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13. Abstract (Maximum 200 Words) <i>(abstract should contain no proprietary or confidential information)</i> The purpose of this research project was to develop conditions and reagents required for effective targeting of the alpha-emitting radionuclide At-211 to metastatic prostate cancer cells. In the studies conducted, three different approaches to "pretargeting" At-211 to human prostate cancer xenografts were evaluated in athymic mice. One of the important questions asked was whether At-211 could be stably attached to a carrier molecule. The results obtained indicate that stable attachments can be obtained for certain types of compounds (i.e. nido-carboranes) but not for others (i.e. arylastatides). Effective tumor targeting was obtained in the studies, but higher than anticipated concentrations of reagents were found in non-target tissues. This finding may be due to the use of intact antibodies (Fc mediated binding). Another problem that became apparent was the fact that large quantities of biotin binding reagents (streptavidin or antibody-streptavidin conjugates) had to be used to offset the endogenous biotin released from body stores. The studies have help to delineate the requirements for pretargeting of At-211, but additional studies are required to optimize this approach.				
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A. INTRODUCTION

A Final Report for grant number DAMD 17-98-1-8500 was submitted on February 15th and I received a letter dated July 11, 2001 indicating that it had been reviewed and found “acceptable as written”. However, due to difficulties in obtaining the quantities of monoclonal antibodies required for the planned studies, some of the studies were not completed within the initial funding period. To complete more of the studies, I asked for, and received a “no-cost” extension of the unspent funds to conduct additional animals studies. This **Supplemental Final Report** describes the additional studies conducted during the period 2/15/01 thru 8/14/01.

It will be difficult to convey the full breadth of the research in this supplemental report. Therefore, it is recommended that the reader obtain a copy of the Final Report to assist in understanding how the supplemental research fits with the overall research project. To help the reader of this report, some information from the Final Report is included, and the Scheme numbering is continued from that report.

The research project was directed at developing a new approach to the therapy of metastatic prostate cancer. The goal of the studies was to develop conditions to effectively target metastatic prostate cancer with an alpha-emitting radionuclide, astatine-211, using an antibody-based targeting system which is termed “pretargeting”. To accomplish that goal, five Technical Objectives were to be addressed. The Objectives that were addressed in the supplemental studies are shown below:

Technical Objective 1: To optimize conditions for “2-step” pretargeting.

Technical Objective 3: To evaluate the localization of At-211 labeled biotin and streptavidin using “2-step” and “3-step” pretargeting protocols.

In the previously submitted Final Report, the results from seventeen animal studies funded by this grant were described. Three additional animal studies were conducted during the extension period. The first supplemental study was directed at the development of an improved clearing agent for use in the “two-step” pretargeting protocol. The second supplemental study was directed at further evaluation of the stability of an astatine-211 labeled *nido*-carborane containing biotin derivative. The third supplemental study was an evaluation of a pretargeting protocol which employed the newly prepared clearing agent and a radioiodinated *nido*-carboranyl biotin derivative. The results of those studies are described below.

B. RESEARCH RESULTS (SUPPLEMENTAL STUDIES)

The results obtained from the research effort are outlined below in sections based on the Technical Objectives that the research related to. The Tasks that were addressed are included under each Objective.

Technical Objective 1: To optimize conditions for “2-step” pretargeting.

The “2-step” protocol initially investigated (from proposed studies) has as a first step injection of a biotinylated monoclonal antibody (mAb) for localization of biotin on cancer cells. After a period of time for tumor localization of the biotinylated mAb, a biotin-binding clearing agent

(usually avidin) is injected. This reagent does not affect the tumor-bound antibody, but rapidly clears the excess biotinylated mAb from blood. After the blood is cleared of biotinylated antibody, a second step is to inject a radiolabeled streptavidin (SAv) to bind with the biotinylated antibody on the tumor cells. Optimization of this protocol involves determining quantities and times required to gain a maximum dose on cancer cells and minimum dose in non-target tissues.

The foregoing explanation has been taken from the Final Report. However, from the results of our studies, and studies reported by other investigators, a two-step pretargeting protocol which utilizes monoclonal antibody-streptavidin (mAb-SAv) targeting of cancer cells, followed by blood clearance of that reagent, then administration of a radiolabeled biotin derivative, appears to have better in vivo characteristics. Thus, the supplemental studies focused on this second "two-step" approach. The supplemental studies were conducted to optimize this protocol are described below. Numbering of the animal experiments is continued from the Final Report. In all of the studies, five mice were used per group (time point).

Task 3: Optimize clearing agent in 2-step pretargeting

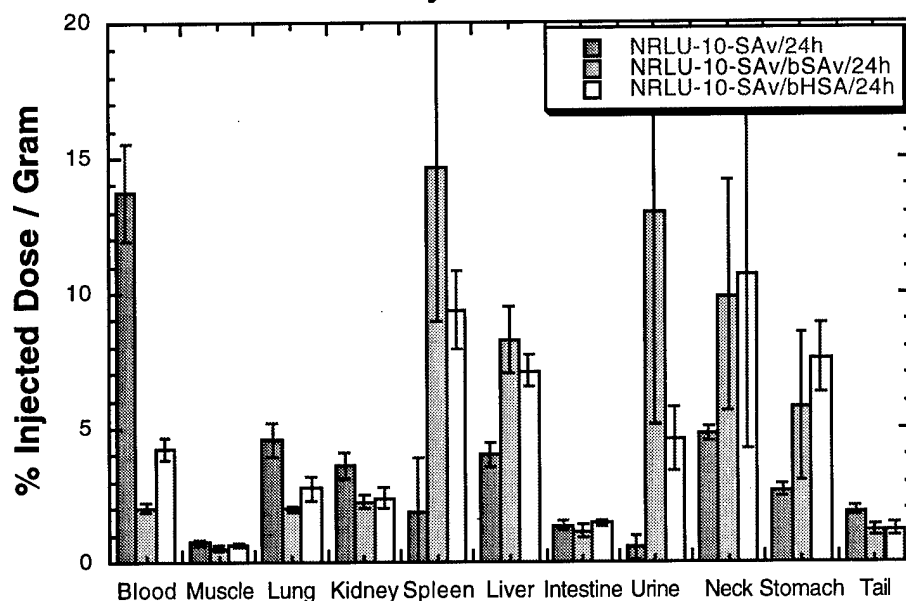
It was apparent from our studies that the biotin-binding "clearing agents" that we previously studied were not optimal. Our initial studies involved the use of biotinylated and neuramidase treated asialoorosomucoid protein. This reagent provided poor clearance of the monoclonal antibody-streptavidin (mAb-SAv) conjugate. We believed that the problem was that there were not enough galactosyl groups present to get efficient clearance via the Ashwell receptors in the liver. In subsequent studies, we investigated the use of biotinylated and galactosylated BSA (bovine serum albumin) and HSA (human serum albumin). These reagents worked more efficiently, but with the protocols for conjugations that we used, were also not optimal.

In the last in vivo experiment reported in the Final Report, we described the development of a new clearing agent, glycosylated streptavidin bound with a biotin trimer [1] that is biotinidase stabilized. The results of that experiment (from Final Report) are described below.

Animal Experiment 17 In the seventeenth animal study, mice were injected with 400 µg of a mAb-SAv conjugate (NRLU-10-SAv). The mAb-SAv conjugate was allowed to circulate for 23 h, then 100 µg of the SAv-biotin trimer conjugate or biotinylated/galactosylated human serum albumin (HSA) was injected. In a control group, no clearing agent was administered. At 1 h after injection of the clearing agent biodistributions were conducted. The results of the tissue distribution are shown in Figure 17. Blood clearance with the SAv-biotin trimer was fairly effective, providing >86% clearance, whereas the biotinylated/galactosylated HSA provided only 71% clearance under the same conditions.

Our goal is to decrease the blood level of biotin binding reagents by >95% (i.e. to less than 1% ID/g). Therefore, in the supplemental studies the above investigation was extended to determine if 2 injections (2 h apart) of clearing agent would improve the total amount cleared from blood. Also, the results obtained in Experiment #17 indicated that our new SAv clearing agent might be superior to biotinylated/galactosylated HSA, but this none of the reagents had been optimized so this was uncertain. Because a publication by Axworthy et al. [2] described an optimized method for biotinylation and galactosylation of HSA, we decided to include biotinylated/galactosylated HSA in the study (using their optimized conjugation conditions). We also felt that it would

Figure 17
Blood Clearance using Biotinylated and Galactosylated SA_v and HSA



be appropriate to include biotinylated and galactosylated avidin, as avidin is much less expensive than SA_v. The biotinylated and galactosylated avidin was obtained in the same manner as streptavidin. The conditions of the experiment and the results obtained are provided below (Animal Experiment 18).

Animal Experiment 18 The eighteenth animal experiment was designed to evaluate two different parameters. Those parameters included: (1) a comparison of biotinylated and galactosylated SA_v, Av and HSA, and (2) a determination of whether 2 injections of clearing agent would improve the blood clearance. The quantities of reagents, i.e. 400 µg of [¹²⁵I]NRLU-10-SA_v conjugate and 100 µg clearing agent, were the same as used in experiment 17. For the first part of the experiment, the clearing agent was administered at 22 h post the injection of [¹²⁵I]NRLU-10-SA_v and the mice were sacrificed 2 h post that (24 h pi NRLU-10 administration). The results are depicted in Figure 18a. It appears that both SA_v and HSA based clearing agents were superior to the Av-based clearing agent. However, the clearance was not as good as targeted, being only 3-4% ID/g. The second part of the study was conducted in the same manner as the first part, except a first injection of clearing agent was made at 20h and a second injection was made at 22h pi [¹²⁵I]NRLU-10-SA_v conjugate. The results of that experiment are shown graphically in Figure 18b. The %ID/g of NRLU-10-SA_v conjugate decreased slightly (about 1%ID/g), but the second injection of Av or HSA-based clearing agents did not change the %ID/g [¹²⁵I]NRLU-10-SA_v obtained with only one injection.

The goal for clearing blood of biotinylated antibody was not attained. The lack of clearing blood of the [¹²⁵I]NRLU-10-SA_v may indicate that the biotin binding pockets were filled with endogenous biotin. However, reports by NeoRx personnel indicate that their dendrimer based clearing agent can result in less than 1% ID/g of the mAb-SA_v conjugate in the blood makes us believe that further studies are needed to improve this parameter. If good blood clearance of the

mAb-SAv is not attained, the subsequently administered radiolabeled biotin will bind with it. This would result in a poor tumor/blood ratio and increased normal tissue radiation doses.

Figure 18a
Biotinylated/Galactosylated Clearing Agent at 22 h
after NRLU-10-SAv injection

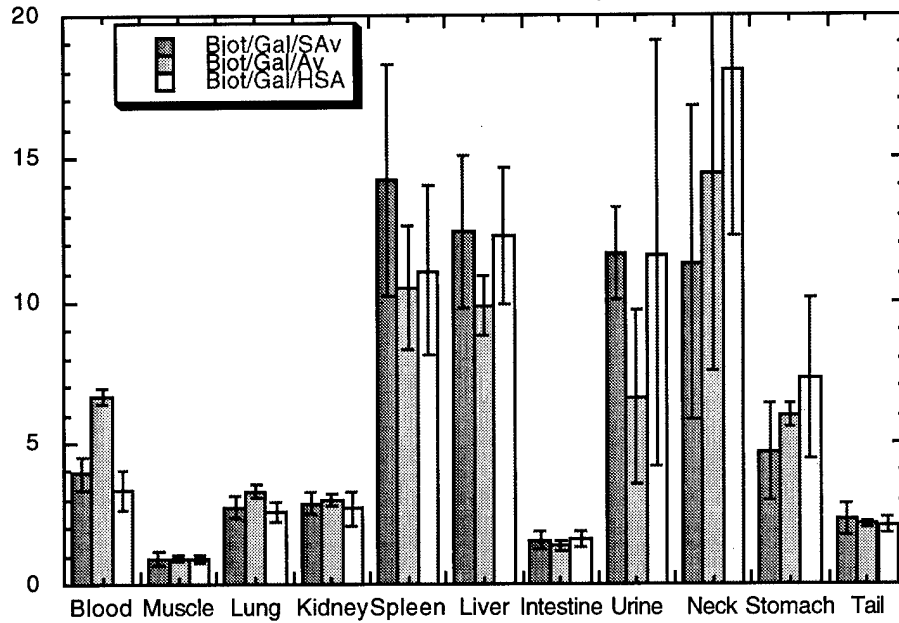
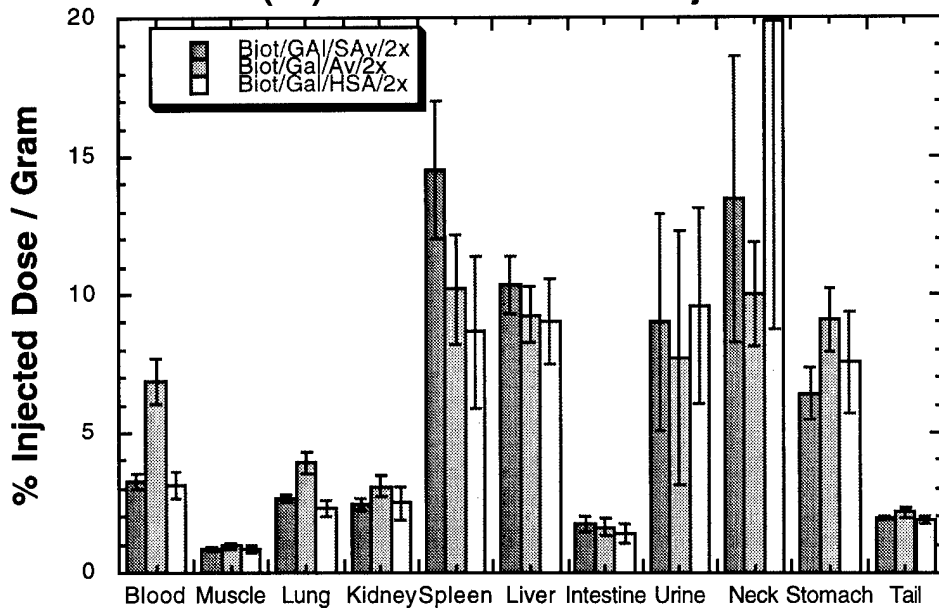


Figure 18b
Biotinylated/Galactosylated Clearing Agent at 20 and 22 h
(2x) after NRLU-10-SAv injection



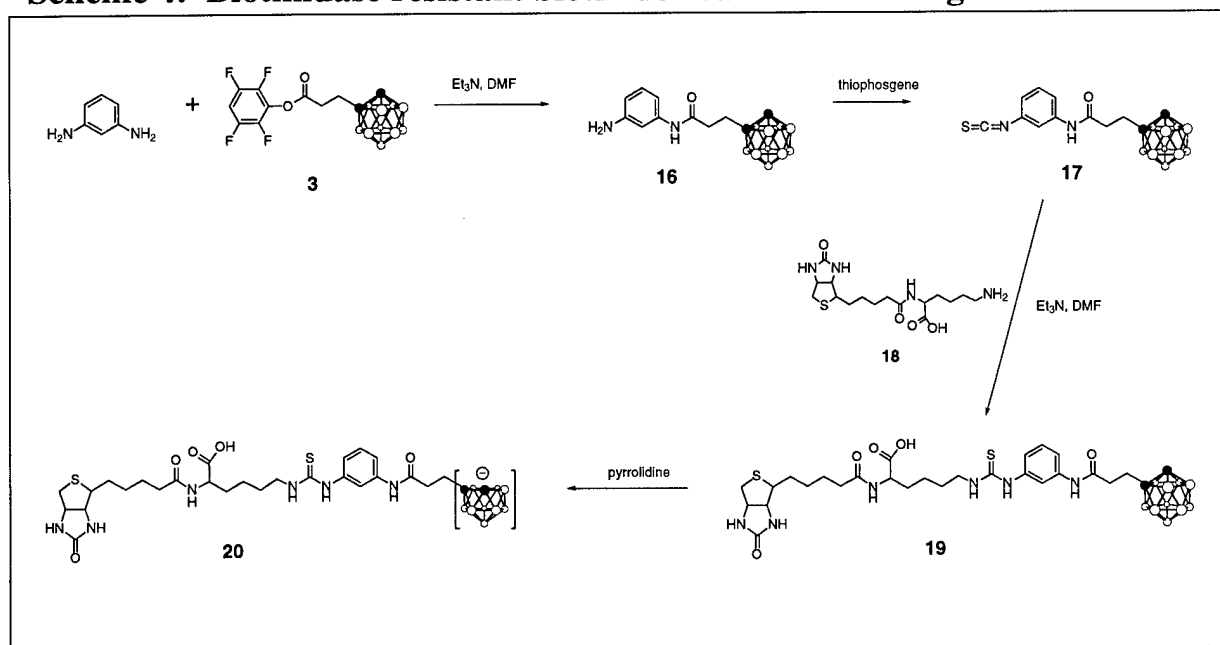
Technical Objective 3: To evaluate the localization of radiolabeled biotin using "2-step" pretargeting protocols.

Some of the following information has been taken from the Final Report to aid the reader in understanding the purpose of our last two animal studies.

Task 12: Optimize 2-Step pretargeting of At-211

We demonstrated that conjugates of biotin and amino acids (α -amine conjugated) which result in having a carboxylate alpha to the biotinamide bond are quite stable to biotinidase. Thus, we prepared a *nido*-carborane containing biotin derivative, **20**, (Scheme 4) which contains a lysine moiety attached to the biotin for biotinidase stability.

Scheme 4: Biotinidase resistant biotin derivative containing *nido*-carborane



Animal Experiment 10 In this experiment the biotin-lysine-*nido*-carborane, **20**, was radiolabeled with I-125 and compared with another biotinidase stabilized derivative that does not contain a *nido*-carborane moiety, biotin-sarcosine-*p*-[¹³¹I]iodobenzoate, **22** (Figures 10a, 10b, and Table 1). As we previously observed for *nido*-carboranes, the blood concentration is much higher for these derivatives than for biotin derivatives that only contain aryl iodides. It seems likely that the slower blood clearance observed for the *nido*-carborane containing biotin derivatives is due to binding with serum proteins. Whether the longer half-life is a problem when short-ranged alpha-

Figure 10a

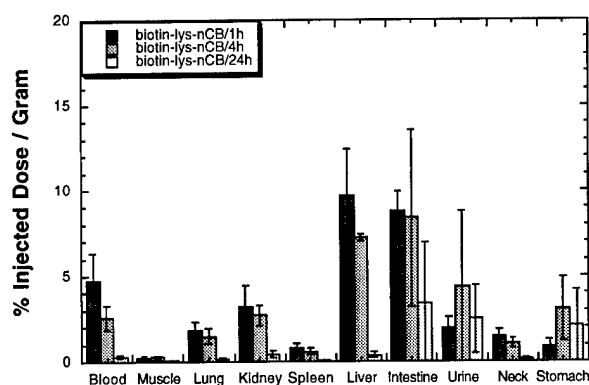
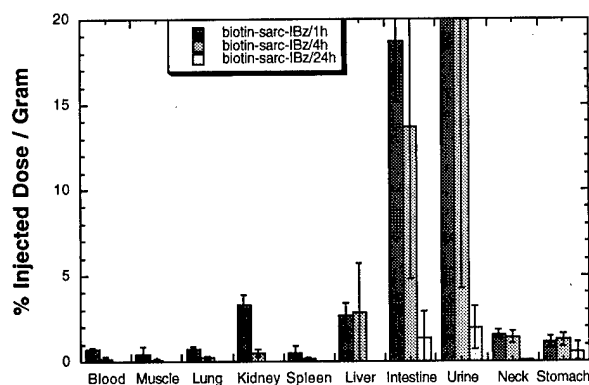


Figure 10b



emitting radionuclides are used is not known yet. The other important observation to be made about the differences in Figures 10a and 10b is that the intestine concentrations are much lower for the *nido*-carborane containing moiety. The reason for this is currently being investigated in other funded studies. The results obtained were encouraging enough to label **20** with At-211 and evaluate its *in vivo* properties.

Animal Experiment 11 Biotin derivative **20** was radiolabeled with At-211 and its biodistribution was compared with I-125 labeled **20** in athymic mice. The results are shown in Figures 11a and 11b. It was very encouraging to see that the At-211 on **20** appeared to be relatively stable to dehalogenase. This statement can be made because the concentrations of At-211 in the lung, spleen and stomach are low and decrease with time. Free astatide localizes in these tissues and increases with time. However, some instability may be noted as the concentration of At-211 is lower in blood than the co-injected I-125, but slightly higher in lung, kidney, and spleen at the three time points.

Figure 11a

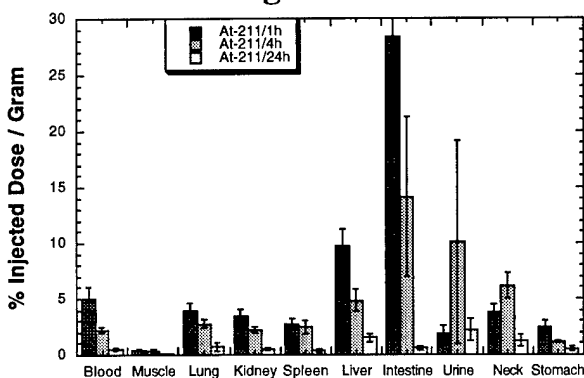
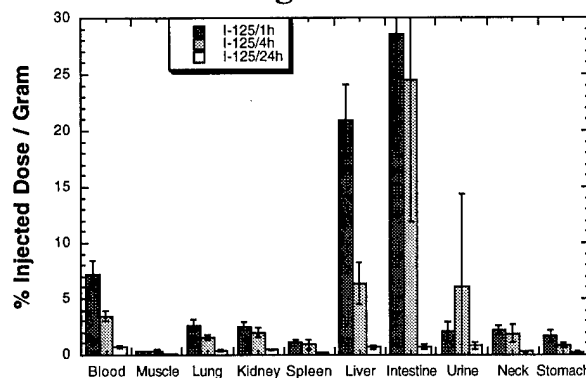


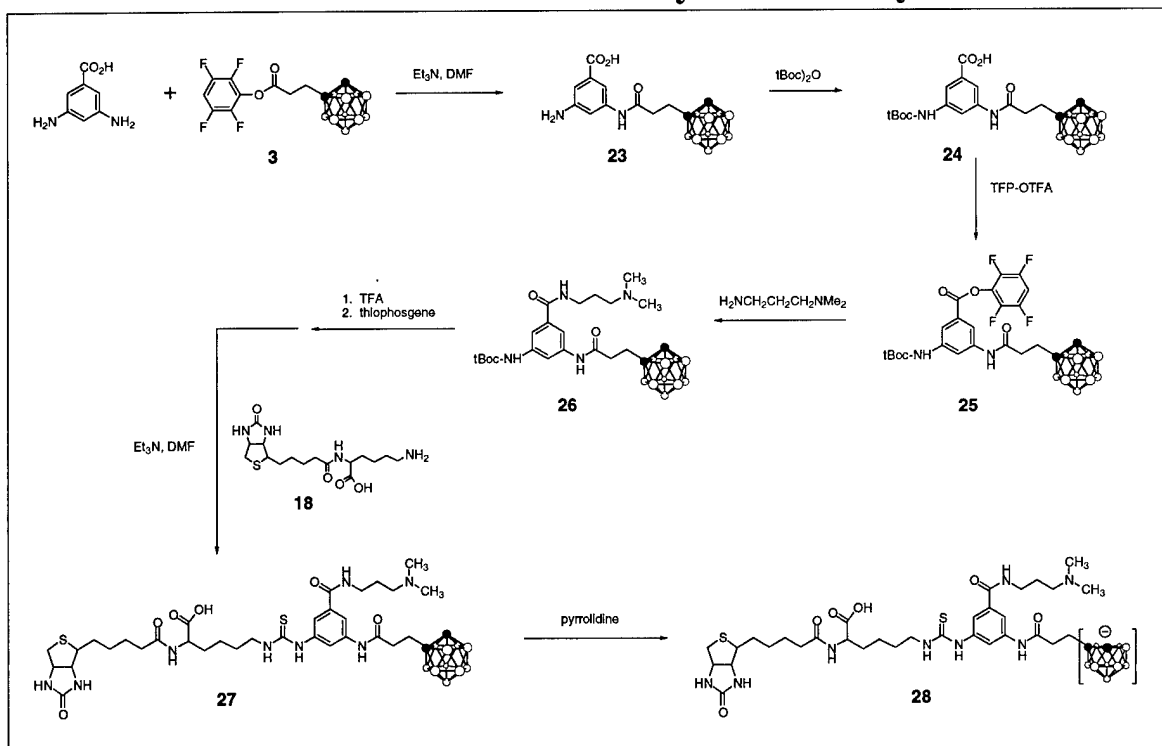
Figure 11b



The results obtained in the above experiment (#11) suggest that we have a biotin derivative which can be used for carrying At-211 to cancer cells *in vivo*. However, it was clear that the *nido*-carboranyl-biotin derivative **20** is not optimal as the *nido*-carborane moiety made it adhere to a glass and metal surfaces, requiring that it be mixed with BSA or HSA prior to injection. In an attempt to decrease the non-specific interaction, we prepared a second *nido*-carborane containing biotin derivative, **28**, as depicted in Scheme 5. It was our hypothesis that an internal

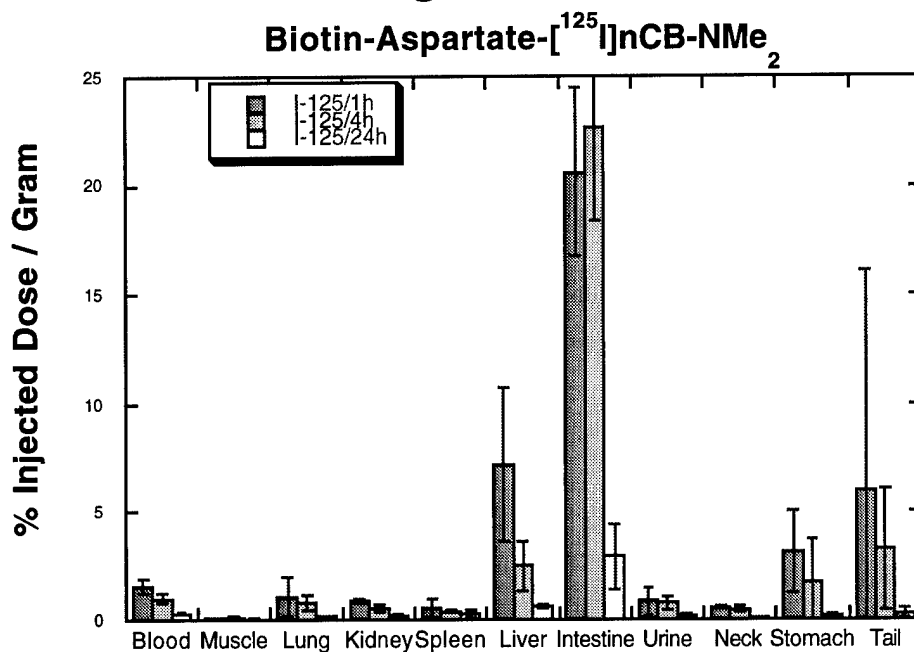
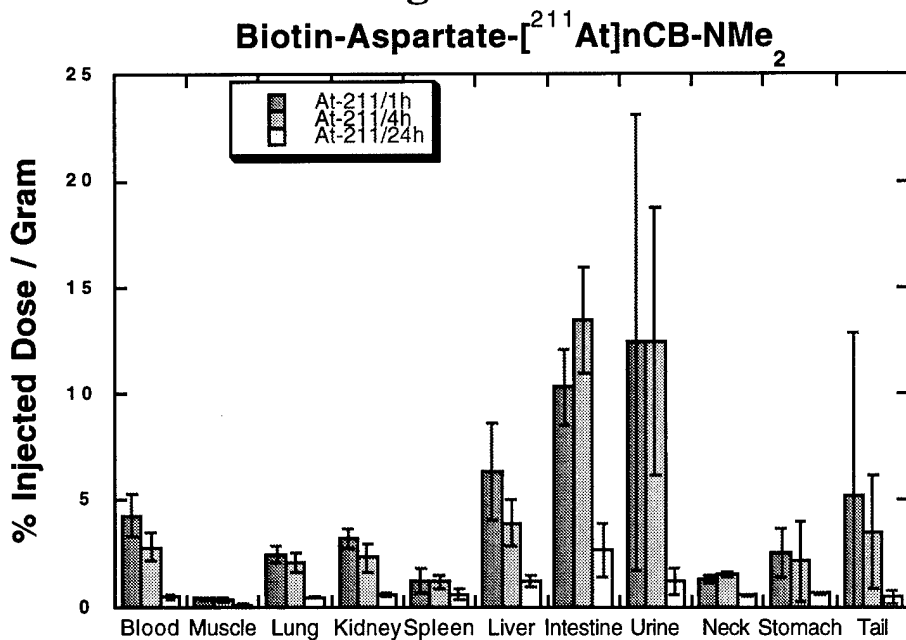
cation (protonated trialkylamine) would decrease the non-specific interaction of the *nido*-carborane.

Scheme 5: Biotinidase resistant biotin derivative containing *nido*-carborane that also contains a dimethylamine moiety



After synthesizing **28**, we conducted radiolabeling experiments and found that it was efficiently labeled with both ^{125}I and ^{211}At . A study was conducted with radioiodinated **28**, but the At-211 labeled **28** was not evaluated prior to the end of the initial funding period. Thus, in the funding extension period, we evaluated the biodistribution of [^{211}At]**28**. That experiment is described below (#19).

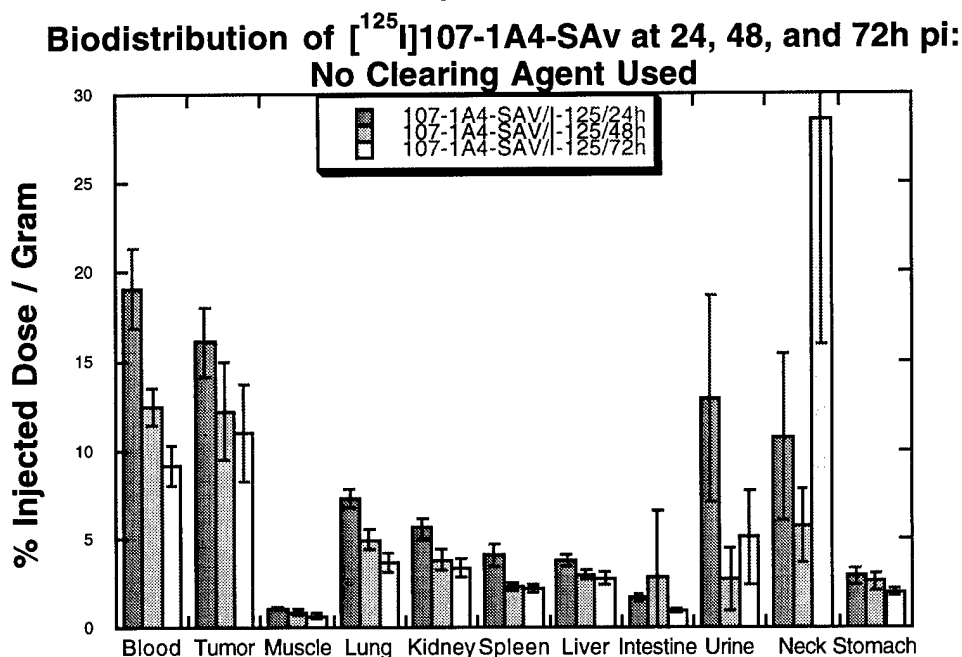
Animal Experiment 19. In the nineteenth animal study, the biodistribution of [^{211}At]**28** was compared with [^{125}I]**28**. A 20 μCi quantity of [^{211}At]**28** and [^{125}I]**28** were mixed and co-injected into 15 athymic mice. The mice were sacrificed at 1, 4, and 24 h pi and the tissue distribution of radioactivity was obtained. To make it easier to evaluate the distributions, separate graphs are provided for [^{125}I]**28** (Figure 19A) and [^{211}At]**28** (Figure 19B). The radioiodinated **28** appears to have a substantial amount of liver clearance and accumulation in the intestines. This result is similar to the what had been seen for the *nido*-carborane containing biotin derivative that did not have a dimethylamine counterion built in (e.g. Figure 11b for [^{125}I]**20**). This fact makes it clear that additional design/synthesis studies have to be conducted to promote renal clearance of *nido*-carborane containing biotin derivatives. Additional studies are warranted, as again, the [^{211}At]**28** appears to be stable to in vivo deastatination (Figure 19b). If it were unstable, increasing amounts of At-211 would have been found in the lung, spleen and stomach at the later time points. This result is exciting and funding has been obtained to conduct additional studies with *nido*-carborane substituted biotin molecules.

Figure 19a**Figure 19b**

Although high liver and intestine concentrations were obtained for [¹²⁵I]28, we decided to conduct an animal study to determine if that compound could be used in a two-step pretargeting protocol. The specifics of that experiment are described below.

Animal Experiment 20 The twentieth animal experiment was designed to evaluate the tumor targeting (LNCaP tumor xenografts) of the *nido*-carborane containing biotin, [^{125}I]28 or a In-111-labeled biotin, [^{111}In]29, in a pretargeting protocol. That protocol was begun by injection of 400 μg of an anti-PSMA (anti-prostate carcinoma antibody) [^{125}I]107-1A4-SAv conjugate, this was followed after 18 h with 200 μg of our SAV-based clearing agent, then after 2 more hours, 1 μg of a radiolabeled biotin was injected. The athymic mice were sacrificed at 24, 48, and 72 h pi mAb-SAv conjugate (or 4, 24, 48 h pi labeled biotin). In the initial part of the study, 15 mice were injected with [^{125}I]107-1A4-SAv and the distribution of this reagent was obtained at 24, 48, and 72 h pi. The data obtained is shown in Figure 20a. The purpose of this experiment was to obtain information about the distribution of antibody-streptavidin conjugate without the clearing agent. As was expected, the blood concentration of mAb-SAv conjugate does not drop appreciably over the 72h. Good tumor localization was obtained and it decreased less than 30% in that time period.

Figure 20a



A second set of 5 mice had the [^{125}I]107-1A4-SAv administered, after 18 h the clearing agent was administered and at 24 h the mice were sacrificed. The tissue distributions from that set of mice are shown in Figure 20b. It is apparent that the clearing agent worked, but it only removed 60%. It is not clear why it was so low. Importantly, the concentration of radiolabeled mAb-SAv conjugate in the tumor xenograft did not decrease significantly.

In a third set of mice, 15 mice were injected with the [^{125}I]107-1A4-SAv conjugate, after 18 h, the biotinylated/galactosylated SAV was administered, and at 20 h 1 μg of [^{131}I]28 was administered. The tissue distribution was evaluated at 4, 24 and 48 h post the administration of the [^{131}I]28. The data obtained is shown graphically in Figure 20c. Although the blood levels are quite low for the *nido*-carborane containing biotin derivative, tumor targeting is not very

Figure 20b

Biodistribution of [¹²⁵I]107-1A4-SAv at 24 h pi without Clearing Agent (left bars) and 4h After Administering Clearing Agent (right bars)

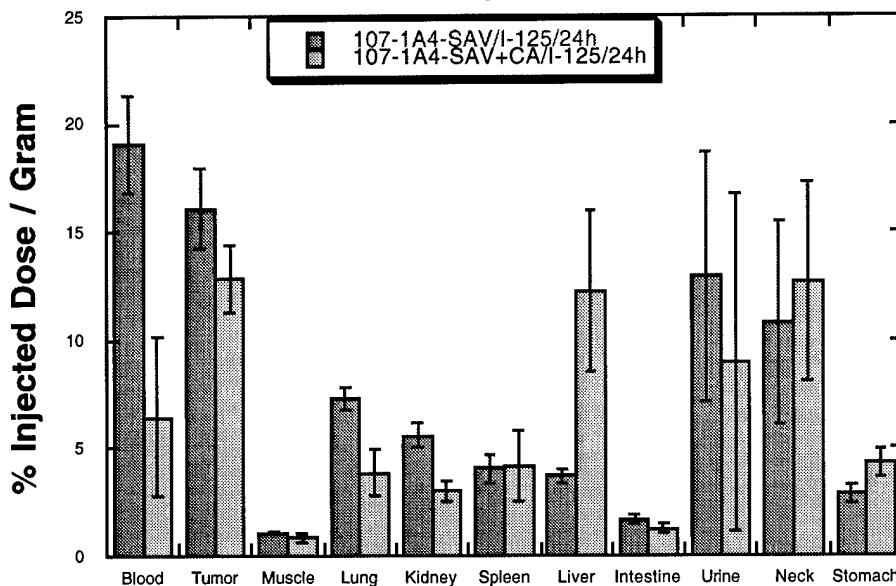
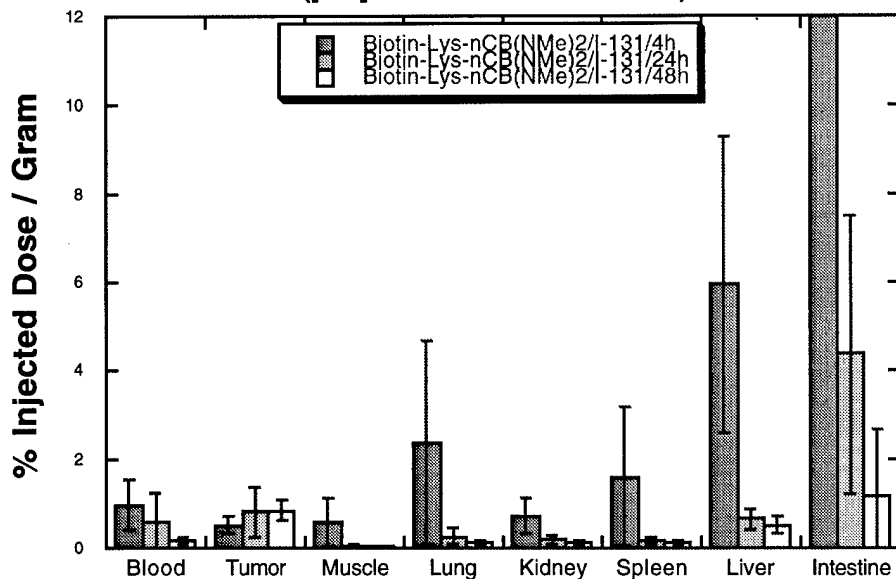


Figure 20c

Biodistribution of [¹³¹I]Biotin-Lys-nCB(NMe₂) at 4, 24, and 48 h pi. ([¹²⁵I]107-1A4-SAv not shown)

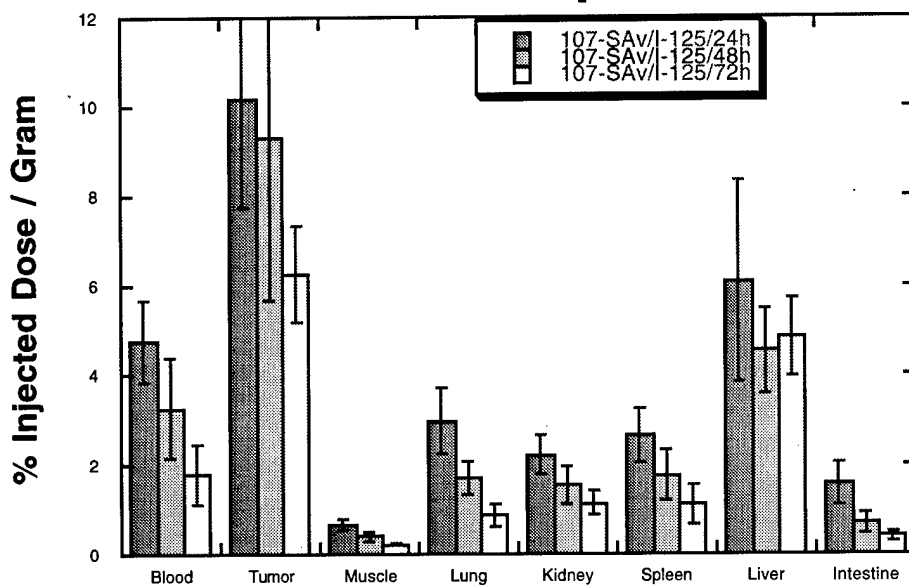


good. If one looks at the tumor targeting of the injected [¹²⁵I]107-1A4 (Figure 20d) was quite good. Although the concentration in tumor (in Figure 20c) is several times higher than the blood at the later time point, the absolute concentration in tumor is very low. This fact coupled with

Figure 20d

Biodistribution of [125 I]107-1A4-SAv at 24, 48, and 72 h pi:

[131 I]Biotin-Lys-nCB(NMe $_2$) not shown



the high liver and intestine concentrations is problematic. As previously noted, further development of the *nido*-carborane containing biotin derivatives will be conducted under funding from another source.

A fourth set of mice (15 mice) were treated as above, except the radiolabeled biotin reagent was one that contains an indium-111 in a DTPA chelate, compound [111 In]29. The data obtained is depicted in Figures 20e and 20f. In this experiment higher %ID/g quantities of the In-111 labeled biotin ([111 In]29) are observed (Figure 20e) than is seen with the [125 I]107-1A4-SAv. The concentration of [111 In]29 in blood is high for a biotin moiety, suggesting that it has bound with the circulating [125 I]107-1A4-SAv. This is most likely caused by the poor blood clearance obtained with the biotinylated/galactosylated SAv.

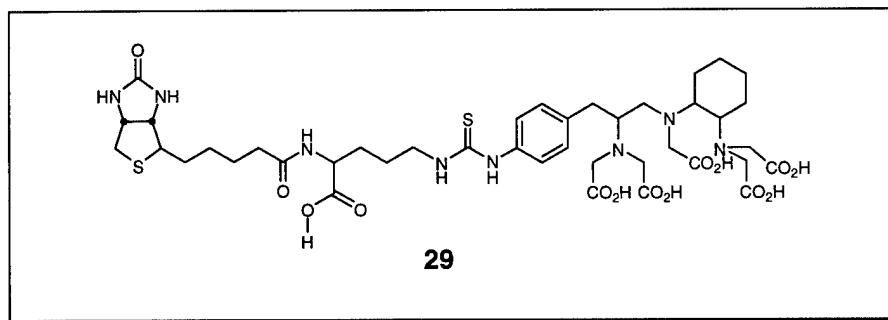


Figure 20e

Biodistribution of [¹¹¹In]Biotin-CHXA'' at 4, 24, and 48 h pi:

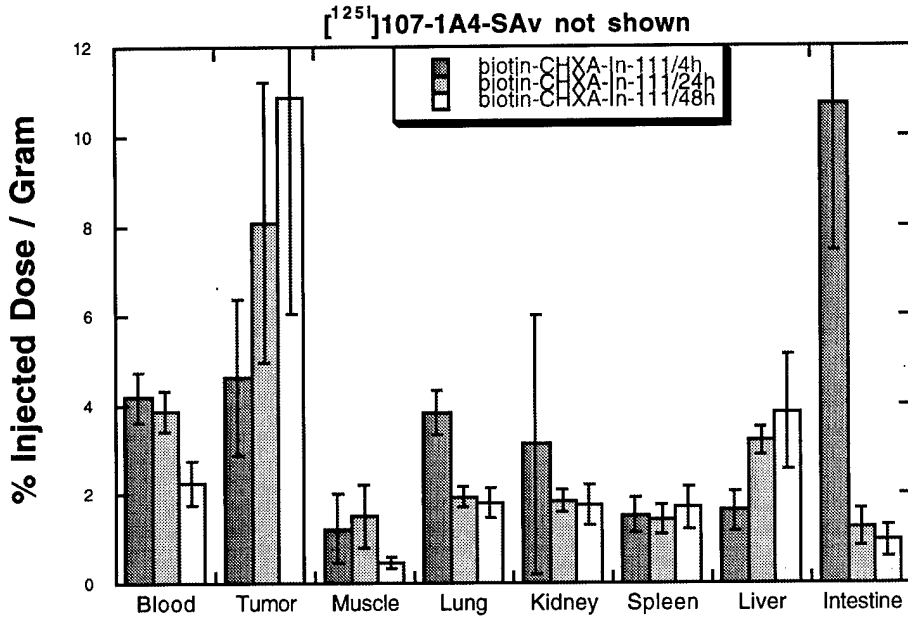
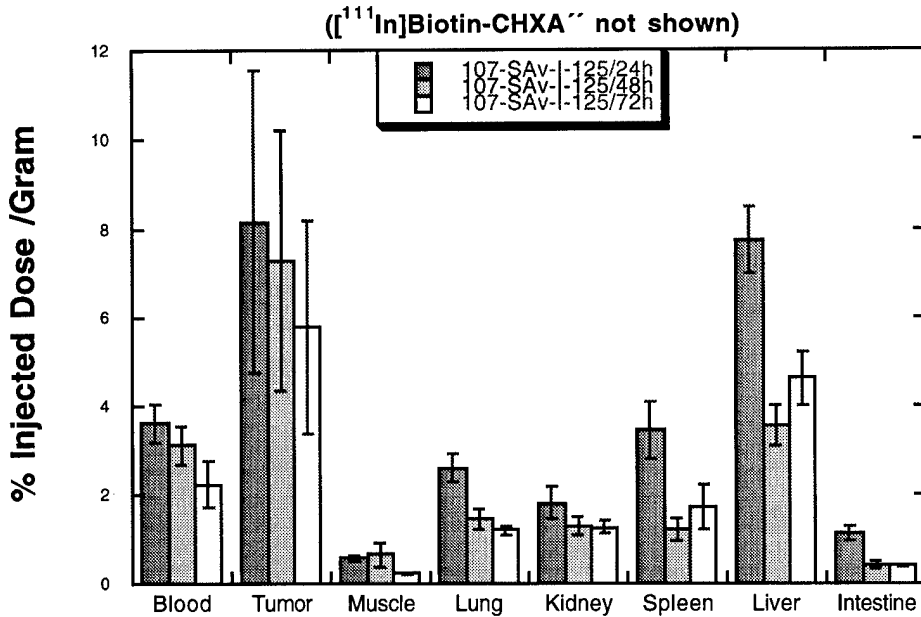


Figure 20f

Biodistribution of [¹²⁵I]107-1A4-SAv at 24,48 and 72 h pi.



C. KEY RESEARCH ACCOMPLISHMENTS (Supplemental Studies):

- Further investigated new clearing reagent made of galactosylated streptavidin and a biotin trimer. This clearing agent does not appear to be optimal for the pretargeting protocol.
- Investigated At-211 labeled *nido*-carboranyl-biotin which has a dimethylamine side group in the hopes that it would improve the problems of non-specific sticking of those compounds, and (perhaps) decrease the liver and intestinal concentrations previously found.
- Conducted a pretargeting protocol in a prostate cancer model and successfully targeted a biotin compound labeled with In-111 to the tumor. Some success was also obtained with a I-131 labeled *nido*-carboranylbiotin derivative, but the absolute quantities of radioactivity in the tumor was low.

D. REPORTABLE OUTCOMES:

No additional manuscripts or presentations have come from the supplemental studies. The information will likely be included in papers to be published in the future.

E. CONCLUSIONS:

We believe that the use of astatinated compound for treatment of metastatic prostate cancer is still a realistic and worthwhile goal. The pretargeting method of delivering radionuclides to cancer cells in patients is a complex system with many variables. Due to this factor, we know that we were optimistic in our original goals. With some additional preliminary studies, we believe that we will be able to investigate the efficacy of therapeutic protocols with At-211. We intend to continue to study this approach and develop reagents that will make its use more feasible. While we did not demonstrate good tumor targeting of At-211 in these studies, we anticipate being able to successfully pretarget At-211 to metastatic prostate cancer cells in mice in the near future.

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G. APPENDICES:

All Figures, including those of reaction schemes, electrophoresis gel scans, and biodistribution graphs have been included in the Body of this report to make it easier to follow. Thus no appendices are included.

H. PERSONNEL THAT WERE PAID ON THIS GRANT

D. Scott Wilbur, Donald K. Hamlin, Reudi Risler, Brian Kegley, Ming-Kuan Chyan, Tim Nguyen, Feng Wan, Robert Vessella