

AD _____

Award Number: W81XWH-06-1-0183

TITLE: Development of Novel Bifunctional Compounds That Induce Apoptosis in Prostate Cancer Cells

PRINCIPAL INVESTIGATOR: John M. Essigmann, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts Institute of Technology
Cambridge, MA 02139

REPORT DATE: March 2008

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. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-03-2008		2. REPORT TYPE Annual		3. DATES COVERED 1 Mar 2007 – 28 Feb 2008	
4. TITLE AND SUBTITLE Development of Novel Bifunctional Compounds That Induce Apoptosis in Prostate Cancer Cells				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0183	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) John M. Essigmann, Ph.D. Robert G. Croy Ph.D. Email: jessig@mit.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Massachusetts Institute of Technology Cambridge, MA 02139				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 We have designed and synthesized a novel compound (11 β) that efficiently triggers apoptosis in prostate cancer cells such as LNCaP. This bifunctional compound was designed to form DNA adducts that are camouflaged by the androgen receptor making them less readily repaired in AR+ prostate cancer cells. The aims of our studies are to investigate the mechanisms by which 11 β is able to trigger apoptosis in target cells. Methods have been developed that permit us to determine the fates of 11 β -DNA adducts in treated cells in culture as well as in tumors growing in animal models. Another objective is to identify the signaling events that lead from DNA adducts to activation of the apoptotic program. Finally we have obtained encouraging results from animal experiments that indicate that molecules such as 11 β may have clinical potential for the treatment of human tumors.					
15. SUBJECT TERMS Chemotherapy, DNA damage and repair, Apoptosis, Steroid Receptors					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusion.....	8
References.....	9
Appendices.....	9

INTRODUCTION

The objective of our research is to develop more effective therapeutics for the treatment of prostate cancers. One novel bifunctional compound (**11 β**) that we have prepared under this program rapidly induces apoptosis in several prostate cancer cell lines in vitro. The **11 β** compound contains a chemically reactive nitrogen mustard linked to a steroid moiety that binds with high affinity to the androgen and progesterone receptor proteins. This compound was designed to create DNA adducts that form tight complexes with these steroid receptors; the formation of a physical complex of the adduct with the receptor makes the adducts difficult to repair in prostate cancer cells, which often over express these receptors. Preliminary studies of **11 β** in cell culture indicated that its effects on prostate cancer cells were different from those of other alkylating agents used in chemotherapy. The apoptotic responses of prostate cancer cells suggested that the **11 β** compound might be a useful agent for chemotherapy. The Specific Aims of our research have been to understand the fate of **11 β** -DNA adducts in treated cells and investigate the mechanisms that lead to apoptosis. We also proposed experiments to assess the antitumor potential of **11 β** in animal models of human prostate cancer.

BODY

Task 1: *Determine if the biochemical changes observed in prostate cancer cells in culture are responsible for the antitumor effects of 11 β in xenograft tumor models.*

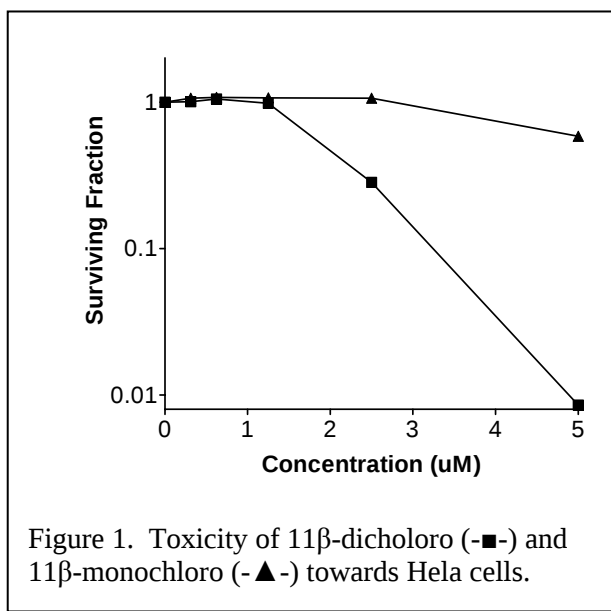
One biochemical change that we proposed as a marker of antitumor response was the DNA damage induced protein p21. Our investigation of p21 responses to 11 β in the prostate cancer cell lines LNCaP, DU145 and PC3 have been completed. We expanded examination of the levels of p21 in the three cell lines up to 24 h after treatment with 11 β . Our present results indicate that p21 responses are robust in LNCaP and PC3 cells, but this was not the case in the DU145 cell line. Therefore, the p21 marker is not appropriate an appropriate biomarker for all of our tumor models. During the next year we will expand our studies to examine additional biomarkers as described in our proposal. These studies will include p70S6K-Thr-389-phosphate along with the c-Myc and Skp2 proteins – all of which showed large changes in concentration in LNCaP cells treated with **11 β** .

Task 2: *Determine the fate of 11 β -DNA adducts in prostate cancer cells and in LNCaP xenograft tumors in animals.*

Using a radiolabeled analog that incorporated one ¹⁴C atom into the linker of our molecule we have used the new technique of Accelerator Mass Spectrometry (AMS) to detect and quantify [¹⁴C]-**11 β** -DNA adducts in treated cells. This permitted us to define the dose-response relationship between the concentration of **11 β** in cell culture media and the level of total DNA adducts in cells.

Despite the extraordinary sensitivity of AMS (ten molecules per cell), a major drawback of this technology is that it is destructive to the sample and hence provides no structural

information. The majority of adducts formed are monoadducts while the ability of **11 β** to form crosslinks has been found by us to be essential for its potent cytotoxic effects. We demonstrated this property by preparing an **11 β -monochloro** compound (**11 β** has two chloroethyl arms whereas the mono-chloro derivative has only one) that was capable of forming only monoadducts. As shown in Figure 1, the **11 β -monochloro** analog that cannot form DNA crosslinks is much less toxic than the **11 β -dichloro** lead compound. These results indicate that optimization in tumor cells of crosslink formation by **11 β -dichloro** would lead to better tumor response and that monitoring the formation of DNA crosslinks and their fate in cells would best correlate with tumor response.



In response to the need to distinguish mono adducts and crosslinks, during the past year we have developed a method based on liquid chromatography-electrospray ionization/tandem mass spectrometry for analysis of **11 β** -DNA adducts. To ascertain the relative amounts of **11 β** -guanine and **11 β** -adenine monoadducts, as well as **11 β** -guanine cross-links *in vivo*, we have employed an Agilent 6410 triple quadrupole electrospray ionization mass spectrometer, whereby selected parent ions, such as **11 β** -guanine or **11 β** -adenine, are fragmented and the fragmentation products, guanine or adenine, are quantified.

To generate standards for optimizing the triple quadrupole mass spectrometry parameters, as well as determining what types of adducts are most likely to form *in vitro*, we treated calf thymus DNA with **11 β -dichloro** in 25% DMSO at 37°C for 12 hr, followed by DNA precipitation with salt and ethanol and a final 70% ethanol wash prior to redissolving in water. Adducts to be analyzed were liberated from DNA by acid hydrolysis (0.2 N HCl 75°C 3 hr) prior to their introduction onto a reversed-phase HPLC column in-line with the mass spectrometer. We have identified mono-adducts of **11 β -dichloro** covalently bound to guanine or adenine (with the other chloroethyl arm of the mustard substituted with a hydroxyl group) as well as an **11 β** derivative bearing 2 guanines (which would result from an inter-strand or intra-strand DNA cross-link).

Positive ESI-MS-MS was used to detect and select **11 β** adducts. Detection of analyte transitions at $m+2/z$ 399.29 $\rightarrow$ 136.00 (adenine) and 407 $\rightarrow$ 152.00 (guanine) provided a extremely sensitive means for adduct analysis. A typical chromatogram is shown in Figure 2.

During the next year we shall refine the mass spectrometric methods to permit quantitative analysis of individual **11 β** -DNA adducts in treated cells and tissues. We shall use the technique of stable-isotope mass spectrometry (SIMS) to determine accurately how the distribution of **11 β** adducts in cellular DNA changes at times following treatment. SIMS utilizes heavy-labeled standards to permit quantitative comparisons of adduct levels between samples. **11 β** -DNA adducts having a +5 increase in mass have been prepared by reacting **11 β** -dichloro with uniformly labeled ^{15}N *E. coli* DNA. These “heavy” adducts will act as internal standards to enable accurate comparison of adduct levels between samples.

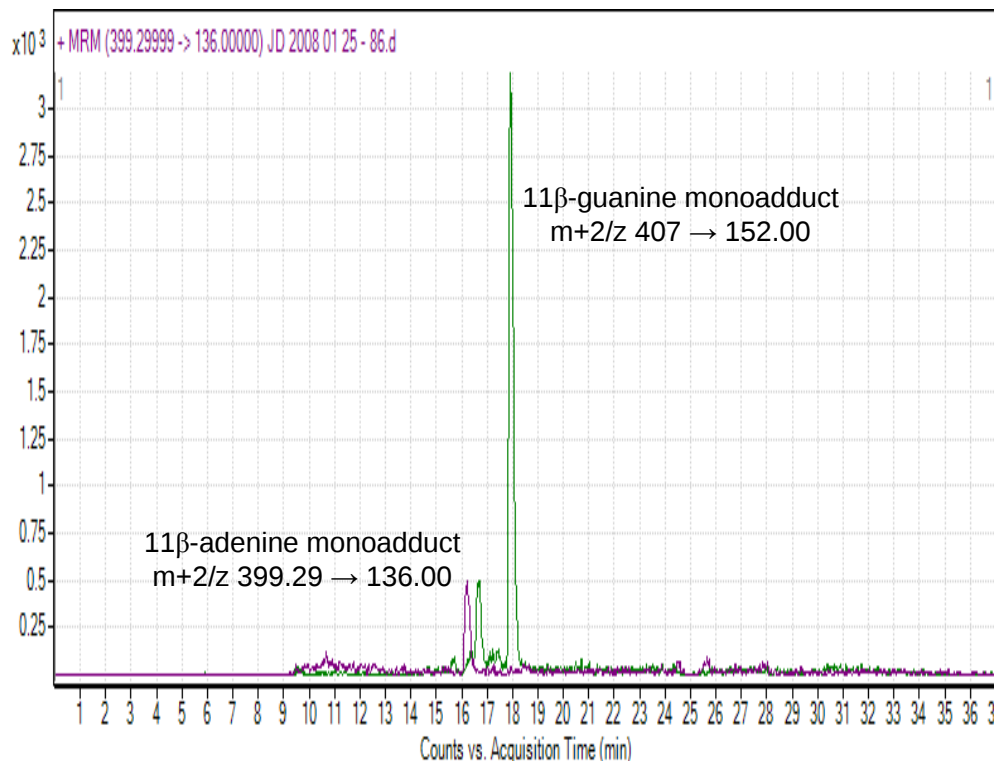


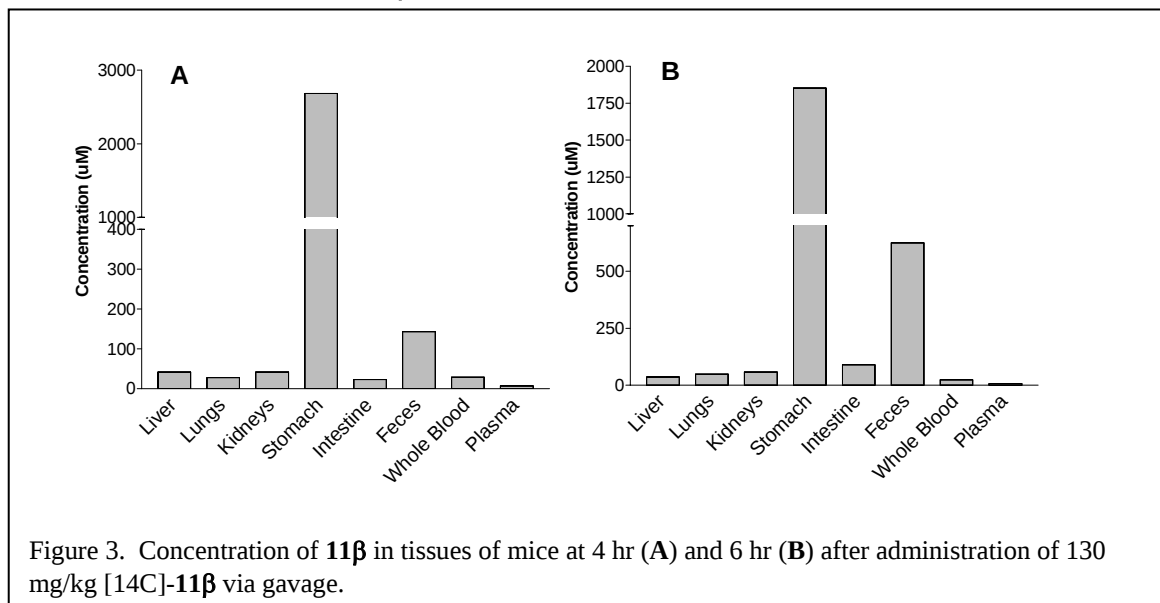
Figure 2. Mass spectrometric analysis of **11 β** -DNA adducts in HeLa cells treated with 10 μM **11 β** -dichloro. The plot shows signal intensity of 136 m/z and 152 m/z fragments of adenine and guanine **11 β** adducts respectively.

We shall continue to utilize the AMS technique to determine levels of **11 β** -DNA adducts in xenograft tumors in animals. AMS provides an extremely sensitive means for direct assessment of bioavailability of **11 β** by measurement of total adduct levels. The complementary tool of ESI-MS-MS technique using SIMS will enable us to examine the fate of specific DNA adduct in tumor and nontarget tissues to determine whether our intended mechanism of action results in increased abundance of monoadducts and crosslinks in tumors.

Task 3: *Formulation of **11 β** in a liposomal vehicle and investigation of its PK and efficacy.* During the past year we investigated the possibility that the **11 β** compound

could be administered orally. This series of experiments is based upon the knowledge that chlorambucil, which is structurally similar to the warhead portion of **11 β** , is an oral drug. Our original plan was to investigate liposomal formulations. We anticipate that work on this portion of the task will resume during the next year. Discussions with several oncologists led us to recognize the advantages of oral administration of the **11 β** compound both in terms of speed in reaching the clinical trial stage and future clinical acceptance. Therefore, we explored the bioavailability of **11 β** in the mouse after oral dosing.

In our initial experiment the **11 β** compound was formulated in an oil/DMSO (10/1)



emulsion. Mice were then administered [¹⁴C]-**11 β** in this formulation via gavage and the tissue distribution of radioactivity were determined. Distributions of radiolabeled **11 β** at 4 and 6 hr after administration are shown in Figure 3.

Thus far, the data indicate that **11 β** has low bioavailability when administered orally in the oil/DMSO emulsion. Most of the compound remained in the stomach and was found in the contents of the intestinal tract (feces) – indicating that under our conditions it was poorly absorbed.

During the next year we shall investigate other formulation vehicles to determine if the oral bioavailability of **11 β** can be increased. We will also resume our efforts to formulate **11 β** in a liposomal carrier.

Task 4: Assess the antitumor activity of **11 β** in additional human prostate tumor models.

As reported in our FY 2004 Progress Report, the **11 β** compound is very effective in preventing the growth of LNCaP cell in mouse xenografts. It is especially encouraging that the dose of **11 β** that was effective in preventing tumor growth did not show significant toxic effects in the animals.

We are now in a position to implement our proposed mechanistic and efficacy studies in xenograft models. Having developed the analytical tools necessary to determine the amount of compound reaching the tumor and assess its relation to antitumor responses, we shall examine the biochemical toxicology of the compound in several prostate tumor models.

We shall also continue to work with two genetically engineered cell lines that are isogenic except for AR status. During the next year we shall test the ability of PC3-neo and PC3-AR to form tumors when injected subcutaneously into nude mice. If these cell lines do grow as xenografts, this model will allow us to determine the role the AR in antitumor responses towards the **11 β** compound *in vivo*.

ACCOMPLISHMENTS

- We have assessed the oral bioavailability of **11 β** in mice and determined that our compound is not well absorbed when administered orally under the conditions tested;
- We have completed our studies on the p21 protein to determine if it can serve as a biomarker of antitumor response;
- We have developed methods to examine both the total level of **11 β** -DNA adducts formed in cells and tissues as well as individual species of **11 β** adducts that may be better correlated with the cytotoxic and therapeutic effects of this compound;
- We have completed toxicity studies isogenic cell lines that differ in AR status and are proceeding to determine the role of the AR in both the cytotoxic effects of **11 β** *in vitro* and tumor responses *in vivo*.

REPORTABLE OUTCOMES

Publications: Hillier, S.M., Marquis, J.C., Zayas, B., Wishnok, J.S., Liberman, R.G., Skipper, P.L., Tannenbaum, S.R., Essigmann, and Croy, R.G. DNA adducts formed by a novel antitumor agent 11 β -dichloro in vitro and in vivo. 2006 Mol. Cancer Ther. 5(4); 977-984.

Degrees Supported:

- Dr. Shawn Hillier has been supported on this grant. He received his Ph.D. in June, 2005. Dr. Hillier was responsible for DNA repair and antitumor studies. He is now employed by Molecular Insight Pharmaceuticals, Cambridge, MA.
- Dr. John Marquis has received support from this grant. He recently left the lab and is now employed by Molecular Insight Pharmaceuticals, Cambridge, MA.
- Mr. Frances Gonzales is a graduate student who has been supported on this grant. He is currently on a medical leave of absence from MIT.

CONCLUSIONS

We continue to be engaged in experiments to define the amounts of DNA damage produced by the **11 β** compound and the fates of this damage in target and non-target cells in culture and tumor xenografts. This information will help us establish the sequence of events that lead to apoptosis. The results we have obtained in animal models are especially significant. The ability of the **11 β** compound to prevent the growth of LNCaP cells in a mouse xenograft model provides evidence of the potential clinical activity of this compound. The activity of **11 β** will now be tested in additional xenograft models of human prostate cancer to assess the range of its antitumor properties.

Our research during the next year will focus on the following goals: (1) continuing our experiments to identify and validate biomarkers such as p21 and the formation of **11 β** -DNA adducts that can be used to calibrate tumor responses to **11 β** in animal models; (2) continuing studies on reformulation of **11 β** focusing both on oral delivery and on liposomes as possible delivery vehicles for the compound and; (3) examining antitumor responses to **11 β** in other animal models of prostate cancer. During the past year we have characterized the toxicity and responses of AR+ and AR- cell lines towards **11 β** and we are now set to initiate our *in vivo* studies. We anticipate that identification of the signaling events originating from DNA adducts will provide valuable biomarkers of antitumor responses as well as clues to the reasons why the **11 β** compound is singularly able to trigger apoptosis, whereas other aniline mustard compounds such as chlorambucil do not.

REFERENCES

None included.

APPENDICIES

None included.