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

Saint Louis, MO 63104

REPORT DATE:

June 2010

TYPE OF REPORT:

Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-06-2010	Final	1 JUN 2007 - 31 MAY 2010

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

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Saint Louis University

Saint Louis, MO 63104

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
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
During the tenure of the award we showed that direct intratumoral injection of zinc killed tumors derived from PC-3 cells. We also demonstrated that direct injection of zinc containing liposomes with attached transferrin as a targeting molecule slowed the growth of PC-3 malignant tumors in mice. These data strongly support the zinc containing targeted liposomes zinc as a therapeutic agent for treatment of prostate cancer. The results clearly showed that zinc reduced tumor size and prolonged survival. These data lend strong preliminary evidence that zinc treatment is efficacious and that liposomes would be an efficacious carrier. Importantly, there was minimal accumulation of zinc in other organs,suggesting the potential for minimal side effects with zinc treatments typical of other cancer chemotherapeutics. The in vivo studies were carried out on tumors derived from PC-3 cells. However, zinc killed many different prostate tumors, e.g. PC-3, LNCap-FGC, LNCaP-r, and DU145 cells. Taken together these data suggest that targeted zinc liposomes may provide a new therapeutic for the treatment of prostate cancer.

15. SUBJECT TERMS
Prostate Cancer, Zinc, Targeted Liposomes

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT **U**	U	c. THIS PAGE U	UU	51	USAMRMC
					19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction:

In the United States alone, Prostate cancer affects 1 in 6 men, making it the most common non-skin cancer in America (1, 2). As many as 30,000 men die from this disease each year in the US, making prostate cancer the second biggest cancer killer of men, behind lung cancer. Androgen deprivation is the most effective non-surgical therapy of localized prostate cancer. Treatments initially result in high responsiveness. However a proportion of patients develop locally advanced or metastatic disease that is refractory to anti-hormone therapy (3, 4). No currently available therapy is able to cure prostate cancer once it has metastasized. Several distinct features of the prostate gland open up unique opportunities for treatment of this cancer. First, the prostate is a nonessential organ, often making complete surgical resection a viable option, albeit one with permanent unpleasant side effects for the patient. Secondly, during early phases of the disease, the malignant prostatic lesions tend to remain focal and restrictively localized to the prostate gland itself. This, combined with the anatomic accessibility of the prostate gland, makes direct intra-tumoral injection of carcinotoxic and carcinostatic agents a real possibility for effective and relatively noninvasive treatment (2). 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 (3, 4) 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.

Decreased levels of Zn have been associated with the growth of prostate cancer cells (5-7). Clinical studies have attempted to evaluate the possibility that Zn replenishment could alter the growth of such cells. Individuals were given high amounts of Zn over the course of several years. In one case, an elevated risk of prostate cancer was noted, in another a decreased risk, and in another, no change (5). A limitation to these studies is the lack of evidence that oral supplementation alters Zn concentrations in the prostate. There is, however, evidence that Zn can inhibit the growth/survival of prostate cells should they become directly exposed to Zn (8, 9).

In the past year (year three) we examined aspects of Zn killing of PC3, LNCaP and DU145 cell lines. These cell lines are distinctly different in their morphology and tissue origin as well as in their gene expression profile for components such as the androgen receptor (10-13). These differences would simulate the genetic and phenotypic heterogeneity characteristic of prostate cancer and thus show the general applicability of a particular treatment (10-13). Significantly, previous studies of these cell lines have demonstrated that they display differential sensitivities to currently used chemotherapeutics (13-15).

We also have explored the effectiveness of direct intra-tumoral injection of liposomes containing zinc acetate into malignant prostatic tumors (7). To our knowledge, this is the first examination of intra-tumoral of a zinc delivery system as a treatment strategy for

prostate cancer, and we feel that these data form powerful preliminary evidence indicating that such a minimally invasive strategy could be efficacious.

Body:

This is the final report on the grant. I have included some of the material from last year to give a better understanding of our progress toward our aims. I have made this clear in the text by indicating the year into the grant the data was acquired.

During the first year of the grant we made significant progress on Aim 1-
Aim 1. Liposome Delivery and Efficacy. We had proposed to compare the efficacies of acidic and non-acidic liposomes (26-28) and the effectiveness of different chemical forms. We also set out to determine the most effective targeting molecule and or combination of molecules. We determined in the first and second year that neutral (non-acidic) liposomes are the most stable and most efficacious liposomes. We also found that many different zinc salts were efficacious. Therefore, we completed this section of Aim1 in years 1 and 2.

During the second year of the grant we initiated studies to study combinations of zinc plus other known therapeutic drugs. Zinc in combination with other well described cancer therapeutic, including taxol were tested. Although these studies were not included in the original proposal we thought that combination therapy might give significant therapeutic benefit and that this idea fit with our original proposal. We have devised isobolagrams from *in vitro* data (data not shown) and our studies suggest that in the addition of zinc lowers the effective dose of taxol. Thus zinc and taxol appear to act synergistically. These data are not completed and we intend to use these data to generate a small Internal Grant to finish the studies for publication.

Testing of Zinc and Zinc Liposomes in Other Cell Lines:

We started these studies in the second year, finished them in the third and the paper has been accepted for publication (Christopher L. Kriedt, Joseph Baldassare, Maulik Shah and Claudette Klein," Zinc functions as a cytotoxic agent for prostate cancer cells independent of culture and growth conditions", Journal of Experimental Therapeutics and Oncology). To test the ability of liposomes to kill other prostate tumor cells, we choose three prostate tumor cell lines; LNCap-FGC, LNCaP-r, and DU145. These cell lines are distinctly different in their gene expresssion profile such as the androgen receptor. LNCap-FGC and LNCaP-r are androgen-sensitive and androgen-resistant line and constitute a model for development of androgen resistance in prostate cancer (16, 17). The three cell lines differ in their intrinsic apoptotic and thioredoxin pathways and p53 status, as well as in their morphology and tissue origin (18, 19). These differences would simulate the genetic and phenotypic heterogeneity characteristic of prostate cancer (18, 19) and thus show the general applicability of zinc treatment. Furthermore, previous studies of these cell lines have demonstrated that they display differential sensitivities to currently used chemotherapeutics. Cisplatin treatment of PC3 and LNCaP results in approximately 10% cell death after a 24 h incubation while similar treatment results in approximately 30% death of DU145 cells

(20, 21). PC3 and DU145 cells are significantly more resistant to doxorubicin and taxol treatment than are LNCaP cells (22, 23), while DU145 cells are the most sensitive to vorinostat, followed by LNCaP cells, with PC3 cells being resistant to such treatment (24, 25).

In year two we found significant cell death occurred within 18 hrs (Data not shown). We then studied zinc concentration responses for several prostate cell lines utilizing either trypan blue exclusion (Figure 1) or MTT assay (Data not shown). Both methods of scoring for cell death gave similar results. PC3 cells displayed 40% and 80% death with 175 uM and 250 uM Zn respectively (Figure 1). At 175 uM and 250 uM Zn, Du145 cells responded similarly to PC3 while both LNCaP cells seemed less susceptible. However, almost complete cell death was achieved with all cell lines using 350 uM Zn (Figure 1). In the experiments shown, Zn was added in the form of ZnSO4. Similar results were obtained when ZnCl2, ZnAc, or Zn perchlorate were used, indicating that Zn is the active ligand (Data not shown).

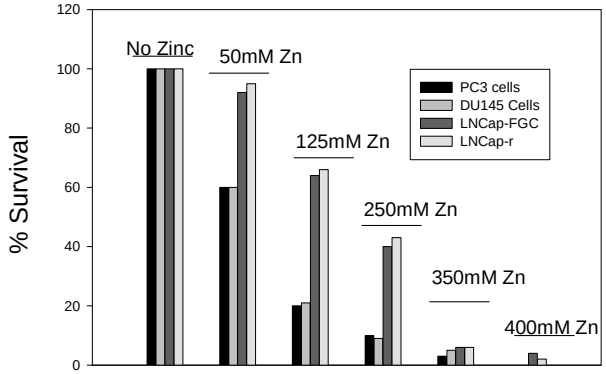


Figure 1 Effect of Zinc on various Prostate Cell lines. *Each cell line was plated at 70% confluency. One day after plating the cells were treated with Zinc (at the indicated conc.) and then 18 hrs after Zinc treatment the cells were stained with trypan blue and scored for trypan blue exclusion*.

In the past year (year three) we showed that cytotoxicity required internalization. When cells were incubated with pyrithione, the heavy metal ionophore, significantly lower Zn concentrations were cytotoxic (Figure 2). Greater than 95% cell death occurred within an 18 h period using 25μM Zn in the presence of pyrithione. Thus much lower concentrations of zinc were required for killing in the presence of the ionophore than in the absence. The data indicate that internalization of Zn is necessary for its cytotoxic effects

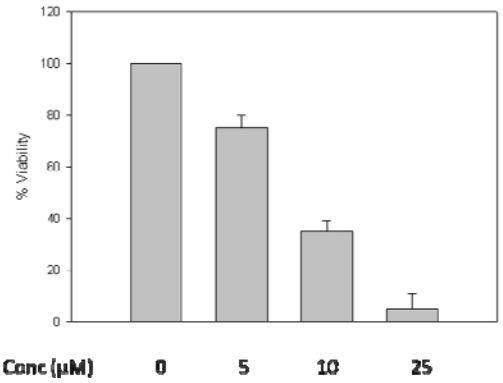


Figure 2. Pyrithione Reduces the Effective Concentration of Zn. *Exponentially growing PC3 cells were incubated with the indicated concentrations of ZnSO4 in the presence of 5μM pyrithione. After an 18 h incubation, cell viability was determined using the MTT assay*

Although monolayer culture systems are commonly used to evaluate the cytotoxic effects of drugs, it is believed that 3-D culture systems more accurately model the behavior of cells in a tumor. With this in mind, we examined the ability of Zn to kill PC3 cells grown in a collagen extracellular matrix ECM. With 3-D cultures crosshatched and grey bars), approximately 40% cell

death was seen with 175 μM Zn and approximately 75% death with 250 μM (Figure 3). Notably, there did not appear to be differences between the responses of cell plated in 2 or 3D. Thus using 2-dimensional and 3-dimensional cultures, we observed that zinc cytotoxicity was independent of both the culture conditions and the rate of cell growth. The attractive properties of zinc cytotoxicity demonstrated in this paper suggest that is can be developed as a novel and effective chemotherapeutic agent for prostate cancer treatment

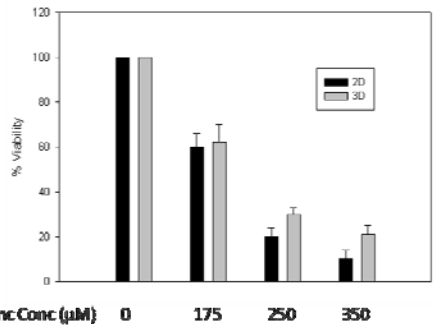


Figure 3 Culture Conditions Do Not Alter the Cytotoxic Effects of Zn. *PC3 cells were plated as 2-D cultures or as 3-D cultures. When cells had achieved a level of either 8 x 105, the indicated concentrations of ZnSO4 were added.* After an 18 h incubation, cell viability was determined using the MTT assay

These data indicate that all cell lines respond to Zn in a time and dose dependent manner. We next determined the ability of zinc to kill a number of cancer cells lines, including PC12, TrampC2, HELA, MCF7, ES2 and SKOV (Table1). While the concentration dependence was different with the different cell lines, treatment with concentrations of 400uM resulted in over 95% cell death for all cell lines tested. These data indicate that zinc treatment can be generalized to other prostate cancer cells and potentially to other cancers, for example breast cancers.as zinc treatment resulted in cell death of ES2 and SKOV cells.

Cell Line	% Survival
PC12	10
TrampC2	8
HELA	2
MCF-7	12
ES2	2
SKOV	3

Table1 Cell survival after treatment with Zinc *Cells were plated at 75% confluency. The following day they were treated with 350uM zinc. and 18 hours after zinc treatment were scored for cell survival by MTT assay*

We have completed these studies and have a submitted a paper (Accepted for publication in *Journal of Experimental Therapeutics and Oncology*). .

Comparison of liposomes prepared by the Reverse Phase Evaporation protocol

During the past three years of the grant we have completed this aim as described below. To compare the Reverse Phase method with the Freeze/Thaw extrusion protocol liposomes were prepared with identical lipid compositions. The liposomes were coupled to transferring as a targeting molecule. We generated zinc entrapped liposomes utilizing established protocols for preparing lipid vesicles. These liposomes contained dioleoyl phosphatidylycholine. For both protocols the vesicles of approximately 100 microns were prepared by passing liposomes through polycarbonate membranes and then 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 4). Cell death was determined at 8 and 18 hours after addition of the liposomes (Figure 4). These data clearly show that liposomes prepared by Freeze/Thaw protocol were more effective than those prepared by the Reverse Phase protocol. These differences are not a result of more efficient coupling of Fab' fragments, because coupling for either preparation was approximately identical (data not shown). We think the Freeze/Thaw preparation may trap zinc more efficiently. However, we have not been successful at obtaining good quantization of trapped zinc concentrations.

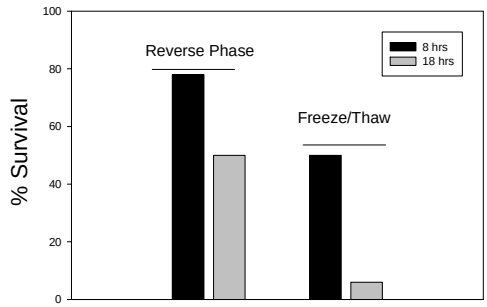


Figure 4 Comparision of different Liposome Preparations-*PC-3 cells were plated at 75% confluency. The following day after platting the cells were incubated 8 and 18 hrs with 250 uliters of Liposomes() prepared either by Freeze/Thaw or ReversePhase protocols After 18 hours the cells were scored for cell survival by either trypan blue exclusion or MTT assay Data not shown).*

We utilized our Freeze /Thaw liposome protocol in future studies in mice (see below). This decision is based on our ability to kill PC-3 cells in cell cultures and our in vivo mouse data (see below, and included paper). We have shown that our Freeze/Thaw liposomes are superior to those prepared by the Reverse Phase protocol.

Testing of Other Targeting Molecules

We proposed to test other targeting molecules (specifically antibodies) and to then employ combinations of these molecules for example transferrin plus antibody to target liposomes to protate tumor cells. These studies have taken longer than expected. Our strategy was to use the hybridoma facility at Saint Louis University to generate media from which we could isolate monoclonal antibody employing affinity purification by passing the media over a protein A column. To initially test our ability to produce, isolate and couple the Fab' fragments to liposomes, we had the hybridoma facility produce 500 ml of media. Antibody screening of the media showed moderate titer (Figure 5). We next tested whether we could successfully isolate the antibody and couple the Fab' fragments to liposomes (16-18). For immunoliposomes, Fab'

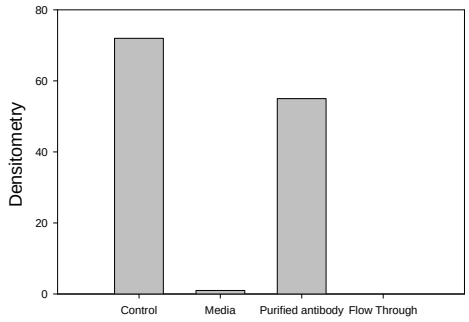


Figure 5 Slot blot screening to assess presence of antibody *Media (500), fractions from protein A column or purified antibody were dotted onto membrane and probed with HRP labeled anti-mouse secondary antibody. Each slot blot was then scanned and analyzed by densitometry. Lane 1 10ul of control antibody, lane 2 100ul of media. Lane 3 10ul of protein A purified antibody, lane 4100ul of flow through of protein A antibody. Blot was visualized by enhanced chemiluminescence*

was covalently conjugated to liposomes after zinc loading. Conjugation was via a thioether linkage between the free thiol near the COOH terminus of the MAb fragment and to maleimide groups at the termini of PEG chains (PEG-MAb linkage) as described (16). To assess coupling efficiency of the antibodies, the liposomes were solubilized in Western blot buffer and run on Western blot against known amounts of antibody (Data not shown). A coupling efficiency of 80% was routinely be achieved by this procedure. Thus we have worked out the techniques for isolating antibody, preparing Fab' fragments and coupling the fragments to Freeze/Thaw prepared liposomes. The next step is to scale up, isolate and test Fab' labeled liposomesn for their ability to kill.

Because we had limited recourses we focused on testing of transferrin labeled liposomes in our Murine Model . We think that this will be the most efficient use of our resources**.**

Determine the Antitumor Activity and Biological Toxicity of Zn in Murine Models of Prostate Carcinoma

We have addressed 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 were performed *in situ* to evaluate the application of Zn compounds (Zn salts) in human zenograft 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.

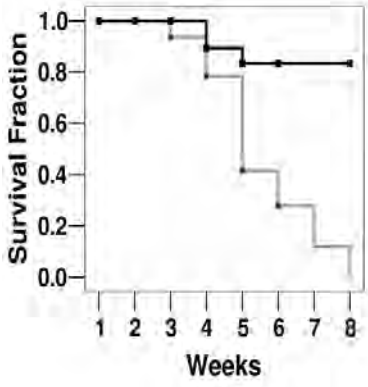


Figure 6. Effect of Intra-Tumoral Zinc Injection on Survival. *Prostate cancer cell xenografts were placed into SCID mice and allowed to grow to a size of 200 mm^3. Every 48 hours for 14 days, mice were then anesthetized and injected with 200 µL of either saline or 3 mM zinc acetate.*

As a measure of the potential usefulness of zinc as a component of prostate cancer chemotherapeutics, we assayed the ability of the intra-tumoral zinc injection protocol to extend the life of animals in our prostate cancer xenograft model (Figure 6). intra-tumoral injection of zinc acetate significantly extended the lifespan of animals in this xenograft model of prostate cancer. Dramatically, although the treatment protocol extended for only two weeks, the enhanced survival of animals in the zinc treatment group was persistent for several weeks beyond (Figure 1). In the control group, all animals had succumbed to the debilitating effects of the growing tumor within eight weeks of the beginning of the treatment protocol. However, in the same time period, 80% of those treated with zinc acetate injections remained alive (Figure 6). This dramatic result was significant ($p = 0.002$) by Kaplan-Meier Survival Analysis and revealed the intra-tumoral injection can halt the growth of prostate cancer *in vivo* with marked in gains in survival.

We also sought to examine the homing of zinc to different tissues, following a single intra-tumoral injection (Figure 7). Although the liver displayed the greatest concentration of zinc, there is no significant difference in zinc levels after zinc administration, although we observed considerable variability between animals. Similarly, there appears to be a reproducible but statistically insignificant accumulation of zinc within the xenograft tumors, even after a single administration (Figure 7). We then extended these observations to conditions of chronic zinc administration and found that our intratumoral zinc injection protocol results in a substantial increase in zinc levels within the tumor xenograft cells, but not in any brain, heart, kidney, or liver. This confirms our supposition that intra-tumoral injection allows for a much higher local concentration of zinc, which in turn may overcome impaired zinc import and thus, increased partitioning of therapeutic zinc into the diseased prostate tissue.

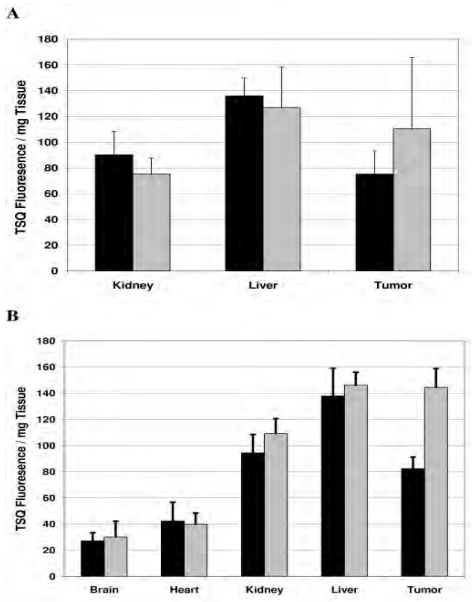


Figure 7 Tissue Zinc Concentration After Acute or Chronic Zinc Administration*. Levels of zinc were measured in specific tissues following either a single (A) or chronic (B) 200 µL injections of 3 mM zinc acetate. Data is presented as an average and errors bars indicate the standard deviation of four mice (n = 4).*

These results show that despite rapid dissipation of zinc into total body water there was a local effect of diminishing tumor growth over time. Although our administration schedule is an impractical method for the treatment of local disease in humans, our studies have established that administration of zinc in the tumor microenvironment can have a potent anti-tumor effect. Direct injection into tumors did result in increasing tumor tissue zinc levels and altered growth over time, an effect that persisted long after zinc injections were ceased.

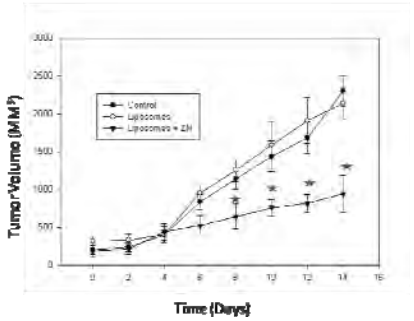


Figure 8 Effect of direct intra-tmoral Injection of zinc liposomes on PC3 growth. *Prostate cancer cell xenografts were placed into SCID mice and allowed to grow to a size of 300 mm^3. Every 48 hours for 14 days, mice were anesthetized and injected. Tumor size was measured at the indicated intervals. Error bars represent average size for 10 mice and "*" represents significance at p<0.05 by ANOVA.*

We next tested whether our zinc entrapped liposomes in our mouse zenograft

model. These experiments were performed in year 3 of the grant. We injected a bolus of PC3 cells subcutaneously into the dorsal region of SCID mice. In a pilot study, we observed that a single dose of zinc liposomes did not affect tumor growth (data not shown). In addition, because previous studies have established that zinc is rapidly distributed in total body water and cleared within 24 hours (see above), we elected to administer repeated doses at 48 hours intervals in order to establish a chronic treatment protocol. When the tumors reached approximately 300 mm^3, treatments were begun: 100 µL of liposomes by direct intratumoral injection every 48 hours for a period of two weeks. Because In a small trial experiment we found that only liposomes containing zinc and a targeting molecule transferrin were effective in arresting tumor growth (Datya not shown), the liposomes were targeted with transferring in addition to containing zinc. After eight days zinc liposomes injections dramatically halted the aggressive growth of PC3 xenografts when compared to non-injected xenografts or xenografts injected with liposomes that do not contain zinc (Figure 8). Because These data indicate that administration ofliposomes containing zinc in have a potent antitumor effect.

KEY RESEARCH ACCOMPLISHMENTS:

(1) Direct injection of liposomes containing zinc slow the growth of tumors in vivo

(2) Zinc treatment is an effective agent for killing different prostate tumor cells including PC-3, LNCap and DU145 cells

(3) Freeze/Thaw combined with extrusion through polycarbonate membranes appears to be an excellent protocol for preparing zinc entrapped liposomes.

(4) Successfully coupled antibody to Freeze/Thaw prepared liposomes

(5) Direct injection of zinc halt the growth of prostate cancer *in vivo* with marked in gains in survival.

(6) Direct injection of zinc does not result in pathology in tissues after zinc administration suggesting that zinc treatment may not lead to significant side effects.

(7) Combinations of zinc plus other cancer therapeutic drugs increases the efficacy of zinc when added alone

REPORTABLE OUTCOMES:

(1) We have applied for an “In House” Presidential Award The grant focuses on the extending our animal studies

(2) We have published two papers:

Shah MR, Kriedt CL, Lents NH, Hoyer MK, Jamaluddin N, Klein C, Baldassare J. "Direct intra-tumoral injection of zinc-acetate halts tumor growth in a xenograft model of prostate cancer", .J Exp Clin Cancer Res. 2009, 28; 84.

Christopher L. Kriedt, Joseph Baldassare, Maulik Shah and Claudette Klein," Zinc functions as a cytotoxic agent for prostate cancer cells independent of culture and growth conditions", Journal of Experimental Therapeutics and Oncology Accepted for publication

CONCLUSION:

During the tenure of the grant we determined a method of preparation of liposomes. The Freeze/Thaw method of liposome preparation is the better preparation for preparation of liposomes containing zinc. We demonstrated that direct injection of zinc dramatically halts tumor growth in a xenograft model of prostate cancer. Furthermore, direct injection into the xenografts did not result in pathology to other organs despite rapid dissipation of zinc into total body water content. These data suggest the use of zinc would likely have limited systemic toxicity. The data also suggest that injection of biogels or depot formulations of zinc may be an alternative strategy to increasing intraprostatic zinc resulting in anti-tumor effect with limited toxicity.

Finally, direct injection of liposomes that contain zinc slows the growth of the tumors when added to prostate cancer cells. Only those liposomes containg zinc and transferring were efficacious. These data are preliminary but suggest the possibility of using liposomes as a vehicle for the delivery of zinc.

REFERENCES:

(1) Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 1999. CA Cancer J Clin 2000; 50, 7.

(2) Landis, S.H.; Murray, T.; Bolden, S.; Wingo, P.A. *et al*. C.A. *Cancer J. Clin*., 1999*, 49,* 8.

(3) Paule B. Reappraisal of the concept of hormone therapy in metastatic prostate cancer and implications for treatment. *Eur Urol* 47, 729–735, 2005.

(4) Rau KM, Kang HY, Cha TL, Miller SA, Hung MC. The mechanisms and managements of hormone-therapy resistance in breast and prostate cancers. *Endocr Relat Cancer* 12, 511–532, 2005

(5) Clark, J.A.; Wray, N.P.; Ashton, C.M. *J. Clin. Oncol*., 2001*, 19,* 72.

(6) Lance, R.S.; Freidrichs, P.A.; Kane, C.; Powell, C.R. *et al*. *Br. J. Urol. Int*., 2001*, 87,* 61.

(7) Shah MR, Kriedt CL, Lents NH, Hoyer MK, Jamaluddin N, Klein C, Baldassare J. J Exp Clin Cancer Res. 2009, 17; 84.

(8) Liang JY, Liu YY, Zou J, Franklin R, Costello L, Feng P.Inhibitory effect of zinc on human prostatic carcinoma cell growth. *The Prostate* 40(3): 200–207, 1999.

(9). Shah M, Kriedt C, Lents N, Hoyer M, Jamaluddin N, Klein C, Baldassare J. Direct intra-tumoral injection of zinc-acetate halts tumor growth in a xenograft model of prostate cancer. *J Exp Clin Cancer Res* 28(1): 84, 2009.

(10) Skjoth IH, Issinger OG. Profiling of signaling molecules in four different human prostate carcinoma cell lines before and after induction of apoptosis. *Int J Oncol* 28(1): 217–29, 2005.

(11) Stewart D, Cooper C, Sikes R. Changes in extracellular matrix (ECM) and ECM-associated proteins in the metastatic progression of prostate cancer. *Reprod Biol Endocrinol* 2, 2, 2004.

(12) Xu W, Ngo L, Perez G, Dokmanovic M, Marks P. Intrinsic apoptotic and thioredoxin pathways in human prostate cancer cell response to histone deacetylase inhibitor. *Proc Natl Acad Sci USA* 103(42): 15540–15545, 2006.

(13) Serafin A, Akudugu J, Bohm L. Drug resistance in prostate cancer cell lines is influenced by androgen dependence and p53 status. *Urol Res* 30(5): 289–294, 2002.

(14) Geldof A, Mastbergen S, Henrar R, Faircloth G. Cytotoxicity and neurocytotoxicity of new marine anticancer agents evaluated using in vitro assays. *Cancer Chemother Pharmacol* 44(4):312–8, 1999.

(15) Chung LW, Baseman A, Assikis V, Zhau HE. Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *J Urol* 173(1): 10–20, 2005.

(16) Van Brussel, J.P., G.J van Steenbrugge, J.C Romijn, F.H Schröder, G.H.J Mickisch European Journal of Cancer 1999, 35; 664

(17) Pousette A, Carlström, K, Henriksson, P, Grande, M, Stege, R The Prostate 1997, 31; 198

(18) John N. Weinstein, Timothy G. Myers, Patrick M. O'Connor, et al. *Science,* 1997, 275; 343

(19) Zorn, K. K. Clinical Cancer Research 2005, 11; 6422

(20) Sánchez C, Mendoza P, Contreras HR, Vergara J, McCubrey JA, Huidobro C, Castellón EA. Prostate. 2009, 69; 1448.

(21) Budman DR, Calabro A, Kreis W. Anticancer Drugs. 2002, 10; 1011.

(22) Lanzi C, Cassinelli G, Cuccuru G, Supino R, Zuco V, Ferlini C, Scambia G, Zunino F. Prostate. 2001, 48; 254

(24) Kortenhorst MS, Zahurak M, Shabbeer S, Kachhap S, Galloway N, Parmigiani G, Verheul M, Carducci MA. Clin Cancer Res. 2008, 14; 6886.

(25) Xu W, Ngo L, Perez G, Dokmanovic M, Marks PA. PNAS U S A. 2006, 103; 15540

(26) Park, J. W., Hong, K., Carter, P., Asgari, H., Guo, L. Y., Keller, G. A., Wirth, C., Shalaby, R., Kotts, C., Wood, W. I., Papahadjopoulos,D., and Benz, C. C. 1999, Proc. Natl. Acad. Sci. USA, 92; 1327

(27) Kirpotin, D., Park, J. W., Hong, K., Zalipsky, S., Li, W. L., Carter, P., Benz, C. C., and Papahadjopoulos, D. 1997, Biochemistry, 36; 66.

(28) Carter, P., Rodrigues, M. L., Park, J. W., and Zapata, G.In: J. McCafferty, H. R. Hoogenboom, and D. J. Chiswell (eds.), Antibody Engineering: A Practical Approach, pp. 291–308. Oxford: IRL Press, 1996.

Direct Intra-tumoral Injection of Zinc-Acetate Halts Tumor Growth in a Xenograft Model of Prostate Cancer

Maulik R. Shah[1,2,*§], Christopher L. Kriedt[3], Nathan H. Lents[4], Mary K. Hoyer[2],

Nimah Jamaluddin[2], Claudette Klein[5*], and Joseph Baldassare[3*]

[1]Department of Otolaryngology, Saint Louis University, Saint Louis, Missouri
[2]Saint Louis University Cancer Center, Saint Louis, Missouri
[3]Department of Pharmacological and Physiological Sciences, Saint Louis University, Saint Louis, Missouri
[4]Department of Sciences, John Jay College, City University of New York, New York City, New York
[5]Department of Biochemistry, Saint Louis University, Saint Louis, Missouri

*These authors contributed equally to this work

[§]Corresponding author

Email addresses:

MRS: shahmr@slu.edu

CK: kleinc@slu.edu

JB: baldasjj@slu.edu

Abstract

Intracellular levels of zinc have shown a strong inverse correlation to growth and malignancy of prostate cancer. To date, studies of zinc supplementation in prostate cancer have been equivocal and have not accounted for bioavailability of zinc. Therefore, we hypothesized that direct intra-tumoral injection of zinc could impact prostate cancer growth. In this study, we evaluated the cytotoxic properties of the pH neutral salt zinc acetate on the prostate cancer cell lines PC3, DU145 and LNCaP. Zinc acetate killed prostate cancer cell lines *in vitro*, independent of androgen sensitivity, in a dose-dependent manner in a range between 200 and 600μM. Cell death occurred rapidly with 50% cell death by six hours and maximal cell death by 18 hours. We next established a xenograft model of prostate cancer and tested an experimental treatment protocol of direct intra-tumoral injection of zinc acetate. We found that zinc treatments halted the growth of the prostate cancer tumors and substantially extended the survival of the animals, whilst causing no detectable cytoxicity to other tissues. Thus, our studies form a solid proof-of-concept that direct intra-tumoral injection of zinc acetate could be a safe and effective treatment strategy for prostate cancer.

Background

In the United States alone, 200,000 men are diagnosed with prostate cancer each year and one out of six men will be diagnosed in their lifetime. As many as 30,000 men die from this disease each year in the US, making prostate cancer the second biggest cancer killer of men, behind lung cancer[1]. However, several distinct features of the prostate gland open up unique opportunities for treatment of this cancer. First, the prostate is a nonessential organ, often making complete surgical resection a viable option, albeit one with permanent unpleasant side effects for the patient. Secondly, during early phases of the disease, the malignant prostatic lesions tend to remain focal and restrictively localized to the prostate gland itself. This, combined with the anatomic accessibility of the prostate gland, makes direct intra-tumoral injection of carcinotoxic and carcinostatic agents a real possibility for effective and relatively noninvasive treatment[2]. In this study, based in part on promising *in vitro* results from our laboratory, we explore the effectiveness of direct intra-tumoral injection of zinc acetate into malignant prostatic tumors.

Zinc is the most abundant trace element in the human body and is vital for the function of many enzymes and proteins in all cells and tissues of the body. There are over 300 zinc-dependent enzymes and zinc is required for the formation of the zinc-finger motif that is an essential component for nearly all transcription factors and many other proteins that bind nucleic acids[3]. It has long been known that chronic insufficient dietary zinc leads to many debilitating developmental defects, but emerging evidence now links

marginally deficient zinc consumption, such as that which affects more than 10% of the US population, to such diseases as anorexia nervosa, Ahlzeimer's Disease, and cancer. Several studies have found that men who consume below the USDA recommended daily allowance (RDA) of 11mg/day are at increased risk of developing prostate cancer[4]. Conversely, other studies have shown that high-dose supplements of zinc can increase the risk of prostate cancer[5]. Thus, the role of dietary zinc in the predisposition to prostate cancer requires further study.

The relationship between dietary zinc and prostate cancer likely stems from the vital role that zinc plays in prostate function. Zinc is known to accumulate in the prostate, and this gland typically harbors the highest concentration of zinc in the body[6]. This is because the secretory cells of the prostate require high levels of zinc to inhibit the enzyme m-aconitase, which normally functions to oxidize citrate during the Krebs cycle. Because citrate is a principle component of seminal fluid, prostate secretory cells do not complete the oxidation of citrate in the mitochondria and the zinc-mediated inhibition of m-aconitase is crucial for the accumulation of citrate in these cells, and thus the subsequent secretion of citrate into seminal fluid[7]. The accumulation of zinc in the prostate epithelium is accomplished by the zinc transporter ZIP1, which is highly expressed in normal prostate tissue[8].

Because zinc is thus antagonistic to the synthesis of ATP in the cells of the prostate gland, it is not surprising that both ZIP1 expression and the accumulation of zinc are markedly attenuated in a cancerous prostate [9]. [10]. Indeed, ZIP1 is considered a prostate tumor suppressor as the inhibition of its function is requisite for malignant transformation, and prostatic zinc

levels have shown an inverse relationship with tumorigenicity [11]. Thus, the restoration of zinc levels in prostate cancer cells is a logical strategy for clinical treatment. Further, zinc has been shown to be required for mitochondrial apoptogenesis in prostate cells *in vitro* [12], and infusions of moderate doses of zinc reliably lead to apoptosis of prostate cancer cell lines [13]. This has led to the hypothesis that clinical administration of zinc could be an effective chemotherapeutic for prostate cancer. However, studies of zinc dietary supplementation for cancer prevention have had mixed results [14, 15].

Recently, vascular delivery of zinc was evaluated as a potential treatment in a mouse model of prostate cancer [6]. Although an increase in apoptosis was observed in the prostate cancer xenografts of the mice receiving high doses of zinc, there was little effect on the overall growth and aggressiveness of the prostate tumors themselves. Because ZIP1 function is known to be impaired in prostate cancer cells, we presume that there was limited homing of zinc to the prostate cancer xenografts. Thus, we reason that a localized infusion of zinc, and thus a greater local concentration, could circumvent the reduced ZIP1 activity and allow greater bioaccumulation of zinc in the diseased prostate. This is important because intravenous doses of zinc are limited due to the cellular toxicity of this heavy metal at super-physiologic concentrations.

In this study, we hypothesize that the direct intra-tumoral injection of zinc could be a safe and efficacious treatment for prostate cancer. To our knowledge, this is the first examination of intra-tumoral zinc delivery as a treatment strategy for prostate cancer, and we feel that these data form

powerful preliminary evidence indicating that such a minimally invasive strategy could be efficacious. Furthermore, because of the preferential accumulation of zinc in prostate tissue, it is conceivable that such a strategy could be entirely free of the debilitating and dose-limiting side effects typical of other cancer chemotherapeutics.

Methods

Cell lines – PC3, DU148, LNCaP cells were originally obtained from ATCC (Rockville, MD). Cells were maintained at 37°C, 5% CO_2 and 95% humidity in DMEM (CellGro, Herndon, VA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine and 100 units/ml penicillin and 1000 ug/ml streptomycin (Invitrogen, Carlsbad, CA).

Animals – NOD/SCID mice at 8 weeks of age were purchased from Charles River Laboratories (Wilmington, MA) and were housed at the Saint Louis University comparative medicine facility. Animals were allowed to acclimate for 2 weeks prior to experimentation. The animals were under the care of a staff veterinarian and managed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Xenografts – PC3 cells grown to 70% confluence were harvested and injected in the dorsum of animals subcutaneously. Each inoculum consisted of 100μL of cell suspension at a concentration of 10^7 cells/ml in phosphate-buffered saline. Tumors were allowed to grow to a size of 300 mm^3 prior to intra-tumoral injection. Tumors were injected with 200μL of 3mM zinc acetate solution every 48 hours. Tumors were measured every 2-3 days with digital calipers. Tumor volume was determined using the following formula: Volume = Length X $Width^2$.

Zinc Measurements - Zinc was quantified in serum and tissues using the TSQ fluorophore (Invitrogen, Carlsbad, CA). 50mM TSQ was prepared in 10mM Tris buffer (ph=8.0). TSQ was added to samples and standard zinc solutions to a final concentration of 10μM in black round-bottom 96 well plates. Endpoint fluorescence was read on a Spectfluor with excitation wavelength of 360 nm and emission wavelength of 535 nm. Tissue zinc levels were measured similarly, after weighing and homogenizing tissue in water by repeated freeze/thaw cycles.

MTT Assay - Cell viability was determined via MTT assay. Briefly, media was aspirated from cells grown in 6 well plates and 1ml of MTT (1mg/ml) solution was added. After 1 hour incubation, MTT solution was aspirated and 0.04 N HCL was added to solubilize the cells and absorbance at 540nM was measured.

Results

Zinc has been shown to be cytotoxic to a variety of cancer cell lines[16-18]. In these studies, different formulations of zinc have been utilized. Unfortunately, *in vivo* measurements regarding the bio-pharmocokinetics of these different zinc salts are lacking. For this study, we have selected zinc acetate as it is pH neutral in aqueous solution with minimal effect on osmalarity, relative to other formulations of zinc. Cytotoxic effects of zinc acetate have not been reported.

In order to examine the general effectiveness of zinc in inducing cell death in prostate cancer cells, we selected three cell lines with distinct properties, representative of the distinct forms in which prostate cancers emerge. For example, PC3 and DU145 cells are androgen-independent, while LNCaP cells are androgen-dependent[19]. The molecular pathways associated with carcinogenesis vary as well between these cell lines[20] as determined by gene expression analysis. For example, PSA is upregulated in PC3 but not expressed in LNCaP. Using markedly different prostate cancer cell lines allowed us to analyze the effect of zinc irrespective of underlying pathways of transformation.

Induction of apoptosis of prostate cancer cells by zinc.

In figure 1, we show that treatment with zinc acetate leads to widespread cell death within 18 hours in three different prostate cancer cell lines (figure 1A). Importantly, cell death is sharply dose-dependent over a

broad range from 100-600μM and the cytotoxicity curves indicate that 300-400μM zinc acetate, depending on cell line, is effective at inducing cell death in ~80% of the cell population within just 18 hours (figure 1A). Having established that zinc acetate has a rapid cytotoxic effect on prostate cancer cell lines, we next established the time course of cell killing *in vitro*. Although only data for PC3 cells are shown, for all three cell lines, 400μM zinc acetate induced cell death quite rapidly, with 50% cell death occurring by 6 hours (figure 1B and data not shown). By 24 hours, greater than 95% of the cells had perished. Interestingly, zinc dose had minimal effect on the kinetics of cell death, as doubling the dose to 800μM zinc only reduced the EC50 by approximately 90 minutes (figure 1B).

Although maximal cytotoxicity is seen within 24 hours with doses of 400μM zinc or higher, we reasoned that longer incubations with lower doses of zinc might also have a cytotoxic effect on prostate cancer cells. Thus, we next evaluated zinc-induced cytotoxicity in PC3 cells at lower doses and found that, surprisingly, at each dose, maximum cell death again occurred by 24 hours with little further cell death thereafter (data not shown). However, prolonged exposure to zinc, even at the lowest dose of 100μM, has a cytostatic effect: cellular proliferation halted and the number of cells remained constant over time (data not shown). Indeed, this cytostatic effect of prolonged exposure to zinc was observed at all doses explored in this study.

Effect of Zinc Acetate on PC3 Xenograft Growth.

Given these promising *in vitro* results, we next examined whether zinc treatments could affect prostate cancer cells *in vivo*. To that end, we

established a human prostate cancer xenograft model by injecting a bolus of PC3 cells subcutaneously into the dorsal region of SCID mice. To date, detailed toxicity reports of zinc acetate in mice are lacking. However, experiments with mice have revealed an LD50 of approximately 50mg/kg for zinc chloride [21]. Because the maximal tolerable dose of zinc acetate has not been established and given that chronic liver changes were observed at the LD50 dose, we elected to use a dose that approximated one-eighth of the LD50, 200μL of 3mM zinc acetate. In a pilot study, we observed that a single dose of zinc acetate had no measurable effect on tumor growth (data not shown). In addition, because previous studies have established that zinc is rapidly distributed in total body water and cleared by renal filtration within 24 hours[22], we elected to administer repeated doses of zinc acetate in 48 hours intervals in order to establish a chronic treatment protocol, while limiting untoward zinc bio-toxicity and stress to animals due to the repeated anesthesia and injection.

When the prostate tumor xenografts reached 300mm^3, treatments were begun: 200μL of 3mM zinc acetate by direct intratumoral injection every 48 hours for a period of two weeks. We selected this somewhat large tumor size for both ease of intratumoral injection, and also for greater accuracy and consistency when using size as an outcome measure. Figure two demonstrates the effect of the zinc injections on tumor growth and it is immediately clear that intratumoral injections of zinc have a profound negative effect on growth of the tumor xenografts. The injection of zinc dramatically halts the aggressive growth of PC3 xenografts and, importantly, the growth

arrest persists after the injection schedule is terminated on the fourteenth day (figure 2). Importantly, the growth of xenografts was unaffected by the anesthesia and injection procedure *per se* as vehicle-injected tumors display growth kinetics indistinguishable from that of non-injected xenografts.

Bioavailability of zinc following intra-tumoral injection

Because of the promising results of arrested prostate cancer cell growth following zinc injection, we next turned our attention to the biodistribution of the zinc in this context. We began with simple subcutaneous injections of zinc acetate in otherwise un-treated SCID mice and found that single injections of zinc result in a rapid increase in serum zinc levels as early as 10 minutes after administration (figure 3A). However, serum zinc levels peak in 90 minutes and return to normal physiological levels within 24 hours (figure 3A). We next examined the pharmacokinetics of intra-tumoral injection of zinc acetate into our prostate cancer xenografts model. The resulting kinetics of zinc distribution are similar: serum zinc levels rise quite rapidly after tumor injection, reaching a maximum within 90 minutes, followed by a steady decline to baseline levels within 24 hours (figure 3B). A significant difference is that peak serum zinc levels are considerably less when injected into tumors then subcutaneously indicating either slower release from tumor tissue or significant uptake into tumor tissue.

We also sought to examine the homing of zinc to different tissues, following a single intra-tumoral injection. As shown in figure 4A, although the liver displayed the greatest concentration of zinc, there is no significant

difference in zinc levels after zinc administration, although we observed considerable variability between animals. Similarly, there appears to be a reproducible but statistically insignificant accumulation of zinc within the xenograft tumors, even after a single administration (figure 4A). We then extended these observations to conditions of chronic zinc administration and found that our intratumoral zinc injection protocol results in a substantial increase in zinc levels within the tumor xenograft cells, but not in any brain, heart, kidney, or liver (figure 4B). This confirms our supposition that intra-tumoral injection allows for a much higher local concentration of zinc, which in turn may overcome impaired zinc import and thus, increased partitioning of therapeutic zinc into the diseased prostate tissue.

Zinc Biosafety

Over the course of our experiments, we were able to limit prostate xenograft growth over a period of two weeks. Even after zinc administration was discontinued, tumor growth was slower than in control animals (figure 2). Importantly, at the dosage delivered to the animals, we did not observe any evidence of biotoxicity during the treatment protocol and no animal death was recorded. Further, a blinded pathologist performed a full post-mortum histological analysis of tissues and uncovered no evidence of tissue toxicity in the animals enrolled in the zinc treatment protocol (data not shown). Liver changes reported by others at the LD50 level were not seen with our substantially lower dosage even with the chronic administration schedule.

Survival of Animals following treatment of prostate cancer xenografts with zinc

As a final measure of the potential usefulness of zinc as a component of prostate cancer chemotherapeutics, we assayed the ability of the intra-tumoral zinc injection protocol to extend the life of animals in our prostate cancer xenograft model. Because they are growing subcutaneously rather than orthotopically xenograft tumors may grow to significant size without causing animal death. For humane reasons, a scoring system was established to assess animal welfare and animals not able to meet two requirements were euthanized. The scoring system consisted of the following: 1. Maintenance of normal weight (Weight loss > 12%); 2. Normal ambulation; 3. Normal grooming; 4. Normal feeding. Importantly, the decision to remove an animal from the protocol due to extreme tumor burden was made by an animal care technician unaware of the treatment group of the particular animal at the time of the decision.

Thus, humane removal of an animal from the protocol was recorded as a death event, and with these data we evaluated survival. As seen in figure five, intra-tumoral injection of zinc acetate significantly extended the lifespan of animals in this xenograft model of prostate cancer. Dramatically, although the treatment protocol extended for only two weeks, the enhanced survival of animals in the zinc treatment group was persistent for several weeks beyond (figure 5). In the control group, all animals had succumbed to the debilitating effects of the growing tumor within eight weeks of the beginning of the treatment protocol. However, in the same time period, 80% of those treated

with zinc acetate injections remained alive (figure 5). This dramatic result was significant ($p = 0.002$) by Kaplan-Meier Survival Analysis and revealed the intra-tumoral injection can halt the growth of prostate cancer *in vivo* with marked in gains in survival.

Discussion

Prostate cancer represents a unique clinical problem with respect to treatment options. 90% of men will present with localized disease [23]. For these men, the current treatment paradigm is prostatectomy or radiotherapy. For men with advanced disease, androgen therapy offers the best opportunity for long term survival. However, treatment may be limited by the androgen responsive nature of the tumor. Given the age at which many men present with prostate cancer and the slow growing nature of this cancer, in many cases, the treatment options may have equivalent morbidity in comparison to the cancer itself. Hence, less invasive methods of treatment with fewer side effects would be very advantageous for men presenting with localized disease.

There is much to suggest that treatment with zinc has real clinical potential. It is solidly established that reduced intracellular zinc levels are necessary for maintaining the malignant phenotype of prostate cancer cells [24] and that malignancy and tumor aggressiveness are inversely proportional to tumoral zinc levels [25]. Thus, the current paradigm for zinc in prostate cancer suggests that loss of intracellular zinc is vital to the transformation of normal prostate tissue into cancerous prostate tissue, likely due to the metabolic effects of zinc in the Krebs cycle. That is, because zinc inhibits m-aconitase, loss of zinc allows for greater energy utilization, supporting the substantially increased cellular metabolism that is necessary for rapid proliferation [26].

Because systemic (i.e. intravenous) injection of zinc has limitations and is poorly targeted to diseased prostate, in this study we evaluated whether increasing zinc bioavailability through direct injection into tumors would impact prostate cancer malignancies. Although repeated intratumoral injections may not be a desirable treatment modality for human prostate cancer patients, we have provided proof of concept that increase of intraprostatic zinc can effectively moderate prostate tumor growth.

In our *in vitro* experiments, we have shown that increasing zinc in the microenvironment to 200-600μM can cause rapid prostate cancer cell death. Cell death was independent of the mechanism of molecular carcinogenesis and independent of androgen sensitivity. Others have reported that the mechanism of zinc associated prostate cancer cell death is apoptotic with a shift in Bax/BCL2 ratios[27] and the morphological changes seen in our studies are consistent with apoptotic cell death. Cell death was also quite rapid indicating that prolonged exposure is not necessary for zinc effects on prostate cancer cells.

Human physiological serum zinc levels are approximately 45 μg/dL. This represents total zinc and not any particular salt form. As such, it is difficult to reconcile levels with molar doses used in our *in vitro* studies, but it is clear that our *in vitro* doses could not be achieved systemically in a whole animal without undue toxicity. However, we hypothesized that, given the rapid nature by which zinc-mediated cell death occurs in prostate cancer cells, the local microenvironment could be altered to a level sufficient to impact

tumor growth whilst avoiding widespread toxicity. Thus, in an attempt to maximize the anti-tumor effect and minimize the biotoxicity, we selected a dose that was approximately 8-fold less than the LD50 toxic dose reported for rodents. Based on the fact that we had no observed tissue biotoxicity, future studies could determine the maximum tolerable dose for direct zinc administration.

Conclusions

Our results showed that despite rapid dissipation of zinc into total body water there was a local effect of diminishing tumor growth over time. Although our administration schedule is an impractical method for the treatment of local disease in humans, our studies have established that administration of zinc in the tumor microenvironment can have a potent anti-tumor effect. Direct injection into tumors did result in increasing tumor tissue zinc levels and altered growth over time, an effect that persisted long after zinc injections were ceased. Our data indicate that methods to increase zinc in the prostate tumor microenvironment could be useful as a way of modulating growth of localized disease. Given rapid physiological clearance of zinc, the use of zinc would likely have limited systemic toxicity. Consequently, injection of biogels or depot formulations of zinc may be an alternative strategy to increasing intraprostatic zinc resulting in anti-tumor effect with limited biotoxicity.

Competing interests

No Competing Interests

Authors' contributions

MRS, CK and JB contributed equally to the design and implementation of the study. MRS was responsible for all statistical analysis. MRS and NHL drafted the manuscript. CLK and MKH equally contributed to carrying out all *in vitro* zinc toxicity studies. CLK performed all of the animal experimentation. NJ and CLK performed all zinc level determinations

Acknowledgements

The authors wish to thank Dr. Craig Lawson for evaluating all of the slides for the biotoxicity studies

This work was supported by DOD Grant pc 061410

References

1. Kamo K, Sobue T: **Cancer statistics digest. Mortality trend of prostate, breast, uterus, ovary, bladder and "kidney and other urinary tract" cancer in Japan by birth cohort**. *Jpn J Clin Oncol* 2004, **34**(9):561-563.
2. Springate CM, Jackson JK, Gleave ME, Burt HM: **Efficacy of an intratumoral controlled release formulation of clusterin antisense oligonucleotide complexed with chitosan containing paclitaxel or docetaxel in prostate cancer xenograft models**. *Cancer Chemother Pharmacol* 2005, **56**(3):239-247.
3. Prasad AS: **Zinc: the biology and therapeutics of an ion**. *Ann Intern Med* 1996, **125**(2):142-144.
4. Heshmat MY, Kaul L, Kovi J, Jackson MA, Jackson AG, Jones GW, Edson M, Enterline JP, Worrell RG, Perry SL: **Nutrition and prostate cancer: a case-control study**. *Prostate* 1985, **6**(1):7-17.
5. Leitzmann MF, Stampfer MJ, Wu K, Colditz GA, Willett WC, Giovannucci EL: **Zinc supplement use and risk of prostate cancer**. *J Natl Cancer Inst* 2003, **95**(13):1004-1007.
6. Feng P, Li TL, Guan ZX, Franklin RB, Costello LC: **Effect of zinc on prostatic tumorigenicity in nude mice**. *Ann N Y Acad Sci* 2003, **1010**:316-320.
7. Costello LC, Franklin RB, Liu Y, Kennedy MC: **Zinc causes a shift toward citrate at equilibrium of the m-aconitase reaction of prostate mitochondria**. *J Inorg Biochem* 2000, **78**(2):161-165.
8. Franklin RB, Ma J, Zou J, Guan Z, Kukoyi BI, Feng P, Costello LC: **Human ZIP1 is a major zinc uptake transporter for the accumulation of zinc in prostate cells**. *J Inorg Biochem* 2003, **96**(2-3):435-442.
9. Desouki MM, Geradts J, Milon B, Franklin RB, Costello LC: **hZip2 and hZip3 zinc transporters are down regulated in human prostate adenocarcinomatous glands**. *Mol Cancer* 2007, **6**:37.
10. Habib FK, Mason MK, Smith PH, Stitch SR: **Cancer of the prostate: early diagnosis by zinc and hormone analysis?** *Br J Cancer* 1979, **39**(6):700-704.
11. Costello LC, Franklin RB: **Novel role of zinc in the regulation of prostate citrate metabolism and its implications in prostate cancer**. *Prostate* 1998, **35**(4):285-296.
12. Costello LC, Franklin RB, Feng P: **Mitochondrial function, zinc, and intermediary metabolism relationships in normal prostate and prostate cancer**. *Mitochondrion* 2005, **5**(3):143-153.
13. Liang JY, Liu YY, Zou J, Franklin RB, Costello LC, Feng P: **Inhibitory effect of zinc on human prostatic carcinoma cell growth**. *Prostate* 1999, **40**(3):200-207.
14. Costello LC, Feng P, Milon B, Tan M, Franklin RB: **Role of zinc in the pathogenesis and treatment of prostate cancer: critical issues to resolve**. *Prostate Cancer Prostatic Dis* 2004, **7**(2):111-117.
15. Gallus S, Foschi R, Negri E, Talamini R, Franceschi S, Montella M, Ramazzotti V, Tavani A, Dal Maso L, La Vecchia C: **Dietary zinc and prostate cancer risk: a case-control study from Italy**. *Eur Urol* 2007, **52**(4):1052-1056.

16. Ronowska A, Gul-Hinc S, Bielarczyk H, Pawelczyk T, Szutowicz A: **Effects of zinc on SN56 cholinergic neuroblastoma cells**. *J Neurochem* 2007, **103**(3):972-983.
17. Dubi N, Gheber L, Fishman D, Sekler I, Hershfinkel M: **Extracellular zinc and zinc-citrate, acting through a putative zinc-sensing receptor, regulate growth and survival of prostate cancer cells**. *Carcinogenesis* 2008, **29**(9):1692-1700.
18. Franklin RB, Costello LC: **Zinc as an anti-tumor agent in prostate cancer and in other cancers**. *Arch Biochem Biophys* 2007, **463**(2):211-217.
19. Sobel RE, Sadar MD: **Cell lines used in prostate cancer research: a compendium of old and new lines--part 1**. *J Urol* 2005, **173**(2):342-359.
20. Yang M, Loda M, Sytkowski AJ: **Identification of genes expressed differentially by LNCaP or PC-3 prostate cancer cell lines**. *Cancer Res* 1998, **58**(16):3732-3735.
21. Bay BH, Wang MC, Yip GW: **Effect of intraperitoneal administration of zinc on C57/6J mouse liver--a light microscopic study**. *Okajimas Folia Anat Jpn* 1998, **74**(6):279-291.
22. Krebs NE, Hambidge KM: **Zinc metabolism and homeostasis: the application of tracer techniques to human zinc physiology**. *Biometals* 2001, **14**(3-4):397-412.
23. Dahm P, Yeung LL, Chang SS, Cookson MS: **A critical review of clinical practice guidelines for the management of clinically localized prostate cancer**. *J Urol* 2008, **180**(2):451-459; discussion 460.
24. Costello LC, Franklin RB: **The clinical relevance of the metabolism of prostate cancer; zinc and tumor suppression: connecting the dots**. *Mol Cancer* 2006, **5**:17.
25. Zaichick V, Sviridova TV, Zaichick SV: **Zinc in the human prostate gland: normal, hyperplastic and cancerous**. *Int Urol Nephrol* 1997, **29**(5):565-574.
26. Singh KK, Desouki MM, Franklin RB, Costello LC: **Mitochondrial aconitase and citrate metabolism in malignant and nonmalignant human prostate tissues**. *Mol Cancer* 2006, **5**:14.
27. Feng P, Li T, Guan Z, Franklin RB, Costello LC: **The involvement of Bax in zinc-induced mitochondrial apoptogenesis in malignant prostate cells**. *Mol Cancer* 2008, **7**:25.

Figures

Figure 1 - Kinetics and Toxicity of Zinc Acetate on Prostate Cancer Cell Lines

Prostate cancer cell lines (Panel A: PC3, DU145, and LNCaP; Panels B and C: PC3) were treated with the indicated concentrations of zinc acetate for either 18 hours (A) or indicated length of time (B and C). Data represent mean cell viability as assessed by MTT assay (n = 3 independent cell populations) and error bars represent standard deviation.

Figure 2 - Effect of Direct Intra-Tumoral Zinc Injection on PC3 Growth

Prostate cancer cell xenografts were placed into SCID mice and allowed to grow to a size of 300mm^3. Every 48 hours for 14 days, mice were then anesthetized and injected with 200μL of either saline (black squares) or 3mM zinc acetate (grey circles). Tumor size was measured at the indicated intervals. Error bars represent average size for 10 mice and "*" represents significance at $p<0.05$ by ANOVA.

Figure 3 - Serum Zinc Levels after Subcutaneous or Intratumoral Zinc Injection

Serum levels were measured at the indicated times following either a subcutaneous (A) or an intratumoral (B) single 200μL injection of 3mM zinc acetate. Data is presented as an average and errors bars indicate the standard deviation of four mice (n=4).

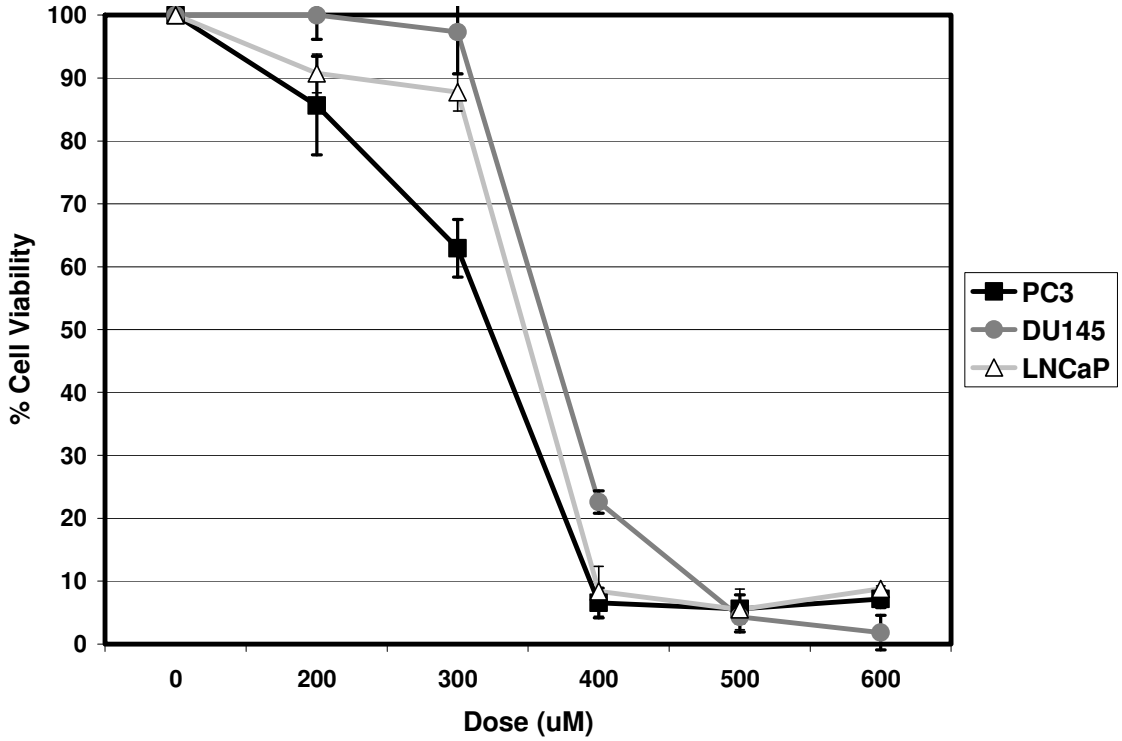
Figure 4 - Tissue Zinc Concentration After Acute or Chronic Zinc Administration

Levels of zinc were measured in specific tissues following either a single (A) or chronic (B) 200μL injections of 3mM zinc acetate. Data is presented as an average and errors bars indicate the standard deviation of four mice (n=4).

Figure 5 - Effect of Intra-Tumoral Zinc Injection on Survival

Prostate cancer cell xenografts were placed into SCID mice and allowed to grow to a size of 200mm^3. Every 48 hours for 14 days, mice were then anesthetized and injected with 200μL of either saline or 3mM zinc acetate. Mice were evaluated by a blinded technician and sacrificed when tumor burden reached predetermined criteria for humane maintenance and care. Difference in mean survival between treatment and control groups was significant ($p<0.002$) by Kaplan-Meier Survival Analysis.

A


100
90
80
70
60
50
40
30
20
10
0
% Cell Viability
0
200
300
400
500
600
Dose (uM)
PC3
DU145
LNCaP

B

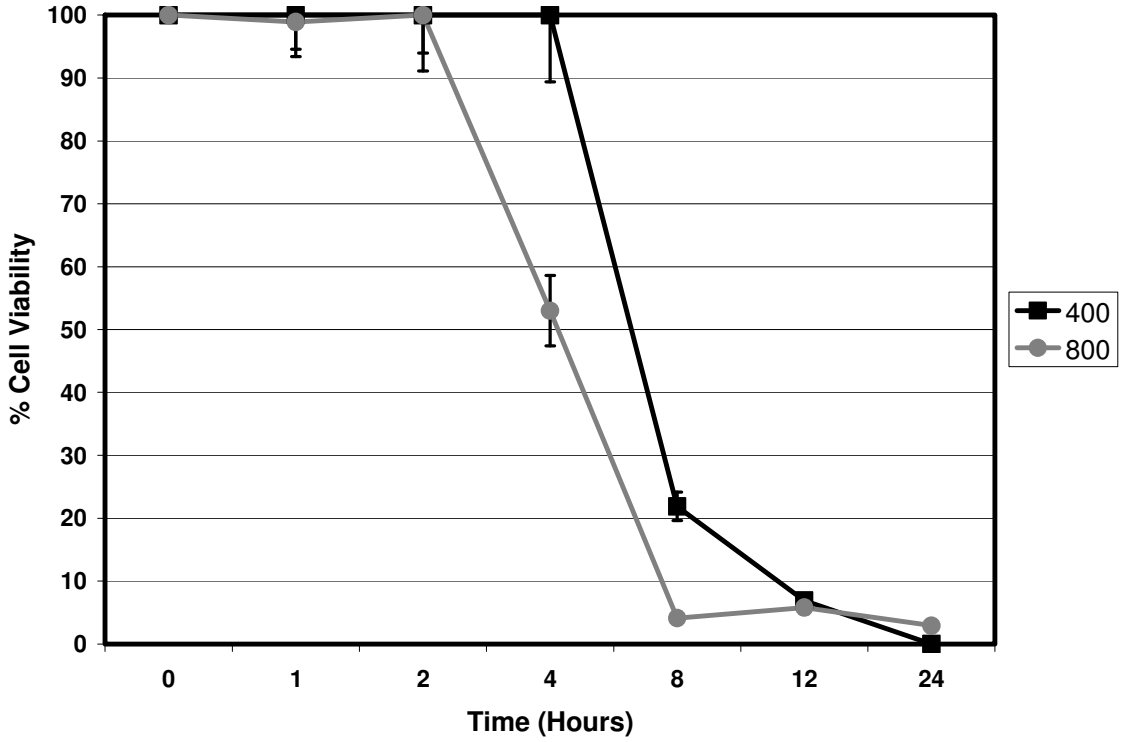

100
90
80
70
60
50
40
30
20
10
0
% Cell Viability
0
1
2
4
8
12
24
Time (Hours)
400
800

Figure 1

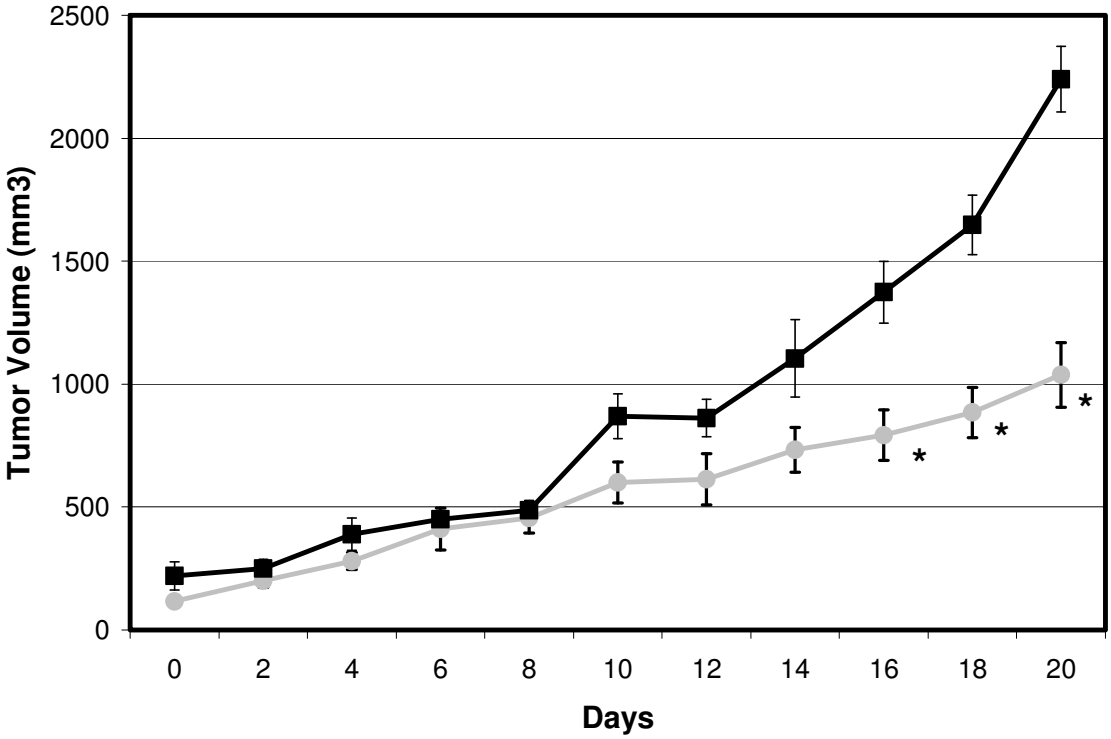
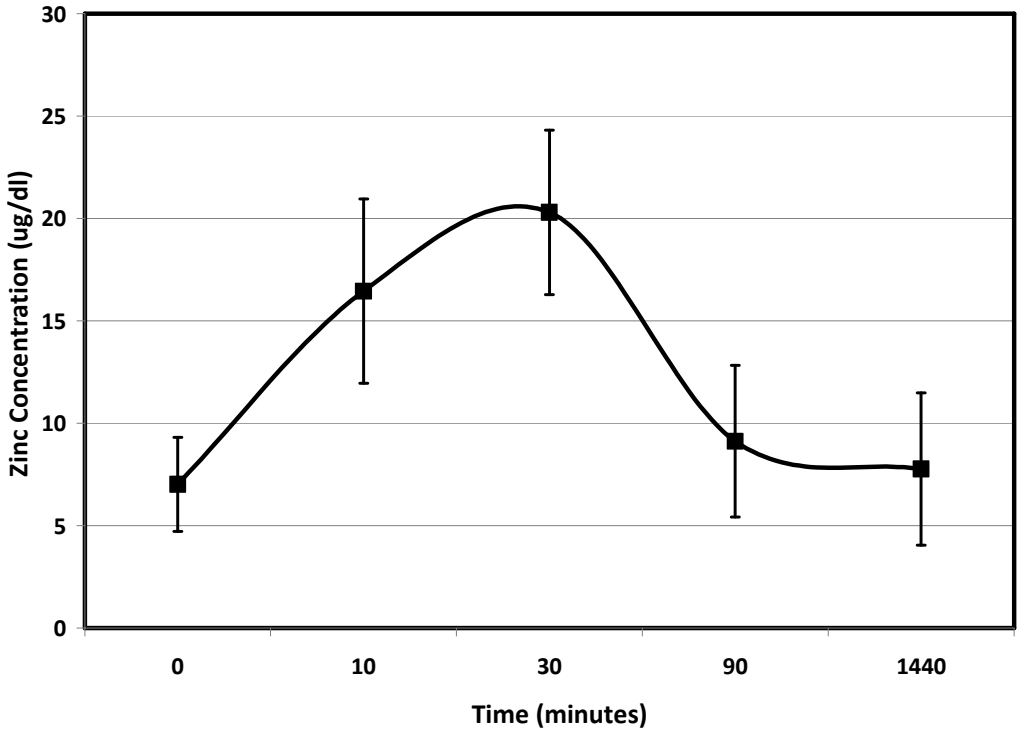

2500
2000
1500
1000
500
0
Tumor Volume (mm3)
0
2
4
6
8
10
12
14
16
18
20
Days
*
*
*

Figure 2

A


30
25
20
15
10
5
0
Zinc Concentration (ug/dl)
0
10
30
90
1440
Time (minutes)

B

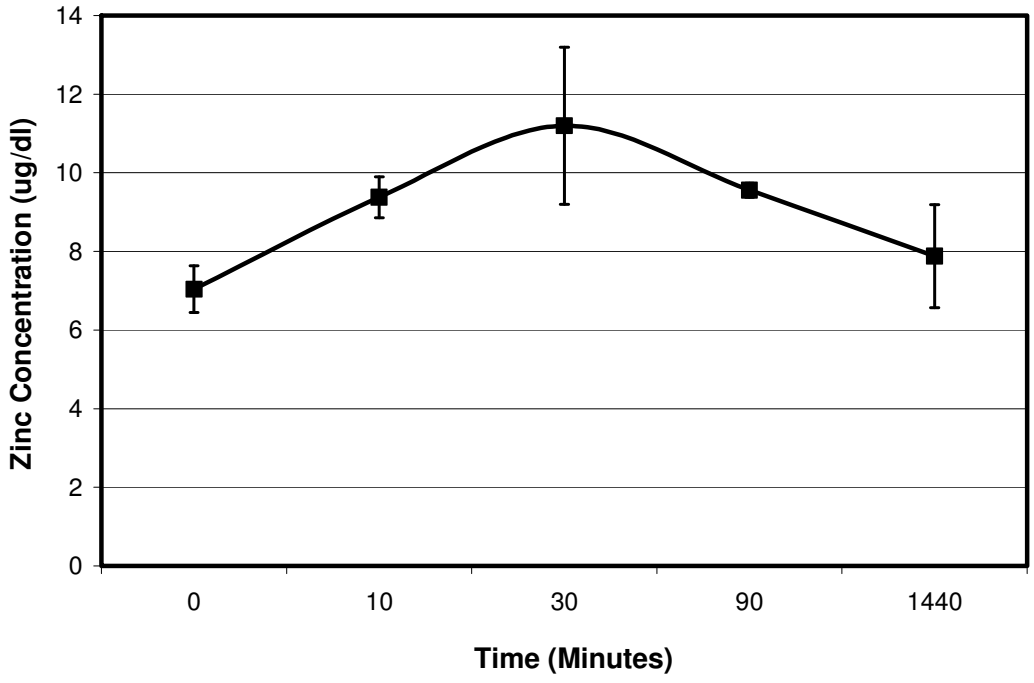
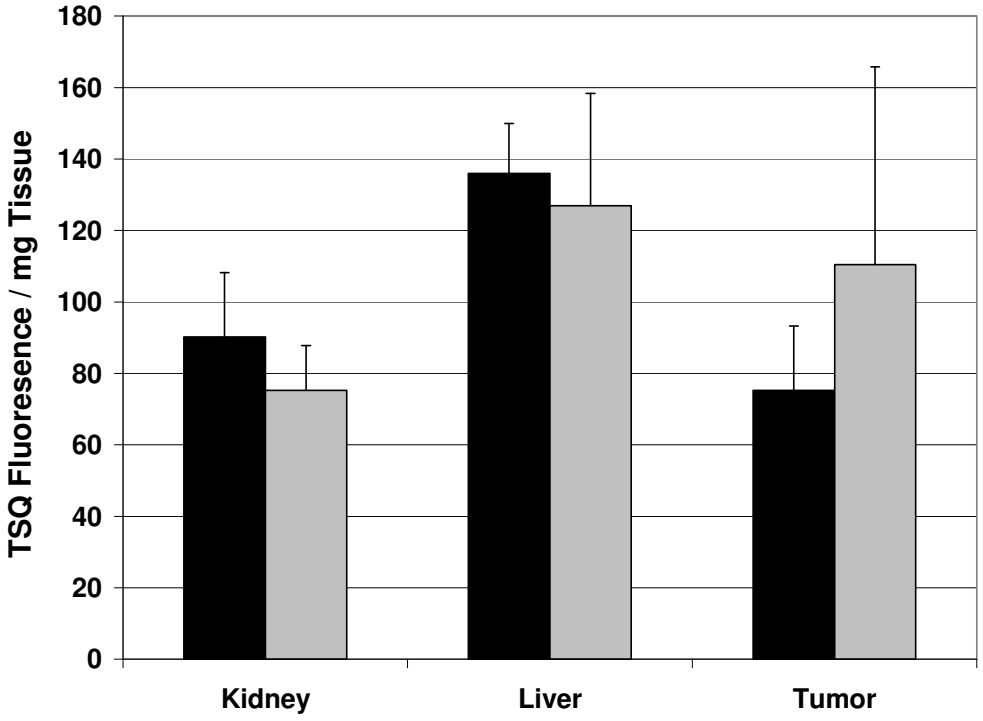

14
12
10
8
6
4
2
0
Zinc Concentration (ug/dl)
0
10
30
90
1440
Time (Minutes)

Figure 3

A


TSQ Fluoresence / mg Tissue
180
160
140
120
100
80
60
40
20
0
Kidney
Liver
Tumor

B

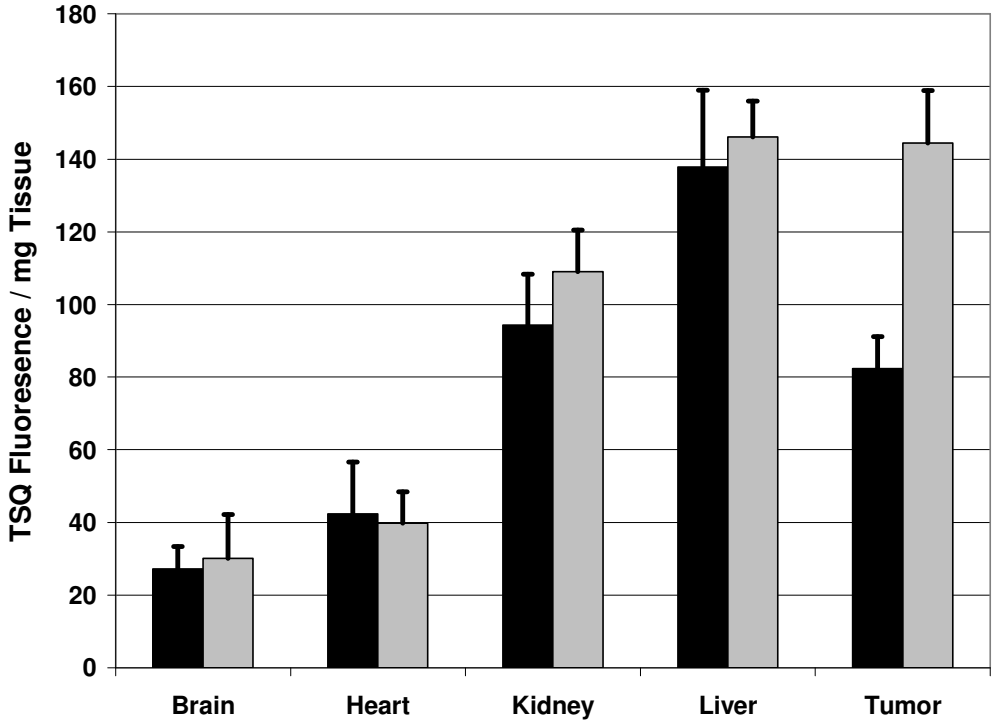

TSQ Fluoresence / mg Tissue
180
160
140
120
100
80
60
40
20
0
Brain
Heart
Kidney
Liver
Tumor

Figure 4

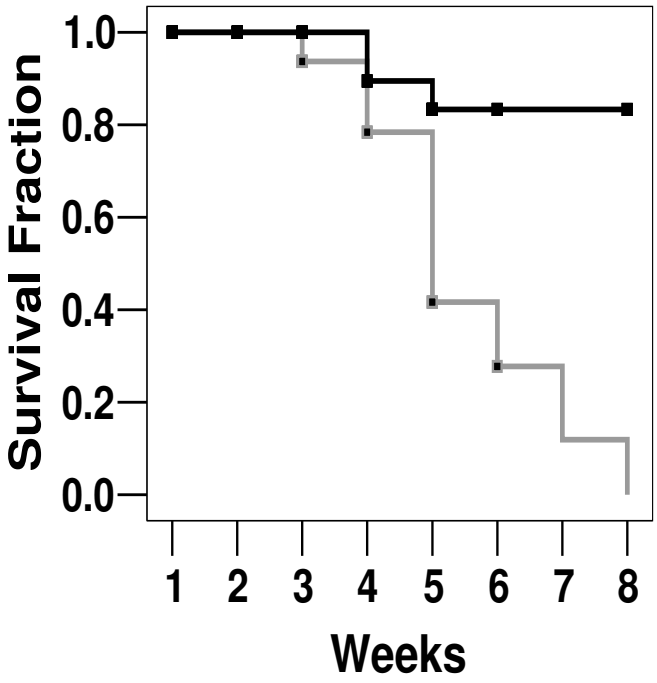


Figure 5

Journal of Experimental Therapeutics and Oncology, Vol. 0, pp. 000–000
Reprints available directly from the publisher
Photocopying permitted by license only

Zinc functions as a cytotoxic agent for prostate cancer cells independent of culture and growth conditions

Christopher L. Kriedt[1], Joseph Baldassare[2], Maulik Shah[3†] and Claudette Klein[1,*]

[1]E.A. Doisy Department of Biochemistry and Molecular Biology
[2]Department of Pharmacological and Physiological Sciences
[3]Department of Otolaryngology, St. Louis University Medical School, St. Louis, MO 63104 USA

**Correspondence to: Claudette Klein, PhD., Professor EA Doisy Department of Biochemistry and Molecular Biology, St. Louis University Medical School, 1100 S. Grand Blvd, St. Louis, MO 63104 USA Telephone: 314-968-8671. Fax: 314-977-9205. E-mail: kleinc@slu.edu*

(Received December 17, 2009; revised March 2, 2010; accepted March 3, 2010)

The effects of zinc on the viability of PC3, LNCaP and DU145 prostate cancer cell lines in vitro were examined. The data indicate that, despite their distinctly different gene expression profiles, morphology and tissue origin, all cell lines responded to zinc in a similar time and dose dependent manner. Experiments using pyrithione indicated that cell death is mediated by internalized zinc. Zinc effects on cells plated as monolayers were compared to its effects on cells plated in a collagen matrix. Although the rate of cell growth in the matrix was delayed compared to cells in 2-dimensional cultures, the cytotoxic effects of zinc were unaltered. Using both 2-dimensional and 3-dimensional cultures, we observed that zinc cytotoxicity was independent of both the culture conditions and the rate of cell growth, results that contrast the activity of the current chemotherapeutics used to treat prostate cancer. The attractive properties of zinc cytotoxicity demonstrated in this paper suggest that is can be developed as a novel and effective chemotherapeutic agent for prostate cancer treatment.

Key words: zinc, prostate, cancer, cell lines, in vitro, chemotherapy

Abbreviations: zinc, Zn; two dimensional, 2-D; three-dimensional, 3-D; extracellular matrix, ECM

INTRODUCTION

Prostate cancer affects 1 in 6 men, making it the most common non-skin cancer in America (1,2). Androgen deprivation is the most effective non-surgical therapy of localized prostate cancer. Treatments initially result in high responsiveness. However a proportion of patients develop locally advanced or metastatic disease that is refractory to anti-hormone therapy (3,4). No currently available therapy is able to cure prostate cancer once it has metastasized. We are investigating the possibility of using Zn as a novel chemotherapeutic for prostate cancer. Our previous studies using IIC9 embryonic fibroblasts, indicated that Zn possesses a number of attractive properties for its use in this regard, including its ability to kill serum arrested cells (5).

Decreased levels of Zn have been associated with the growth of prostate cancer cells (6-8). Clinical studies have attempted to evaluate the possibility that Zn replenishment could alter the growth of such cells. Individuals were given high amounts of Zn over the course of several years. In one case, an elevated risk of prostate cancer was noted, in another a decreased risk, and in another, no change (6,9,10). A limitation to these studies is the lack of evidence that oral supplementation alters Zn concentrations in the prostate. There is, however, evidence that Zn can inhibit the growth/survival of prostate cells should they become directly exposed to Zn (6,11,12). Here we examined aspects of Zn killing of PC3, LNCaP and DU145 cell lines. These cell lines are distinctly different in their morphology and tissue origin as well as in their gene expression profile for components such as the androgen receptor (13-16). These differences would simulate the genetic and phenotypic heterogeneity characteristic of prostate cancer and thus show the general applicability of a particular

treatment (13-16). Significantly, previous studies of these cell lines have demonstrated that they display differential sensitivities to currently used chemotherapeutics (16-18).

We also compared the effects of Zn on cells grown in 2-D and 3-D cultures to assess if/how the ECM and cell architecture could influence the outcome of Zn treatment. Differences in the responses to currently used anticancer drugs have been demonstrated in cells under these two types of culture systems (18-22). Given the important regulatory role of the ECM in tissue morphology and signaling, 3-D conditions are believed to more favorably model the events in early oncogenesis as they occur in tumor models (23,24). Thus, 3-D cultures allow for more facile characterization of drug properties e.g. dose-responses, than using a more complex xenograph model (23,24). The data reported here indicate that Zn is a potent cytotoxic agent when internalized and that, unlike current therapeutics, the cytoxicity of Zn is independent of cell type and growth characteristics.

MATERIALS AND METHODS

Tissues culture media was purchased from GIBCO. FCS was purchased from Sigma. All other reagents were purchased from Sigma.

Cell Lines and Culture Conditions

PC3, LNCaP and DU145 cells were maintained as exponentially growing cells in DMEM supplemented with 10% FCS. To monitor growth, cells were harvested by trypsinization at the times indicated, stained with trypan blue, and counted on a hemocytometer. Trypan blue staining confirmed that untreated cells were >95% viable. 3-D cultures were established by plating cells at the indicated densities in a collagen matrix that was established according to the published literature (25). Briefly, under sterile conditions, 0.75 ml collagen solution containing 0.7 ml twice concentrated medium, 0.26 ml H_20, 0.09 ml 0.1N NaOH, 0.2 ml FCS and 0.5 ml type I collagen (2mg/ml in 18 mM HAc) were mixed on ice and added to 35 mm Petri dishes. Then 0.25 ml of a cell suspension (0.4×10^6 cells/ml) was added to the collagen solution. To monitor growth in such cultures, the ECM was digested with collegenase and the cells retrieved by centrifugation. The cell pellet was resuspended in medium, stained with trypan blue and counted. Again, trypan blue staining confirmed 95% viability of untreated cells. All growth experiments were performed a minimum of two times, with duplicate samples.

MTT Assay

For 2-D cultures, MTT was added to the medium at a final concentration of 0.5 μg/ml. After 45 min., the media was removed, the insoluble formazan product dissolved, and the OD570 determined as previously described (5). For 3-D cultures, cells were released from the collagen matrix as described above prior to the addition of MTT. All MTT experiments were performed a minimum of three times, with triplicate samples.

RESULTS

Zn is cytotoxic to a variety of prostate cancer cell lines

The effects of Zn on the viability of PC3, LNCaP and DU145 prostate cancer cell lines are shown in Figure 1. All cell lines that were incubated with Zn for 8h (Fig. 1A) showed modest cell death at 175 μM, with about 40% death at 250 μM. With 350 μM Zn, it appeared that LNCaP cells were more susceptible to the toxic effects of Zn, however this did not hold true for longer incubation times. After 18 h, PC3 cells displayed 40% and 80% death with 175 μM and 250 μM Zn respectively (Fig. 1B). At 250 μM Zn, DU145 cells responded similarly to PC3 while LNCaP cells seemed less susceptible. However, almost complete cell death (>90%) was achieved, independent of cell line, using 350 μM Zn for 18 h. In the experiments shown, Zn was added in the form of $ZnSO_4$. Similar results were obtained when chloride, acetate or perchlorate salts were used, indicating that Zn is the active component (data not shown).

Zn cytotoxicity requires its internalization

The data indicate that all cell lines respond to Zn in a time and dose dependent manner. We chose to continue our studies with PC3 cells. When cells were incubated with pyrithione, the heavy metal ionophore, significantly lower Zn concentrations were cytotoxic. Figure 2 shows that under such conditions, approximately 25% cell death was noted after an 8-h incubation with 25 μM Zn. More dramatic responses were seen with longer incubation times, with cell death

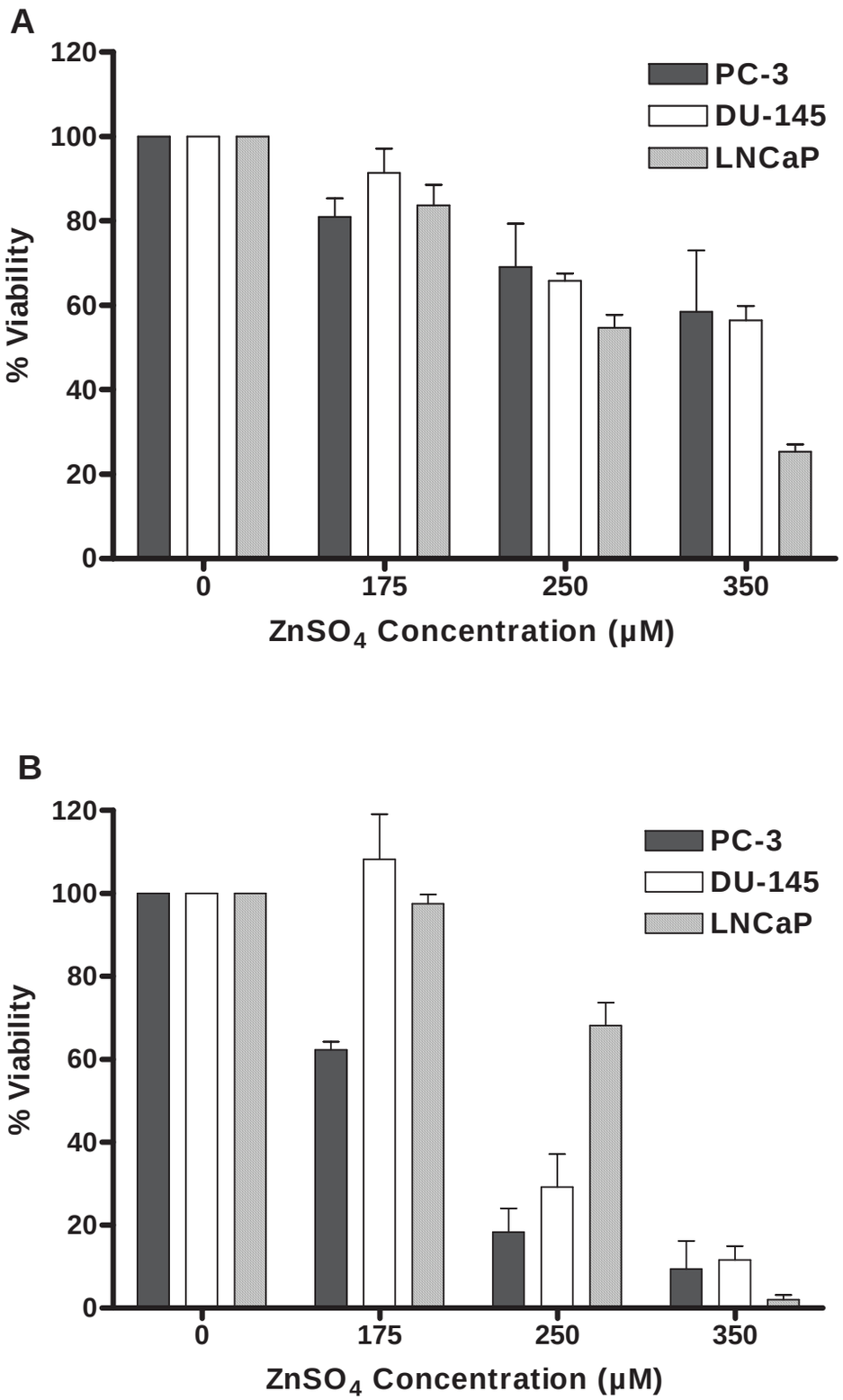


Figure 1. Effect of Zn on the Viability of Varied Prostate Cancer Cell Lines. Exponentially growing PC3, DU145 and LNCaP cells were treated with the indicated concentrations of $ZnSO_4$ for either 8 h (A) or 18 h (B). At that time, cell viability was determined by the MTT assay as described in Materials and Methods.

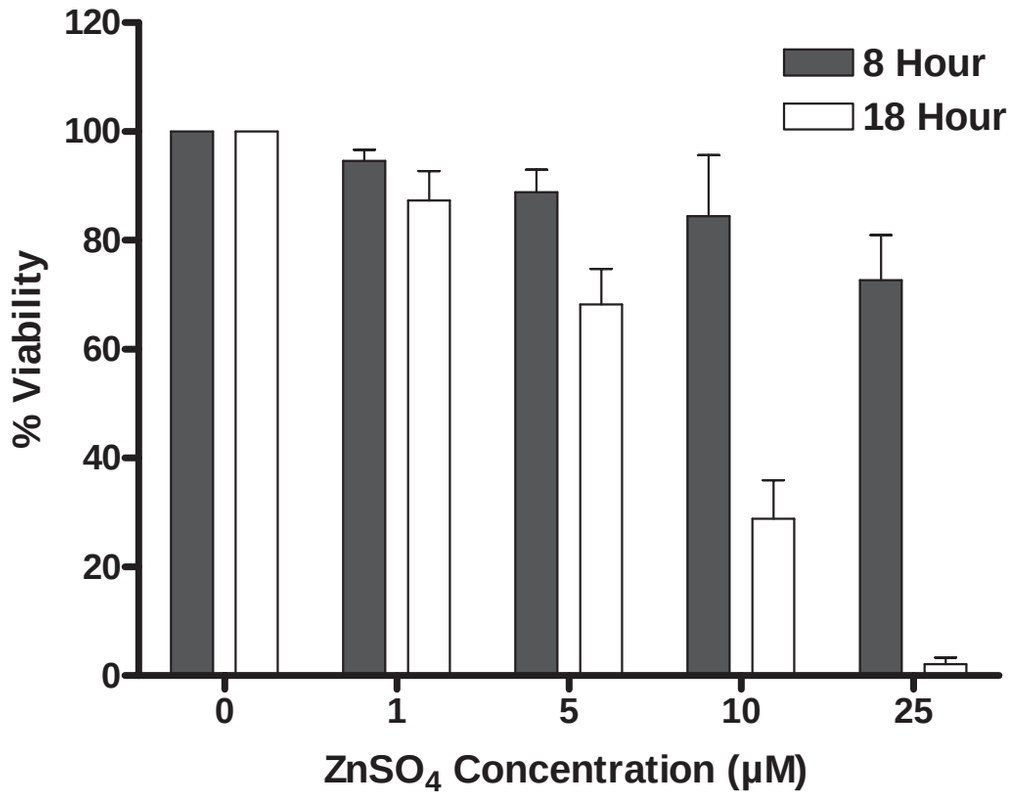


Figure 2. Pyrithione Reduces the Effective Concentration of Zn. Exponentially growing PC3 cells were incubated with the indicated concentrations of $ZnSO_4$ in the presence of 5µM pyrithione. After an 8h (filled bars) or 18 h (clear bars) incubation, cell viability was determined using the MTT assay described in Materials and Methods.

being noted with concentrations as low as 1µM Zn. Greater than 95% cell death occurred within an 18 h period using 25µM Zn in the presence of pyrithione. The data indicate that internalization of Zn is necessary for its cytotoxic effects.

Zn cytotoxicity is not affected by cell growth conditions

Although monolayer culture systems are commonly used to evaluate the cytotoxic effects of drugs, it is believed that 3-D culture systems more accurately model the behavior of cells in a tumor (19,23). With this in mind, we examined the ability of Zn to kill PC3 cells grown in a collagen ECM. First we compared the growth properties of cells in 2-D versus 3-D cultures. Figure 3A shows that cells plated in an ECM at relatively low density underwent a lag period of approximately 6-7 days, after which they doubled in approximately 3 days. Cells seeded at a higher density did not exhibit a significant lag period and displayed a doubling time of 3 days by day 2. Cells plated as a monolayer (Figure 3B) displayed similar behaviors with respect to both the initial plating densities and the presence or absence of a lag period. Cells in the 2-D culture conditions, however, did exhibit a faster doubling time, of approximately 1.5 days.

Since our goal was to compare the toxicity of Zn in monolayer and ECM cultures, we first determined if any differences could reflect the different growth rates of the cells under these two conditions. Thus, we examined the effects of Zn on cells that had reached two different cell densities; one that would test the response of cells at their maximal growth rate (8×10^5) and another when cells were nearing stationary phase (1.2×10^6). The data in Figure 4 demonstrate the results obtained after an 18-h incubation. With 3-D cultures (crosshatched and grey bars), approximately 40% cell death was seen with 175 µM Zn and approximately 75% death with 250 µM. Notably, there did not appear to be differences between the responses of cell plated at the different cell densities. Additionally, the behavior of cells plated in the ECM mimicked that seen with cells in 2-D cultures at the same densities (filled and clear bars). Thus, it appeared that neither

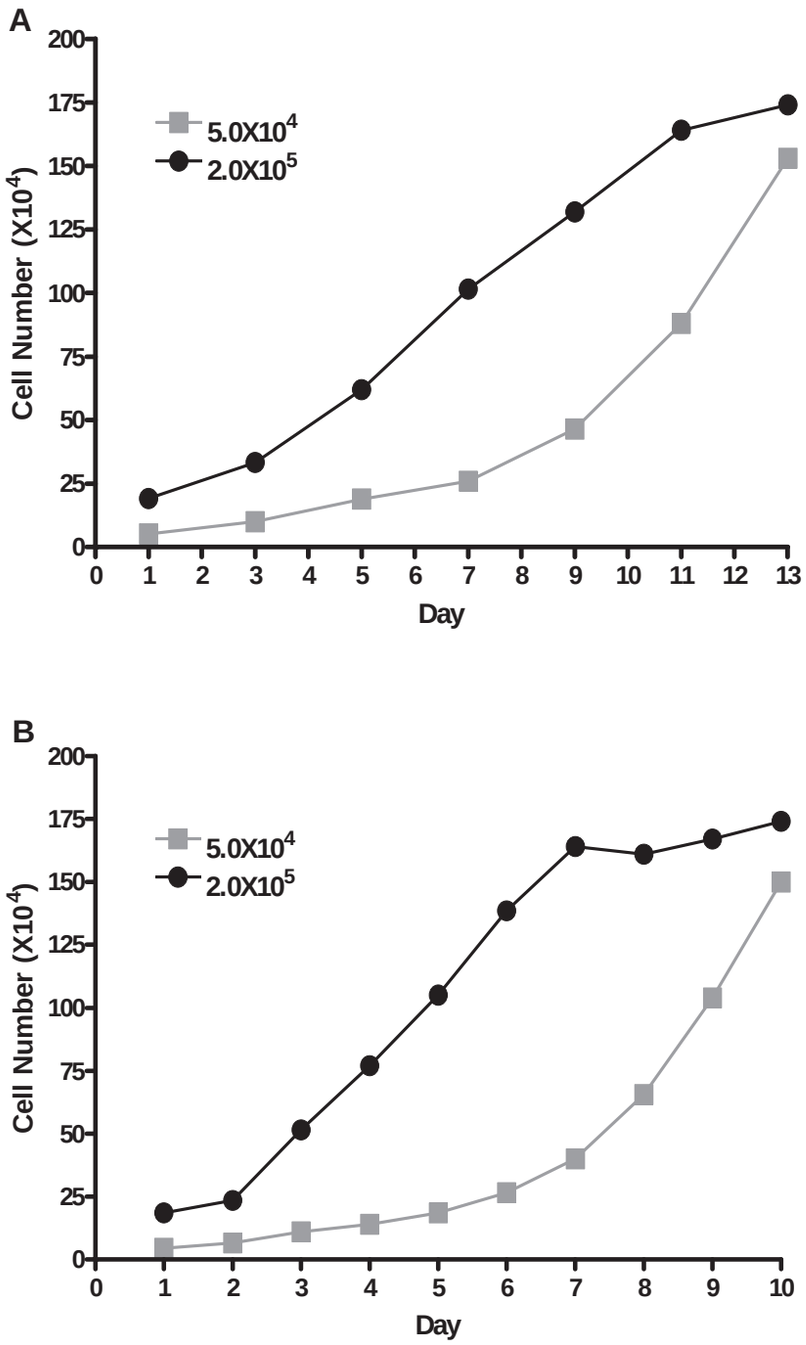


Figure 3. Effect of Culture Conditions on Cell Growth. PC3 cells were plated in an ECM (A) or as monolayers (B) at either 5 x 10^4 cells (squares) or 2 x 10^5 cells (circles). Cell growth was monitored on the indicated days as described in Materials and Methods.

the plating conditions nor the growth rate affect cell sensitivity to Zn.

To confirm that cell death in 3-D cultures required Zn internalization, we performed experiments in the presence of pyrithione. Figure 5 shows the results obtained when cells were grown in the ECM until they reached the indicated densities and then treated with Zn in the presence of pyrithione (crosshatched and grey bars). The results are compared with those obtained with 2-D cultures treated at the same cell densities (filled and clear bars). In all cases, death was observed with Zn concentrations lower than those required in the absence

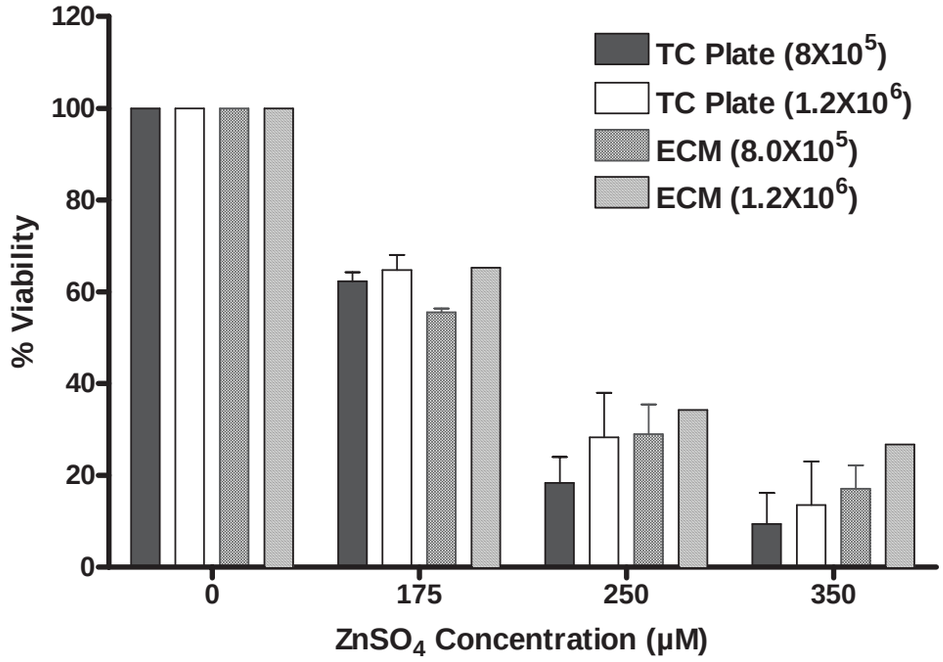


Figure 4. Culture Conditions Do Not Alter the Cytotoxic Effects of Zn. PC3 cells were plated as 2-D cultures (filled and clear bars) or as 3-D cultures (crosshatched and grey bars). When cells had achieved a level of either 8 x 10^5 (filled and crosshatched bars) or 1.6 x 10^6 (clear and grey bars), the indicated concentrations of $ZnSO_4$ were added. After an 18 h incubation, cell viability was determined using the MTT assay as described in Materials and Methods.

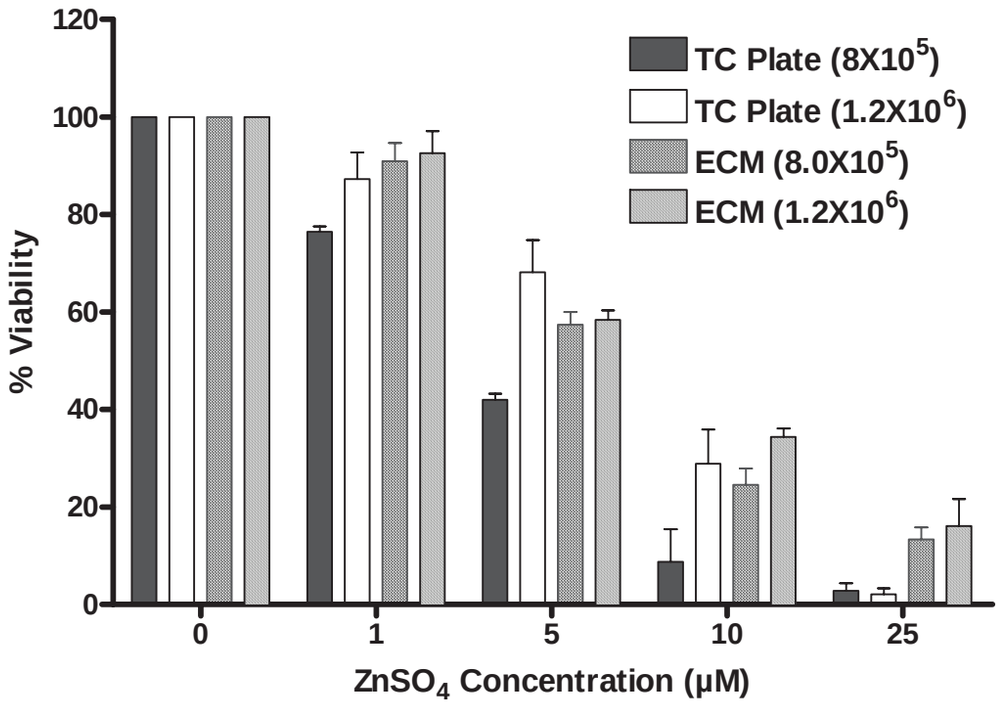


Figure 5. Intracellular Zn Mediates Cell Toxicity Under All Culture Conditions. PC3 cells were plated as 2-D cultures (filled and clear bars) or as 3-D cultures (crosshatched and grey bars). When cells had achieved a level of either 8 x 10^5 (filled and crosshatched bars) or 1.6 x 10^6 (clear and grey bars), 5µM pyrithione and the indicated concentrations of $ZnSO_4$ were added. After an 18 h incubation, cell viability was determined using the MTT assay as described in Materials and Methods.

of pyrithione. A slightly greater sensitivity of the lower density 2-D cells was observed in this experiment but, in general, cell death was detectable with 1 μM Zn and almost complete with 25 μM Zn under all culture conditions.

DISCUSSION

The attractive properties of Zn cytotoxicity demonstrated in this paper suggest that is can be developed as a novel and effective chemotherapeutic agent for prostate cancer treatment. One attractive feature of this reagent is that cell death is achieved rapidly, with a time-course of hours, as opposed to days/weeks needed with other therapeutics. For example, Zoledronate and Docetaxel, currently used chemotherapies for prostate cancer, result in approximately 60% death of PC3 cells after a 72 h incubation (26), compared with the > 95% cell death with Zn after an 18 h incubation. Another attractive feature is that the effectiveness of Zn is not altered by the cells micro-environment: Whether cells were tested in 2-D or in 3-D cultures, they responded similarly to Zn treatment. 3-D cultures have provided some important insights into the behavior of cancer cells, including cellular proliferation, differentiation, and gene expression and the role of intercellular and cell-ECM interactions in these processes. Comparisons of these two types of culture systems have shown critical differences in the behavior of cells and, importantly, in their responses to currently used anticancer drugs (18-22). This contrasts with the relatively uniform responses to Zn that we observed in PC3 cells under varied culture conditions, and, more recently, in a xenograph model (12). That the cytotoxic effects of Zn appear independent of the model system suggests that other features e.g. synergy with other therapeutics, its molecular mechanism of action, can be readily studied in the simpler and more defined conditions of 2-D cultures.

Using cells grown in ECM and as monolayers, we were also able to demonstrate that the potency and efficacy of Zn killing was not influenced by the growth rate of the cells. PC3 cells grow twice as slowly in the ECM as when tested as monolayers, yet they show the same dose and time dependence for Zn killing. By using cells near/at stationary phase to even further limit their growth rate, we observed that the time and dose dependency of Zn killing was still unaltered in either culture condition. Thus the rate of cancer cell growth is not a factor in determining the outcome of Zn treatment, a result that contrasts the activity of many of the current chemotherapeutics directed toward rapidly growing cells. This is of particular interest in the treatment of prostate cancer, which is often a more slowly growing cancer.

Another attractive feature of Zn treatment is that it appears to be equally effective against a variety of prostate cancer cells. Here we have examined its potency and efficacy in killing three different, well-studied prostate cancer cell lines, PC3, DU145, and LNCaP. These cell lines are distinctly different in their gene expression profile for components such as the androgen receptor and psa. They also differ in their intrinsic apoptotic and thioredoxin pathways, as well as in their morphology and tissue origin (13-16). These differences would simulate the genetic and phenotypic heterogeneity characteristic of prostate cancer (13-16) and thus show the general applicability of a particular treatment. Previous studies of these cell lines have demonstrated that they display differential sensitivities to currently used chemotherapeutics. Cisplatin treatment of PC3 and LNCaP results in approximately 10% cell death while similar treatment results in approximately 30% death of DU145 cells (20). PC3 and DU145 cells are significantly more resistant to doxorubicin and taxol treatment than are LNCaP cells (17,27), while DU145 cells are the most sensitive to vorinostat, followed by LNCaP cells, with PC3 cells being resistant to such treatment (15). In contrast, all of the cell lines displayed similar sensitivities to Zn, and did so in relatively short time periods. Given the role of p53 in determining drug sensitivity (16,28-31), it is also of interest to note that the similar responsiveness of LNCaP (p53 wild type), DU154 (p53 mutant) and PC3 (p53 null) cells indicate that the cytotoxic effects of Zn are independent of p53 status.

Although the concentrations of Zn necessary to induce cell death within an 18-h period appear somewhat high, it is clear from our experiments using pyrithione that this reflects the concentrations necessary to allow for sufficient Zn uptake. This interpretation is consistent with the findings of Albrecth et al (2008) who, upon incubating these different cell lines with 75 μM Zn for 24 h, showed no significant change in their Zn content. Thus, higher external concentrations are necessary to change the intracellular levels of Zn. Notably, in agreement with our data, Albrecth et al (2008) observed no loss of cell viability under conditions where no change in intracellular Zn concentrations occurred. It is also of interest to note that, under normal conditions, cells of the prostate gland contain high levels of Zn, much of which is present in the cytosol where it is believed to be bound to citrate (6). A general finding in the case of prostate cancer is that Zn homeostasis is altered such that the cellular content of Zn is significantly reduced (6). The reasons behind the reduced levels of cellular Zn are not yet known but our data suggest that conditions that allow for those levels to increase can result in an effective cancer treatment. In our experiments, Zn uptake was facilitated by the presence of the heavy

metal ionophore, pyrithione. Under those conditions, Zn concentrations could be dramatically reduced, over 100-fold to low μM concentrations, to achieve almost complete cell death in 18 h. We are currently developing alternative conditions/vehicles to facilitate Zn delivery into a cell and evaluate its effectiveness as a cytotoxic agent.

CONCLUSIONS

The attractive properties of Zn cytotoxicity demonstrated in this paper suggest that Zn can be developed as a novel and effective chemotherapeutic agent for prostate cancer. One attractive feature of this reagent is that cell death is achieved rapidly, with a time-course of hours, as opposed to days/weeks needed with other therapeutics. Additionally, the potency and efficacy of Zn killing is not influenced by the growth rate of the cells, a feature of particular interest in light of the fact that often times, prostate cancers are slow growing. Another attractive feature of Zn treatment is that, in contrast to other chemotherapeutics, it appears to be equally effective against a variety of prostate cancer cells and not influenced by the growth conditions. Relatively low doses of Zn are required under conditions that facilitate Zn internalization, suggesting that its use as a therapeutic would unlikely have adverse side-effects once conditions/vehicles that assure its uptake by the cells are developed.

ACKNOWLEDGEMENTS

This research was supported by a grant from the DOD.

REFERENCES

1. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. *CA Cancer J Clin* 50, 7–33, 2000.
2. Wingo PA, Landis S, Ries LA. An adjustment to the 1997 estimate for new prostate cancer cases. *Cancer* 80, 1810–1813, 1997.
3. Paule B. Reappraisal of the concept of hormone therapy in metastatic prostate cancer and implications for treatment. *Eur Urol* 47, 729–735, 2005.
4. Rau KM, Kang HY, Cha TL, Miller SA, Hung MC. The mechanisms and managements of hormone-therapy resistance in breast and prostate cancers. *Endocr Relat Cancer* 12, 511–532, 2005.
5. Klein C, Creach K, Irintcheva V, Hughes K, Blackwell P, Corbett J, Baldassare J. Zinc induces ERK-dependent cell death through a specific Ras isoform. *Apoptosis* 11(11): 1933–1944, 2006.
6. Costello LC, Feng P, Milon B, Tan M, Franklin RB. Role of zinc in the pathogenesis and treatment of prostate cancer: critical issues to resolve. *Prostate Cancer Prostatic Dis* 7, 111–117, 2004.
7. Franklin RB, Milon B, Feng P, Costello LC. Zinc and zinc transporters in normal prostate and the pathogenesis of prostate cancer. *Front Biosci* 10, 2230–2239, 2005.
8. Huang L, Kirschke CP, Zhang Y. Decreased intracellular zinc in human tumorigenic prostate epithelial cells: a possible role in prostate cancer progression. *Cancer Cell Int* 6, 10, 2006.
9. Chang ET, Hedelin M, Adami, HO, Gronberg H, Balter KA. Re: Zinc supplement use and risk of prostate cancer. *J Natl Cancer Inst* 96, 1108–1109, 2004.
10. Jarrard DF. Does zinc supplementation increase the risk of prostate cancer? *Arch Ophthalmol* 123, 102–103, 2005.
11. Liang JY, Liu YY, Zou J, Franklin R, Costello L, Feng P. Inhibitory effect of zinc on human prostatic carcinoma cell growth. *The Prostate* 40(3): 200–207, 1999.
12. Shah M, Kriedt C, Lents N, Hoyer M, Jamaluddin N, Klein C, Baldassare J. Direct intra-tumoral injection of zinc-acetate halts tumor growth in a xenograft model of prostate cancer. *J Exp Clin Cancer Res* 28(1): 84, 2009.
13. Skjøth IH, Issinger OG. Profiling of signaling molecules in four different human prostate carcinoma cell lines before and after induction of apoptosis. *Int J Oncol* 28(1): 217–29, 2005.
14. Stewart D, Cooper C, Sikes R. Changes in extracellular matrix (ECM) and ECM-associated proteins in the metastatic progression of prostate cancer. *Reprod Biol Endocrinol* 2, 2, 2004.
15. Xu W, Ngo L, Perez G, Dokmanovic M, Marks P. Intrinsic apoptotic and thioredoxin pathways in human prostate cancer cell response to histone deacetylase inhibitor. *Proc Natl Acad Sci USA* 103(42): 15540–15545, 2006.
16. Serafin A, Akudugu J, Bohm L. Drug resistance in prostate cancer cell lines is influenced by androgen dependence and p53 status. *Urol Res* 30(5): 289–294, 2002.
17. Geldof A, Mastbergen S, Henrar R, Faircloth G. Cytotoxicity and neurocytotoxicity of new marine anticancer agents evaluated using in vitro assays. *Cancer Chemother Pharmacol* 44(4):312–8, 1999.
18. Chung LW, Baseman A, Assikis V, Zhau HE. Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *J Urol* 173(1): 10–20, 2005.
19. Doillon CJ, Gagnon E, Paradis R, Koutsilieris M. Three-dimensional culture system as a model for studying cancer cell invasion capacity and anticancer drug sensitivity. *Anticancer Res* 24(4): 2169–77, 2004.
20. Xu F, Burg K. Three-dimensional polymeric systems for cancer cell studies. *Cytotechnology* 54(3): 135–143, 2007.
21. Faute MA, Laurent L, Ploton D, Poupon MF, Jardillier JC, Bobichon H. Distinctive alterations of invasiveness, drug resistance and cell–cell organization in 3D-cultures of MCF-7, a human breast cancer cell line, and its multidrug resistant variant. *Clin Exp Metastasis* 19(2): 161–167, 2002.

22. O'Connor KC. Three-Dimensional Cultures of Prostatic Cells: Tissue Models for the Development of Novel Anti-Cancer Therapies. *Pharm Res* 16(4): 486–93, 1999.

23. Hoffman RM. To do tissue culture in two or three dimensions? That is the question. *Stem Cells* 11(2): 105–11, 1993.

24. Pampaloni F, Reynaud E, Stelzer E. The third dimension bridges the gap between cell culture and live tissue. *Nature Rev Mol Cell Biol* 8, 839–845, 2007.

25. Bell E, Ivarsson B, Merrill C. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc Natl Acad Sci USA* 76:1274–1278, 1979.

26. Ullén A, Lennartsson L, Harmenberg U, Hjelm-Eriksson M, Kälkner KM, Lennernäs B, Nilsson S. Additive/synergistic antitumoral effects on prostate cancer cells in vitro following treatment with a combination of docetaxel and zoledronic acid. *Acta Oncol* 44(6): 644–50, 2005.

27. Theyer G, Schirmböck M, Thalhammer T, Sherwood ER, Baumgartner G, Hamilton G. Role of the MDR-1-encoded multiple drug resistance phenotype in prostate cancer cell lines. *J Urol* 150(5 Pt 1):1544–7, 1993.

28. Giovannetti E, Backus HH, Wouters D, Ferreira CG, van Houten VM, Brakenhoff RH, Poupon MF, Azzarello A, Pinedo HM, Peters GJ. Changes in the status of p53 affect drug sensitivity to thymidylate synthase (TS) inhibitors by altering TS levels. *Brit J of Cancer* 96(5):769–775, 2007.

29. Xue C, Haber M, Flemming CM, Marshall G, Lock R, MacKenzie K, Gurova K, Norris M, Gudkov A. p53 Determines Multidrug Sensitivity of Childhood Neuroblastoma. *Cancer Res* 67, 10351, 2007.

30. Zhang Z, Li M, Wang H, Agrawal S, Zhang R. Antisense therapy targeting MDM2 oncogene in prostate cancer: Effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. *Proc Natl Acad Sci USA* 100(20):11636–11641, 2003.

31. Fojo T. p53 as a therapeutic target: unresolved issues on the road to cancer therapy targeting mutant p53. *Drug Resistance Updates* 5(5): 209–216, 2002.

32. Albrecht AL, Somji S, Sens MA, Sens DA, Garrett, SH. Zinc transporter mRNA expression in the RWPE-1 human prostate epithelial cell line. *Biometals* 21(4):405–16, 2008.