

AD_____

Award Number: W81XWH-07-1-0289

TITLE: Targeted Zinc Delivery: A Novel Treatment for Prostate Cancer

PRINCIPAL INVESTIGATOR: Joseph J. Baldassare, Ph.D.

CONTRACTING ORGANIZATION: Saint Louis University
St. Louis, MO 63103

REPORT DATE: June 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				<i>Form Approved</i> 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 30-06-2008		2. REPORT TYPE Annual		3. DATES COVERED 1 JUN 2007 - 31 MAY 2008	
4. TITLE AND SUBTITLE Targeted Zinc Delivery: A Novel Treatment for Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-07-1-0289	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Joseph J. Baldassare, Ph.D. Email: baldasjj@slu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Saint Louis University St. Louis, MO 63103				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 At present, treatment for patients with advanced metastatic prostate disease or who progress to metastatic disease is limited. We proposed to initiate studies to develop and comprehensively evaluate targeted zinc loaded liposomes as a therapeutic for the treatment of men refractory to current treatment options. In the past year we have evaluated a number of zinc compounds and have shown that zinc acetate is suitable for entrapment into transferrin targeted liposomes. We have also developed a human xenograft model and have shown that intratumor injection of zinc solutions arrest the growth of the subcutaneous PC-derived tumors. This result is exciting and shows the potential of zinc as a prostate cancer therapeutic.					
15. SUBJECT TERMS Liposomes, targeted, zinc, prostate, cancer, chemotherapy					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	8	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	7
References.....	7
Appendices.....	8

INTRODUCTION:

Prostate cancer remains the most common non-cutaneous malignancy in the Western world and is the second highest cause of cancer death in males, after lung cancer (1). American men have a one-in-six chance of developing the disease (2). Despite these grim statistics, which underline the importance of prostate cancer as an enormous medical and socioeconomic problem, surprisingly little progress has been achieved by current treatment regimens (3). Current non-surgical treatments of localized prostate carcinoma, a leading cause of cancer deaths in men, can be treated with standard therapies many of which depend on androgen deprivation (4-6). Treatments directed toward androgen deprivation initially result in high responsiveness (7-9). However a proportion of patients develop locally advanced or metastatic disease that is refractory to anti-hormone therapies (10-13). Those patients generally undergo either radiation or cytotoxic chemotherapy or a combination therapy. While these modalities are effective in some men with advanced prostate cancer, at present there is currently no curative therapy for patients that are resistant to these treatments. We proposed to focus on development of novel anti-cancer strategies, based on our recent data showing that zinc kills all types of tumor cells (14) including prostate cancer cells. In light of the observations that zinc is toxic to prostate cells but that oral supplementation of zinc is an ineffective means of regulating the levels of zinc in the prostate, we proposed to develop targeted zinc-containing liposomes to prostate cells and, as a result, elicit selective killing of those cells.

BODY:

In the past year we have made significant progress on Aim 1- **Aim 1. Liposome Delivery and Efficacy.** We had proposed to perform experiments that compared the efficacy of acidic and non-acidic liposomes, and the effectiveness of different chemical forms of Zn and the possible synergistic effects with other known chemotherapeutic agents. Finally we proposed to determine the most effective targeting molecule and or combination of molecules.

We initially tested a number of chemical forms of zinc and a number of liposome preparations. Commercial available preparations including zinc sulfate, zinc chloride, citrate and zinc acetate were tested for their ability to kill PC3 cells when added to cells in culture. The dose dependencies and time course of PC3 cell death with different zinc salts were quantified by trypan blue exclusion and confirmed by the MTT cell death assay. While zinc chloride was the most effective when added directly to PC3 cells, zinc acetate was the most effective when incorporated into liposome preparations (Data not shown). Analysis of the liposome preparations show that the effectiveness of zinc acetate was a result of significant increase in trapping of zinc acetate when compared to other zinc salts. In addition to commercial preparations, we have tested a number of organic zinc compounds. To generate these compounds we have established a collaboration with a synthetic organic chemist Dr Paul Jelliss, Associate Professor of Chemistry, Department of Chemistry, Saint Louis University. Dr. Jelliss has synthesized a number of pyridine based zinc and phthalic ester zinc complexes. These compounds were found to be effective, but not sufficiently more potent to justify their use as therapeutics. These experiments indicate that zinc acetate is the most effective as a therapeutic and will be used in future experiments to test different liposome preparations.

In our preliminary data we had generated liposomes that effectively killed prostate derived PC-3 cancer cells. We have confirmed these results (Figure 1). PC-3 cells incubated with zinc and targeted with transferrin kill PC-3 cells (Figure 1). Importantly untargeted liposomes do not kill PC-3 cells (Figure 1). Furthermore, incubation of PC-3 cells in which the transferrin receptor has been downregulated with targeted zinc entrapped

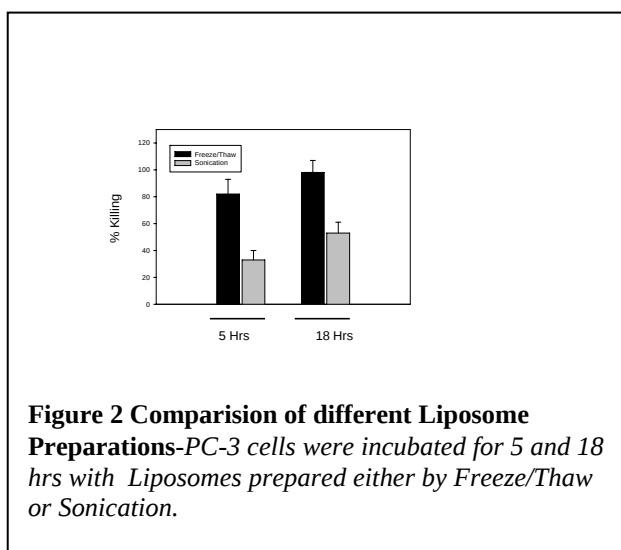
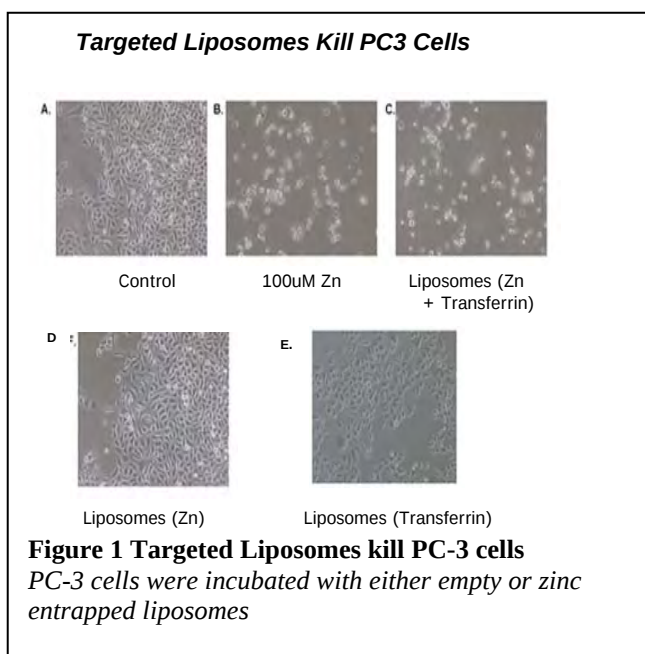
liposomes of transferrin are not killed (Data not shown). These data clearly demonstrate effective killing of PC-3 cells require targeting molecules.

While these liposomes did kill they did not take advantage of the acidic conditions present in endosomes. To test whether pH sensitive liposomes containing pH-sensitive phosphatidylethanol increased efficacy, we tested whether incorporation of poly(ethylene glycol)-diortho ester-distearoyl glycerol (POD) significantly affected cell killing. Surprisingly we found no difference between pH-sensitive and insensitive liposomes suggesting that the neutral liposomes readily release their contents.

Because the studies described above suggested that zinc trapping was a significant determinant of zinc-containing liposomes to kill PC-3 cells in culture, we next tested different methods for preparing the liposomes. We had proposed to generate zinc entrapped liposomes utilizing several established protocols for preparing lipid vesicles (Liposomes: A Practical Approach. Second Edition.

Practical Approach Series, Volume 264. Edited by Vladimir Torchilin and Volkmar Weissig) including a) Reverse Phase Evaporation, b) limited Sonication and c) multiple Freeze/ Thaw. At present only Sonication and Freeze/Thaw preparations have been generated. These liposomes contained either low melting phosphatidylcholine lipids (dioleoyl phosphatidylcholine) or high melting (disteryl phosphatidylcholine), and unilamellar vesicles of approximately 100 microns were prepared by passing a suspension of liposomes through polycarbonate membranes and passing the final preparation through G200 Sephadex column to remove untrapped zinc. Transferrin (CD71) was used as a targeting agent to deliver Zn into PC3 cells (Figure 2). IIC9 embryonic fibroblasts which express low levels of CD71 were used as a control for toxicity of the liposomes. Cell death was determined at both 5 and 18 hours after addition of the liposomes (Figure 2). These data clearly show that liposomes prepared by Freeze/Thaw were more effective than those prepared by the Sonication protocol. The zinc loaded liposomes did not kill IIC9 cells or PC-3 cells that had been pretreated with transferring to down regulate the transferring receptor (Data not shown).

The above studies were performed on cells in 2D cultures. To test for any possible effects of culturing in 3D, we also cultured PC-3 cells in a collagen matrix and examined the ability of zinc and zinc loaded liposomes to kill. No significant different was found when between PC-3 cells cultured in 2D and 3D (Data not shown). We intend to finish these studies and submit them for publication in the coming year.



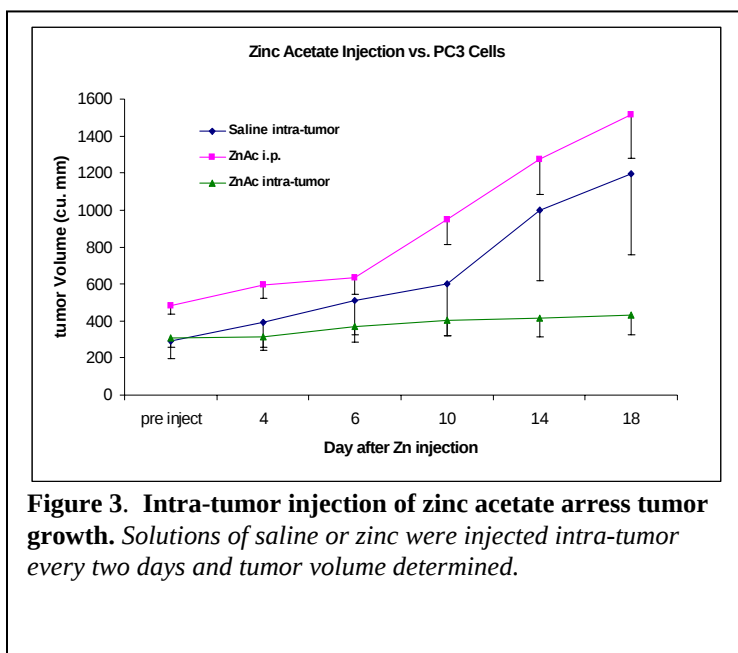
While considerable progress on Aim 1 has been attained during the first year we have not examined other prostate cells, for example LnCaP as proposed in our application. These studies will be carried out in the coming year and should demonstrate that our strategy can be generalized to other prostate cancer cells. In the coming year we also will examine the efficacy of liposomes prepared by the Reverse Phase Evaporation protocol and will test other targeting molecules. Finally we proposed to employ combinations targeting molecules for example transferrin plus antibodies directed against CD97. We expect to complete these studies in the following granting period.

We also have begun to address the ability of zinc to kill tumor cells in a murine prostate carcinoma model. **Aim 2 Determine the Antitumor Activity and Biological Toxicity of Zn in Murine Models of Prostate Carcinoma.** Experiments will evaluate *in situ* the application of Zn compounds (Zn salts and Zn-loaded targeted liposomes) in human xenograft and syngeneic murine prostate cancer models to evaluate the efficacy of both direct intra-tumoral injection and systemic administration of these compounds on tumor size, histological characteristics and animal survival.

To test whether zinc can kill PC-3 cells *in vivo*, PC-3 cells were injected into the dorsum of immunodeficient (nude) animals (1-3). Zinc acetate solutions were administered at dosage ranges determined in Aim 1 and anti-tumor activity monitored by evaluation of tumor size over time and ultimately animal survival (Figure 3). While injections of zinc acetate i.v. did not affect growth of the tumors, intra-tumor injection of 0.5 mM zinc markedly arrested tumor growth (Figure 3). We have also collected tissue and blood samples to test the half-life, biodistribution and toxicity of the Zinc solutions. In addition we have collected organ samples to study for signs of toxicity. Initial studies indicate that the

half-life of zinc is rather short, less than 3 hours suggesting that at these concentrations zinc acetate will not result in severe toxicity. We are in the process of evaluating these results and analyzing the blood and tissue samples for submission of this work in the journal Cancer Research.

These studies clearly demonstrate that direct injection of zinc into established subcutaneous PC-3 tumors arrested growth of the tumor. Preliminary analysis of tissue samples suggest that these concentrations were not toxic suggesting that higher concentrations would be tolerated. In the coming year we want to establish the effective therapeutic dose of zinc. After we have established the therapeutic dose we next will generate targeted zinc loaded liposomes and test their efficacy in our mouse model. Finally we will examine the anti-tumor activity after systemic administration of targeted Zn liposomes at various dosages on mice with established subcutaneous tumors and also mice with pulmonary metastases. These results are exciting and suggest the potential of zinc as a therapeutic.



KEY RESEARCH ACCOMPLISHMENTS:

- (1) Zinc kills PC-3 cells
- (2) Transferrin targeted zinc loaded liposomes kills PC-3 cells in culture
- (3) Zinc kills PC-3 cells cultured in collagen gels (in 3D).
- (4) Direct injection of zinc arrest growth of prostate tumors in a human xenograft murine prostate cancer model

REPORTABLE OUTCOMES:

- (1) We have applied for a NIH R21 grant utilizing the technology developed from this grant. The grant is focused on the screening of aptamers against novel surface molecules.
- (2) Two papers are in preparation

CONCLUSION:

We have demonstrated that zinc loaded targeted liposomes can effectively kill PC- cells cultured in culture and in collagen gels. These data demonstrate that zinc can be entrapped in liposomes at concentrations that kill tumor cells. These studies also indicate that targeting of the liposomes is an effective strategy for generating selective treatment of cells. Our data also show that liposomes composed of readily available lipid are effective. Finally we have demonstrated that intra-tumor injection of zinc into subcutaneous tumors (PC-3 cells) arrest growth of the tumor. This is an exciting result and demonstrates the potential of zinc as a cancer therapeutic. What we have not accomplished is demonstrating that zinc loaded vesicles can arrest growth in mice. While we have been successful at killing PC-3 cells in culture with zinc loaded vesicles, our initial studies with liposomes in our human xenograft and syngeneic murine prostate model. Analysis of our liposome preparations suggest that either we need to increase the concentration of zinc entrapped or scale up the amount of concentration of liposomes. Both approaches will be attempted. We have begun studies with reverse phase preparations and preliminary data suggests that these entrap significant higher concentrations. Finally, we are in the process of scaling up the amounts of liposomes that the *in vivo* experiments require.

REFERENCES:

- (1) Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 1999. *CA Cancer J Clin* 2000;50:7±33
- (2) Landis, S.H.; Murray, T.; Bolden, S.; Wingo, P.A. *et al. C.A. Cancer J. Clin.*, 1999, 49, 8-31.
- (3) Clark, J.A.; Wray, N.P.; Ashton, C.M. *J. Clin. Oncol.*, **2001**, 19, 72-80
- (4) Lance, R.S.; Freidrichs, P.A.; Kane, C.; Powell, C.R. *et al. Br. J. Urol. Int.*, **2001**, 87, 61-65.
- (5) Huland, H. *Eur. Urol.*, **2001**, 39 (Suppl. 1), 3-9
- (6) Beyer, D.C.; Shapiro, R.H.; Puente, F. *Int. J. Radiat. Oncol. Biol. Phys.*, **2000**, 48, 1583-1589
- (7) Murphy, G.P.; Mettlin, C.; Menck, H.; Winchester, D.P. *et al. J. Urol.*, **1994**, 152, 1817-1819

- (8) Zincke, H.; Oesterling, J.E.; Blute, M.L.; Bergstralh, E.J. *et al. J. Urol.*, **1994**, *152*, 1850-1857
- (9) Hanks, G.E.; Hanlon, A.; Schultheiss, T.; Corn, B. *et al. J. Urol.*, **1994**, *152*, 1775-1780
- (10) Keuppens, F.; Whelan, P.; Carneiro de Moura, J.L.; Newling, D. *et al. Cancer*, 1993, *72* 3863-3869.
- (11) Dijkman, G.A.; Janknegt, R.A.; De Reijke, T.M.; Debruyne, F.M. *J. Urol.*, **1997**, *158*, 160-163
- (12) Klotz, L. *Cancer*, 2000, *15* (12 Suppl.), 3009-301
- (13) Paul, R. and Breul, J. *Drug Saf.*, 2000, *23*, 381-390
- (14) Klein, C, Creach, K, Irintcheva, V, Hughes, KJ, Blackwell, PL, Corbett, JA and Baldassare, JJ. *Apoptosis*, 2006, *11* :1933-44

APPENDICES:

None