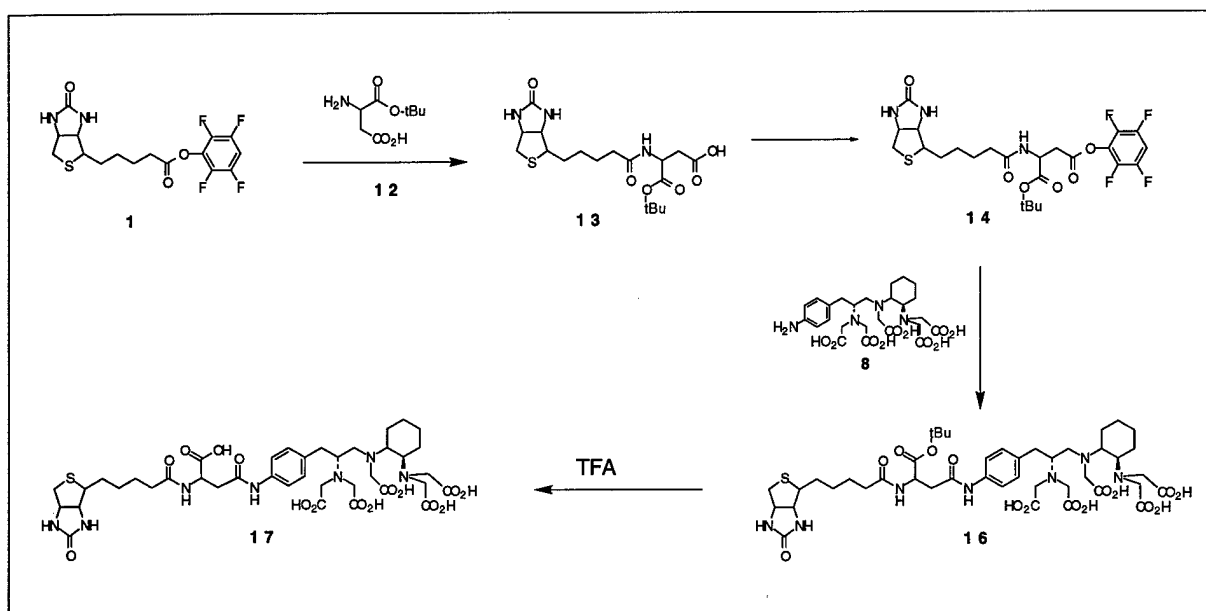


Figure 2: Scans of chromatograms from BiCl₃ chelation of biotin-sarcosine-DOTA**A: At 10 min reaction time – no Bi chelation****B: At 10 min reaction time – about 50% reaction****C: At 24 hour reaction time – complete chelation**

Our continuing studies with radiolabeled biotin derivatives have produced new information about what derivatives might be more favorable for *in vivo* use. The N-methylglycine (sarcosine) adduct of biotin was previously targeted because it was shown that this moiety effectively blocked the biotin cleaving action of the serum enzyme, biotinidase [4-6]. However, we have observed that the N-methyl group causes the dissociation of biotin from avidin and streptavidin to be greatly increased (unpublished results). As our optimal biotin derivatives have very high binding affinities with avidin and streptavidin, we conducted studies to determine which (of several different) biotin derivatives retained the very slow dissociation found in unaltered biotin. We found that the length of side chain and the biotinamide moiety must be

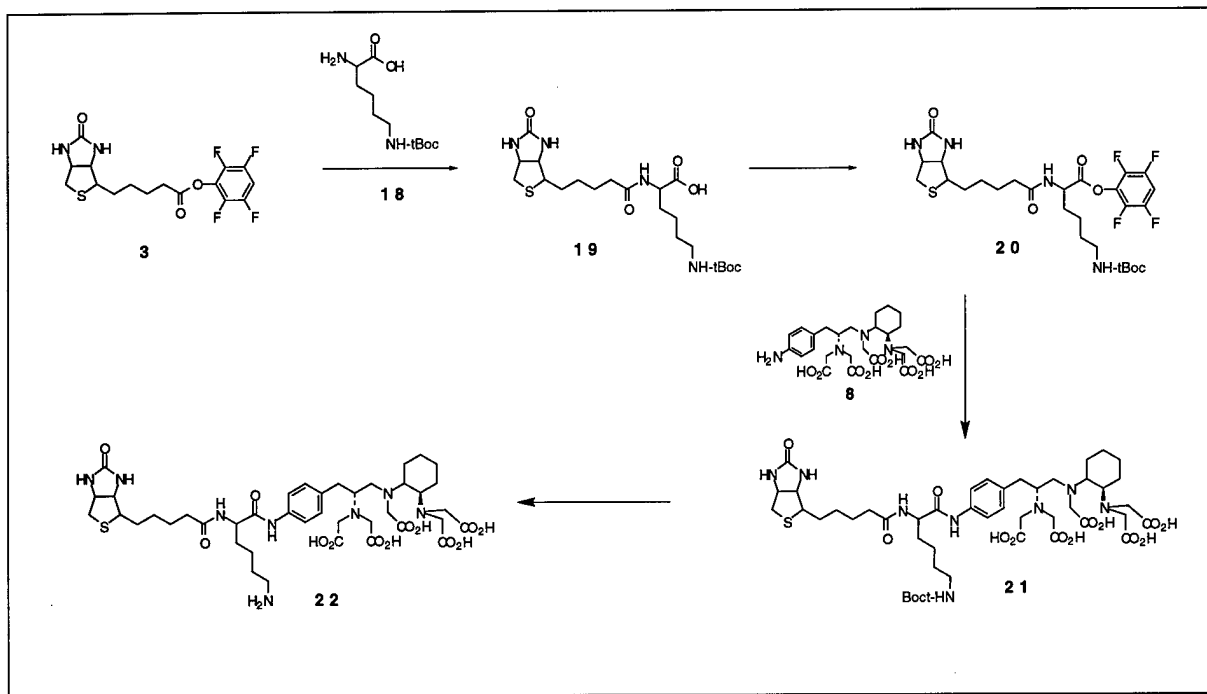
retained to obtain high binding affinities. Further, we have found that biotin derivatives with bulky substituents alpha to the biotinamide blocked biotinidase activity with only minimal increase in the dissociation rates. Therefore, we felt it was important to prepare additional biotin-CHX derivatives to have the optimal compound for the studies. Preparation of a biotin-aspartate-CHX-A'' derivative, **17**, and a biotin-lysine-CHX-A'' derivative, **22**, were prepared as shown in Schemes 2 and 3.

Scheme 2: Biotin-CHX-A'' derivative which contains aspartic acid for biotinidase stabilization



These compounds have been prepared and fully characterized. They are stored as the t-butyl ester **16** and N-tBoc, **21**, protected derivatives, but can be prepared for bismuth-213 labeling by treatment with neat trifluoroacetic acid. Radiolabeling of **17** and **22** with Bi-213, and evaluation of the labeled derivatives in athymic mice is planned for the near future.

Scheme 3: Biotin-CHX-A'' derivative which contains lysine for biotinidase stabilization



Task 2: Obtain ²²⁵Ac/²¹³Bi generator and evaluate/optimize the labeling of biotin derivatives.

Task 3: Evaluate distribution / in vivo stability of ²¹³Bi labeled biotin derivatives

These tasks have not been completed at this time as they were delayed until we had the new biotin derivatives prepared for testing. We anticipate these tasks to be completed in the next month or two.

Objective 2: Evaluate Biotinylated Antibody and Streptavidin Co-Localization and Blood Clearance

Task 4: Obtain humanized BrE-3 from Dr. Ceriani (Walnut Creek, CA)

The proposal that was written had little effort spent on obtaining an anti-breast antibody as I received a letter from Dr. Roberto L. Ceriani of the Cancer Research Institute of Contra Costa that indicated up to 100 mg of huBrE-3 would be provided for the study (letter attached as Appendix Item 1). Early in the studies I wrote a letter to Dr. Ceriani to make arrangements to obtain the antibody. I got no response, but I felt that he was a busy person so I did not aggressively pursue it. My timeframe for getting the antibody was at 6 months into the project as

it was not needed until then. As that time approached, I called his office, sent several e-mails, and wrote another letter. I have obtained no response. I have tried many times since then to reach him via e-mail (internet <http://mypage.ihost.com/CancerResearchInstitute>) and by phone (925-943-1167), but have not spoken with him directly and have not received any responses thus far. I have recently tried again to contact Dr. Ceriani, but without success. (This has been the most frustrating situation that I have ever run up against!).

After it was clear the huBrE-3 would not be obtained, I began to look for another anti-breast cancer antibody. I spoke with Dr. Alan Fritzberg at NeoRx Corporation (Seattle) about breast cancer antibodies and he indicated that their pan-carcinoma antibody NR-LU-10 had detected breast cancers. He indicated that they would collaborate by providing the antibody, but that a material transfer agreement had to be put in place between NeoRx and the University of Washington. The paperwork was put together and sent to our Office of Technology Transfer (OTT). The OTT found the wording of the material transfer agreement to not be acceptable and NeoRx would not change the wording. Therefore, I could not obtain the antibody from them. I looked for another breast cancer antibody and decided that the L6 antibody that was in several publications of Dr. DeNardo [7-11] might be a good candidate for our studies. This antibody was owned by Bristol-Myers Squibb, but recently was acquired by a new startup company in Seattle, Seattle Genetics. Dr. Perry Fell of Seattle Genetics agreed to provide the antibody for our studies, and we were successful in getting a material transfer agreement in place. They kindly provided 100 mg of L6 for the studies.

Task 5: Begin to grow up MCF-7 cells for use in single cell evaluations

MCF-7 cells were obtained and grown up for use in Dr. Vessella's laboratory for use in these studies.

Task 6: Biotinylate the antibody and evaluate the number of biotins on it.

The L-6 that obtained was biotinylated and assessed for the number of biotins present. It was found that there were 1.3 biotins / antibody by the HABA test. Before this parameter was optimized, it was important to test the cell binding of biotinylated L6 with MCF-7 cells vs non-biotinylated L6.

Task 7: Radioiodinate biotinylated L6 and evaluate binding with MCF-7 cells.

Cell immunoassays were conducted on aliquots of 1×10^6 cells, with 2 assays / sample. The cells were either fresh or thawed and adjusted to 1×10^6 viable cells if revived from frozen aliquots. After addition of radiolabeled L6 or radioiodinated/biotinylated L6, the cells were incubated at 37°C for 45 min on a cell rotator. In the first experiment, the amount of L6 required

to saturate the MCF-7 cells was examined. To obtain this, increasing quantities of radioiodinated L6 were added to fresh MCF-7 cells in an attempt to determine when the antibody was in excess. It was found that the radioiodinated L6 bound only to the extent of 2.6%. This result was troubling, so another experiment was conducted where a non-specific radioiodinated antibody, MOPC-21 was compared with radioiodinated L6 and radioiodinated/biotinylated L6. The results were not good. The MOPC-21 bound 4.6-5.3 %, the L6 bound 5.2-6.8 % and the L6-biotin bound 2.8-3.6 %. This meant that there was no binding of L6 to MCF-7 cells.

The previous result prompted us to test the radiolabeled L6 against a number of other breast cancer cell lines (ductal and epithelial lines – all cells are in tissue or cell culture bank at ATCC). The cell lines tested were:

| Cell Line | Binding |
|--------------------------------|---------|
| SK-BR-3 (passage 3, Vessella), | 2.6% |
| BT-20 (passage 292), | 4.2% |
| T47D (passage 91), | 2.6% |
| ZR-75-1 (passage 1, Vessella) | 2.8% |
| MDB0M8-134VI (passage 83) | 4.9% |

The results clearly indicate that there is no specific binding of the radioiodinated L6 to the breast cancer cells tested. We have contacted Seattle Genetics and asked them whether the antibody batch that they gave to us might be bad (it has been stored for several years) and whether there might be another breast cancer cell line that it binds with. We have not gotten a reply yet.

With the prospect that the L6 may not be a good candidate antibody, we have gone back to NeoRx Corporation and opened discussions again about obtaining NR-LU-10 for our studies. They have indicated that they would allow the University of Washington to provide a material transfer agreement and they will sign it. That process is underway at this time. We anticipate having NR-LU-10 in the laboratory within the next month. We will test it against the cell lines listed and any breast cancer cell lines that NeoRx has shown it to bind with.

Task 8: Prepared a biotinylated blood clearance reagent and characterize. Demonstrate binding with streptavidin.

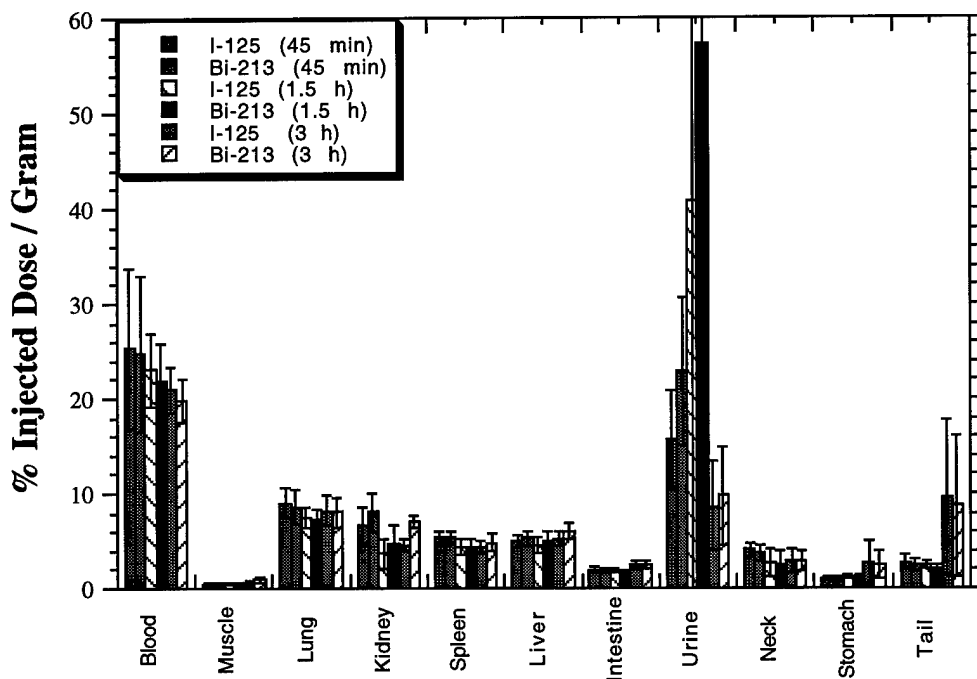
We have prepared biotinylated asialoorosomuroid protein [12] and have shown that it clears streptavidin from blood.

Task 9: Evaluate blood clearance of radiolabeled antibody-streptavidin conjugate using the biotinylated clearing agent.

This task has not been completed as the antibody to be used has not been determined (see previous explanation). We were interested in the possibility of using a 2-step pretargeting protocol that has the ²¹³Bi attached to streptavidin (targeting biotinylated antibody) rather than the 2-step protocol where ²¹³Bi is on a biotin derivative (targeting the antibody-streptavidin conjugate) so we conducted an animal study with ²¹³Bi-labeled succinylated streptavidin vs radioiodinated chelated streptavidin. Succinylated streptavidin has been shown to not localize to kidney as the native streptavidin does [13,14].

In the study, streptavidin was demetallated, conjugated with isothiocyanato-benzyl-CHX-A'', and succinylated. It was again demetallated and stored for use. A quantity of this material was labeled with ¹²⁵I and another quantity was labeled with Bi-213. The radiolabeled succinylated/CHX-A'' conjugated streptavidin was injected into athymic mice that did not have tumor xenografts. Mice were sacrificed at 45 min, 1.5 h, and 3 h after injection. The times were chosen as they represent 1, 2, and 4 half-lives of Bi-213. A bar graph of the data obtained is provided in Figure 3.

Figure 3: Biodistribution of ²¹³Bi- and ¹²⁵I-labeled Succinylated Streptavidin



The primary goal of this experiment was to determine how stable the ^{213}Bi label was on streptavidin over the period of 4 half-lives. It is apparent from the data that the ^{213}Bi is very stable on the succinylated streptavidin. This experiment indicates that it may be possible to use succinylated streptavidin as the carrier of ^{213}Bi if the cancer cells are readily accessible to the blood, which may be the case for metastatic cancer cells.

Task 10: Place cells in subrenal capsule for use in tumor localization studies.

Studies were conducted to determine if MCF-7 would grow in subrenal capsules, the metastatic model that we are going to use in these studies. Twenty animals were injected with 2×10^5 cells under the renal capsule. Of those injected, 15 animals grew observable tumors (a 75% take rate). The animals (groups of 4) were assigned a time for sacrifice to assess tumor burden (at 2,3,4,5, and 6 weeks post implantation). The tumor burdens were assessed via three methods.

1. Tumor volume (ovoid mass in 3 dimensions). This was calculated by the formula: length x width x height x 0.5236, which is the most common formula for subcutaneous tumors.
2. Change in kidney mass. This measurement is based on the weight of the implanted kidney vs non-tumor bearing kidney. The problem with this measurement is that there may be a lot of apoptosis occurring and the kidney can decrease in weight rather than increase.
3. Surface area of lesion (length x width). This seemed to be the most accurate as depth was difficult to judge due to infiltration of the cells into the cortex, whereas the surface of the capsule was easy to measure.

A graphical representation of results obtained using the three methods of measurement out above is included as Appendix Item 2. The graphs show that there is no clear trend in size of tumor with time from implantation.

Task 11: Evaluate Tumor Localization of biotinylated antibody and Co-localization with Streptavidin.

This task has not been completed as we do not have an antibody that is appropriate at this time.

Objective 3: Evaluate binding and toxicity of ^{213}Bi -labeled biotin in an in vitro tumor cell model using the pretargeting method.

Tasks 12: Evaluate cell survival of MCF-7 cells in culture when treated with biotinylated antibody / streptavidin and biotinylated antibody and ^{213}Bi -labeled biotin.

As we do not have the antibody for this system, we have conducted studies with another antibody CA12.10 (a canine anti-CD45 antibody reactive with hematopoietic cells). In those studies we demonstrated that the antibody directly labeled with ^{213}Bi provided very efficient cell killing when labeled at high specific activity (RBE about 10). At low specific activity, cell survival was the same as that found for a non-specific labeled antibody. With saturation of the cells, all of the cells were killed. The results were compared with a dose-equivalent response of external beam gamma irradiation. The data obtained is provided as Appendix Item 3.

Task 13: Continue cell survival studies with Bi-labeled biotin

This task has not been worked on yet. We will conduct the studies after we have identified an antibody to use.

Task 14: Calculate doses delivered and examine the biological effectiveness of the doses provided by ^{213}Bi .

A portion of this task has been done as described above. More studies will be done with the next ^{213}Bi labeling experiments.

C. KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized three biotin-CHX-A'' derivatives and a biotin-DOTA derivative for labeling with ^{213}Bi .
2. Tested some of biotin derivatives for labeling with stable bismuth and ^{213}Bi .
3. Obtained MCF-7 cells, grew them in culture, and implanted in subrenal capsules for the animal model to be used.
4. Attempted to obtain huBrE-3 antibody from Dr. Ceriani (did not get response). Obtained L6 antibody from Seattle Genetics for testing. Also working on obtaining NR-LU-10 antibody as a backup.
5. Successfully succinylated streptavidin and evaluated its in vitro and in vivo characteristics with various levels of succinylation (done in concert with other studies).
6. Biotinylated and radioiodinated L6. Found that it did not bind with several different breast cancer cell lines. Further studies to explain this are underway.

7. Prepared biotinylated asialoorosomuroid protein and tested it as a clearing agent for streptavidin and/or antibody-streptavidin conjugates.
8. Determined that the ^{213}Bi -label is very stable over three half-lives when labeled streptavidin is injected into mice.
9. Determined that ^{213}Bi -labeled antibody is very cytotoxic with a RBE of approximately 10 when it is used at a very high specific activity.

D. REPORTABLE OUTCOMES:

There have been no submitted manuscripts or presentations to date on this research effort. However, it is anticipated that a manuscript will be prepared and submitted for publication within the next year.

E. CONCLUSIONS:

The first year of studies has been severely hampered by the fact that Dr. Ceriani did not provide the antibody that he had stated (in a letter – Appendix Item 1) he would. We have had difficulty in obtaining an alternate antibody, but feel this will be accomplished shortly. We have prepared some new biotin-CHX (DTPA) derivatives based on results from other studies that have been conducted in our laboratory. We have not tested these compounds yet as their synthesis has only recently been accomplished. We have conjugated CHX-A'' to streptavidin and have ^{213}Bi labeled it to determine if the ^{213}Bi label was stable enough for use on this carrier molecule. It was found to be stable, therefore, we will test this compound as well as the biotin derivatives. As expected, our studies of the radiotoxicity of the ^{213}Bi have shown that it is very cytotoxic on single cells.

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G. APPENDICES:

Appendix Item 1: Letter from Dr. Ceriani assuring us that we will get huBrE-3 for the studies

Appendix Item 2: Graphs of results obtained using different methods of measurement of the MCF-7 subrenal capsule size.

Appendix Item 3: Cell Survival data for external beam irradiation, irradiation with low and high specific activity ^{213}Bi -labeled antibody, and ^{213}Bi -labeled non-specific antibody.

APPENDIX ITEM 1



Cancer Research Institute of Contra Costa
"great oaks from little acorns grow"

June 12, 1997

Dr. Scott Wilbur
Department of Radiation Oncology
Radiochemistry Group
University of Washington
2121 N. 35th Street
Seattle, WA 98103-9103

Dear Dr. Wilbur:

In response to your letter of June 3, 1997, and its contents, I wish to assure my supply of antibody huBrE-3 (up to 100mg) to be used in your project "Preclinical Evaluation of a Targeted Alpha-Emitting Radionuclide in Radiotherapy of Breast Cancer" in accordance with the conditions of your request. This antibody cannot be used for any commercial purpose and cannot be administered to humans.

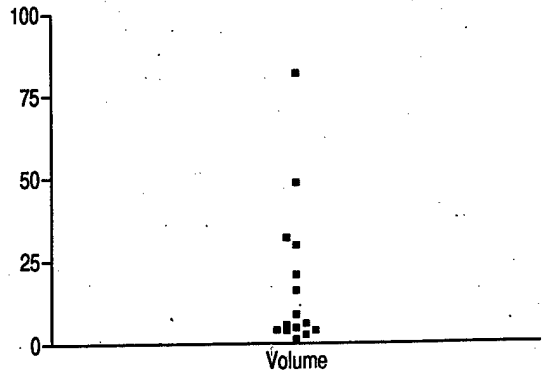
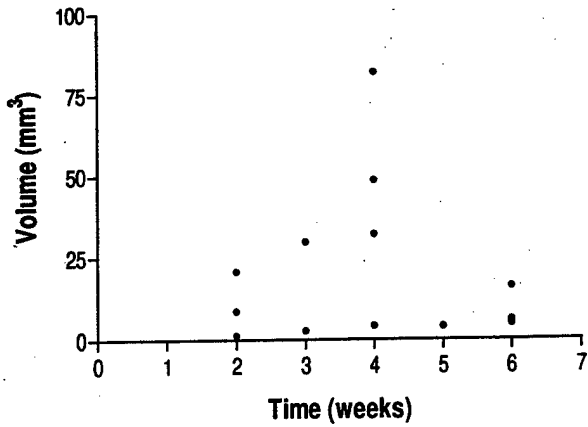
I believe your project proposes a very interesting new use for our antibody and I look forward to a successful collaboration.

Sincerely,

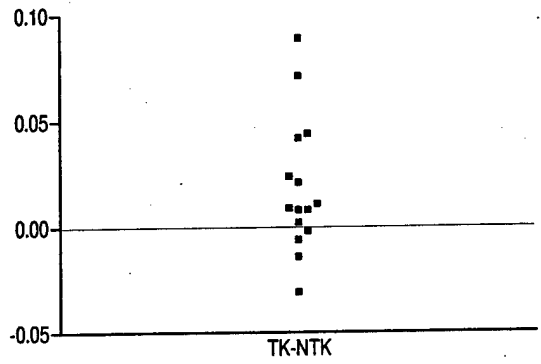
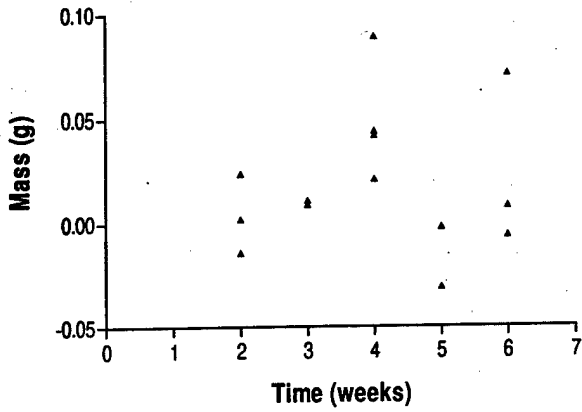
Roberto L. Ceriani, M.D., Ph.D.
Principal Investigator
RLC/ljdb
file 5/a:103.ltr

APPENDIX ITEM 2

Volume vs. Time



Δ Kidney Mass vs. Time



Surface Area vs. Time

