AWARD NUMBER: W81XWH-13-1-0386

TITLE: Imaging Prostate Cancer (PCa) Phenotype and Evolution

PRINCIPAL INVESTIGATOR: Jason A. Koutcher

CONTRACTING ORGANIZATION: Sloan Kettering Institute for Cancer Research
                                 New York, NY 10065

REPORT DATE: October 2015

TYPE OF REPORT: Annual Report

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

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
October 2015	Annual	30 Sep 2014 - 29 Sep 2015

4. TITLE AND SUBTITLE

Imaging Prostate Cancer (PCa) Phenotype and Evolution

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0386

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

E-mail: koutchej@mskcc.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH
New York, New York 10065

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal of the project is to investigate the potential of inhibiting iron metabolism to inhibit prostate cancer growth. Specifically, we will study Deferiprone, an iron chelator, and focus on its effect on aconitase in prostate tumors. It has been shown that changes in citrate metabolism at the level of mitochondrial aconitase, is an early change in carcinogenesis in the prostate. This change in metabolism is detectable by magnetic resonance. The project includes both in vitro and in vivo studies to determine its potential utility for clinical translation. Our findings to date include that deferiprone in 4 cell lines has an IC50 (inhibitory concentration for 50% of cells) of about 50uM (typical serum levels after standard dose = 100uM), inhibits tumor growth in vivo in 2 transgenic tumor models, and results in decreased tumor iron concentration as measured by MRI. We have had difficulty with monitoring in vivo glucose metabolism and thus switched to using iron imaging as a non-invasive surrogate for monitoring DFP activity. In year 3 we will finish developing and evaluating aconitase knockdown cell lines to test the hypothesis that DFP's iron chelating activity decreases activity and expression of aconitase.

15. SUBJECT TERMS					
Deferiprone, iron, prostate cancer, metabolism, magnetic resonance					

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	**b. ABSTRACT** Unclassified	**c. THIS PAGE** Unclassified	Unclassified	10	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS:

1. **Introduction:** Normal prostate peripheral tissue has low mitochondrial aconitase (m-Acon) activity. This has been associated with zinc-induced inhibition of m-Acon in the peripheral epithelial cells. Activation of m-Acon is an early biochemical change during prostate cancer development and has been associated with a down-regulation of zinc transporters. This leads to a shift from citrate-producing to a citrate-oxidizing malignant phenotype, which has been extensively observed in different human prostate cancer cell lines. Thus, in the clinical setting, high levels of citrate are typically observed in normal prostate epithelia while essentially nondetectable in prostate cancer tissue, particularly in high grade tumors.

Aconitase is an iron-sulphur enzyme that catalyzes the two-step isomerization of citrate to isocitrate, and has two isoforms: m-Acon (mitochondrial aconitase) and c-Acon (cytoplasmic; additionally functions as iron regulatory protein 1, when the iron levels in the cell become low). The unique $[Fe_4S_4]^{2+}$ cluster of aconitase reacts directly with the substrate and contains a labile iron atom, which must be replaced occasionally. Thus, when the iron levels in the cell become low, the cluster cannot be regenerated and the enzyme becomes inactive: $[Fe_3S_4]^+$. In fact, iron is present in many enzymes and is essential for cancer metabolism, cell growth, and proliferation and thus has been suggested as a potential chemotherapeutic target. Iron is present in many enzymes, in addition to aconitase, and thus this is not the only potential mechanism but we focused on aconitase because of its importance in prostate cancer. It is noted that aconitase is only one of multiple iron containing enzymes that could be effected by changes in iron concentration

Deferiprone (DFP) is an orally available iron chelator used in the clinic, primarily for the treatment of thalassemia and Friedreich ataxia. DFP readily enters cells and reaches the major intracellular sites of iron accumulation. In addition, this agent has been shown to impair the activity of m-Acon by removing iron from the mitochondria, and also to inhibit growth of some but not all cell lines.

2. Keywords: Deferiprone, aconitase, metabolism, tricarboxylic acid cycle, magnetic resonance

3. Accomplishments:

Major Goals:

Aim 1. Determine if m-acon shRNA knockdown and inhibition with Deferiprone, an m-acon inhibitor, decreases murine PCa TRAMP C2 and MycCaP cell proliferation, migration, and invasiveness. Determine if knockdown of m-acon and Deferiprone inhibit TCA cycle activity, glycolysis and lactate, and increase citrate levels in murine PCa TRAMP C2 and MycCap cells.

Aim 2A: Determine *in vivo,* if orthotopic tumors formed from shRNA-m-acon transfected TRAMP C2 and MycCap cells, or wild type (WT) tumors treated with Deferiprone, have slower growth, than control tumors (derived from cells with scrambled shRNA or untreated WT cells).
Aim 2B: Using MRS, determine *in vivo* if orthotopic PCa tumors formed from shRNA-m-acon transfected TRAMP C2 and MycCap cells, or wild type (WT) tumors treated with Deferiprone, have higher levels of citrate and lower levels of choline and lactate than control tumors.
What was accomplished: Aim 1 – we have shown that treatment with DFP decreases cell proliferation and migration and inhibits TCA cycle (metabolism). Similarly in vivo (Aim 2), we

have shown that DFP inhibits tumor growth. We were not able to monitor metabolism in vivo but since this was a surrogate, we are measuring the effects of DFP by measuring iron metabolism/concentration in tumors. This does not represent a change in goal but a change in methodology since the goal remains to develop a non-invasive imaging method to monitor the effect of DFP. We have demonstrated that in the MycCaP, treatment with DFP decreases tumor iron concentration. Studies in the TRAMP C2 are pending. Details are provided below. We have not yet done the studies with the shRNA and are still developing those cells. Thus Aims 1 and 2 are about 60% complete

Year 1: Accomplishments in Year 1 (summarized previously) included demonstration that DFP inhibited prostate cancer cell growth at low (clinically achievable) concentrations. DFP had a growth inhibitor effect in both TRAMP C2 and Myc-Cap cell lines after a two day incubation period. The half maximal inhibitory concentration (IC_{50}) of DFP was about 50 uM in both cell lines. Deferiprone was also shown to inhibit cell migration in TRAMP C2 cells. Metabolic changes associated with cell growth inhibition during the first 24h of exposure to DFP were measured using our cell perfusion system. The most important findings were 1) DFP led to a 6-fold decrease in $1\text{-}^{13}C$-glucose uptake from the extracellular medium after 5h perfusion, 2) DFP essentially blocked the synthesis of $4\text{-}^{13}C$-glutamate after 10 h exposure which would be expected if it inhibited aconitase activity or expression. On the other hand, no changes were detected at any time in the rate of incorporation of $2\text{-}^{13}C$-acetate (derived from ^{13}C-labeled citrate exported to the cytosol) into fatty acids, which would be expected if aconitase was inhibited.

Table 1: IC50 Measurements	
Cell lines	IC 50 value
Myc-CaP	54.41± 2.9
TRAMP-C2	50.68 ± 1.4
Myc-CaP/CR	52.41 ±3.4
TRAMP-C2 5 NC	54.08 ±1.9

In Year 2 we focused on in vivo studies of the effect of DFP on prostate tumor growth although we also performed some in vitro studies. We combined docetaxel with DFP (in vitro) which doubled the sensitivity of MycCaP cells to docetaxel (IC_{50} decreased from 44.7±2.6 to 24.0 ± 3.1 nM), suggesting its potential use in enhancing cytotoxic therapies (subtask 9). Similar and more impressive results were also found in breast cancer cell lines which are being pursued separately.
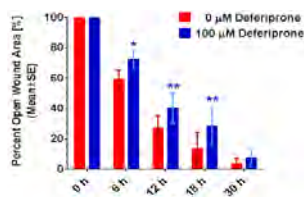

Fig. 1. Effect of 100 uM DFP on migration of TRAMP C2 cells. *= p<0.05;**=p<0.01

We expanded our in vitro study to 2 additional cell lines, recognizing the need to study castrate resistant prostate cancer (CRPC) cell lines. We obtained from Roswell Park (Dr. Leigh Ellis) a castrate resistant PCa cell line derived from the MycCaP cell (MycCaP/CR). Both the MycCaP/CR and the human CRPC cell line (22rv1) had IC50 values of approximately 50 uM after 48 hours exposure to DFP, similar to the MycCaP and TRAMP C2 (Table 1).

Figure 1 shows   the effect of 100uM on migration of TRAMP C2 cells and shows a statistically significant effect on migration inhibition. The test was done by performing a "scratch" on an almost confluence plate of cells and determining the rate at which the "scratch" is closed and demonstrates that cells migrate more slowly during treatment with DFP. Similar results were obtained with the MycCaP tumor cells (subtask 9).

We are in the process of generating aconitase knockdown tumor cells and have made significant progress and are now obtaining single cell clones for expansion (subtask 3) and study. These has been somewhat of a bottleneck but we have made progress in this area over the last few months.

In addition to the above in vitro studies, ongoing in vitro studies include a) studies of the effect of DFP on cell migration in the cell lines noted in Table 1 and Western blots on the effect of DFP on these cell lines.

The Effect of DFP on PCa Growth In Vivo: Previous studies have used DFP at a dose of 24 mg/kg/day to 150 mg/kg twice daily and also 200mg/kg. We chose a conservative dose of 150 mg/kg/day by gavage. Tumor bearing mice were treated with DFP or saline chronically until tumors became too large and mice required sacrifice.   Since tumors were implanted orthotopically we measured tumor volumes by MRI weekly.
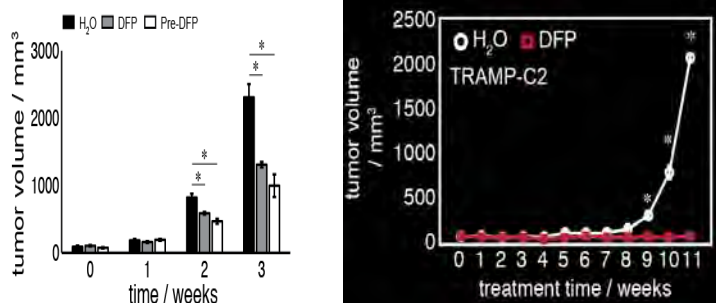
Fig. 2: Effect of DFP on in vivo growth of MycCaP (left) and TRAMP C2 tumors. By weeks 2 and 3, tumor growth of the MycCaP treated with DFP is significantly slower than control. *=$p<0.05$. In the TRAMP C2 (right), no tumor growth is noted over 11 weeks treatment with DFP. *=$p<0.001$.

Figure 2 demonstrates the effect of chronic DFP administration on tumor growth. Significant decreases in tumor growth were noted in both the MycCaP and TRAMP C2. In the MycCaP tumor, we studied the effect of DFP administered after tumor inoculation and also the effect of DFP administered as a chronic therapy before tumor administration but no differences in effect were noted. By two weeks of DFP therapy in MycCaP tumors, there was a significant decrease in tumor growth rate, which was even greater by three weeks (subtasks 14-18).

The effect of DFP on TRAMP C2 was extremely impressive. Tumors were monitored for up to 11 weeks at which point control mice had tumors that required sacrifice so the study was terminated. Almost all the TRAMP C2 tumors treated with DFP failed to show any growth.

Figure 3. FeMRI as a label-free probe of systemic Fe(III). (a) T_2^*-weighted MRI of 0-0.3 mg $Fe(NO_3)_3$ g^{-1} H_2O. (b) R_2^*=$1/T_2^*$ vs. mg Fe(III) g^{-1} with linear best fit (n=3 phantoms, +/- s.e.m.). (c) [Fe(III)] parametric map from MRI of the phantom in (a) Dashed circle shows position of H_2O sample, color bar corresponds to mg Fe(III) g^{-1}

While DFP was effective in both tumors, clearly the efficacy was far greater in the TRAMP C2 (Fig. 2 (right)). The investigators feel that the difference in effect between these two cell lines is an important issue and needs to be pursued and preliminary studies are planned, in addition to plans to obtain funding to continue this avenue of research.

In vivo metabolism studies have been challenging. While the in vitro studies performed in Year 1 were very informative, signal to noise was a major issue in vivo. We attempted to test this initially by studying tumors that were injected subcutaneously wherein signal to noise is much higher since the tumor is directly in the radiofrequency coil. This was only marginally successful in that we could generate data with modest/adequate signal to noise but could not demonstrate an effect with DFP. We thus undertook an alternative approach to mapping the effects of DFP (subtasks 16A, 17A, 18A) in orthotopic tumors as proposed in the application.
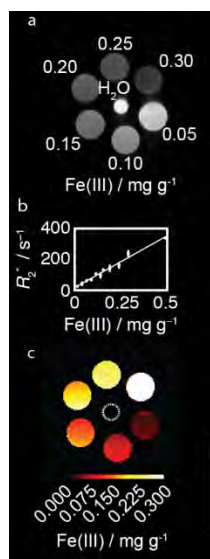
The goal of the metabolism studies was to provide a non-invasive surrogate method to monitor the drug target to explain the successes and failures of novel drugs and optimize dose and schedule. MRI is used clinically to measure liver and heart iron concentrations (1-3); we hypothesize that DFP's mechanism of action is to deplete the tumor of iron. Decreases in tumor iron concentration induced by DFP are expected to be detectable by MRI using spin echo T2 (spin-spin relaxation), gradient echo T2* (apparent T2), and other methods. We are also hypothesizing that DFP could work on tumor cells, tumor associated macrophages, or both types of cells. Current methods to non-invasively assess and monitor tumor associated macrophages (TAM) infiltration by MRI is limited by the contrast agent itself due to variable cell uptake (4-6), limited lifetime of cellular label (<1 week) (7-8), and because exogenous iron contrast agents influence TAM phenotype and infiltration because of the dependence of macrophages on iron (9). These problems are likely to be true in tumor cells also. Iron particles alter macrophage function since they induce ferritin and immunological alterations (10,11). Thus a non-perturbing, non-invasive method to measure iron in tumors would be important in developing and optimizing schedules for DFP and similar to the metabolic studies, could be a non-invasive surrogate to measure the target of DFP.

We performed studies on phantoms of iron solutions of different concentration to derive an iron calibration curve for T2* images (Fig. 3). This provided a calibration allowing us to relate signal intensity on a T2* weighted image to tissue iron content. Figure 4 shows images obtained of MycCaP orthotopic tumor bearing mice. On the left is the control mouse and on the right a mouse treated w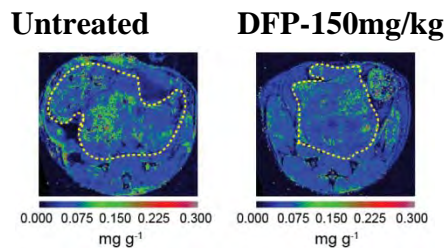ith DFP. Regions of high R2* (1/T2*) corresponding to regions of high iron are reduced in the tumor in the DFP treated mice. Our preliminary studies (not shown) suggest that this is due to less macrophage accumulation which we are studying. Studies with TRAMP C2 are ongoing (Tasks 15, 16A)

Currently we are writing up both the in vitro and in vivo results although a few more experiments are in progress and required prior to submission

Untreated　　**DFP-150mg/kg**

Fig. 4. R2* images of MycCaP tumors. Yellow line delineates the tumor. Left: untreated. Right: treated with DFP. Notice that in the untreated animal there are regions of high R2* (high iron) due to presence of iron. Areas of high R2*(high iron) are less prevalent in treated group.

References:

1. St. Pierre, T. G., Clark, P. R. & Chua-Anusorn, W. Measurement and mapping of liver iron concentrations using magnetic resonance imaging. *Ann. N. Y. Acad. Sci.* **1054**, 379-385 (2005).

2. Garbowski, M. W. *et al.* Biopsy-based calibration of T2[*] magnetic resonance for estimation of liver iron concentration and comparison with R2 Ferriscan. *J. Cardiov. Magn. Reson.* **16** (2014).

3. Fischer, R. & Harmatz, P. R. Non-invasive assessment of tissue iron overload. *Hematology* **2009 No. 1**, 215-221 (2009).

4. Daldrup-Link, H. E. *et al.* Imaging of tumor associated macrophages with clinically-applicable iron oxide nanoparticles. *Clin. Cancer Res.* **17**, 5695-5704 (2011).

5. Narunsky, L., Oren, R., Bochner, F. & Neeman, M. Imaging aspects of the tumor stroma with therapeutic implications. *Pharmacol. Ther.* **141**, 192-208 (2014).

6. Weissleder, R., Nahrendorf, M. & Pittet, M. J. Imaging macrophages with nanoparticles. *Nat. Mater.* 13, 125-138 (2014).

7. Bulte, J. W. M. In vivo MRI cell tracking: clinical studies. *A. J. R.* 193, 314-325 (2009).

8. Neuwelt, A. *et al.* Iron-based superparamagnetic nanoparticle contrast agents for MRI of infection and inflammation. *A.J.R.* 204, w302-w313 (2014).

9. Corna, G. *et al.* Polarization dictates iron handling by inflammatory and alternatively activated macrophages. *Haematologica* 95, 1814-1822 (2010).

10. Laskar A, Eilertsen J, Li W, Yuan XM. SPION primes THP1 derived M2 macrophages towards M1-like macrophages. Biochem Biophys Res Commun. 2013 ;441(4):737-42

11. Shen CC, Liang HJ, Wang CC, Liao MH, Jan TR Iron oxide nanoparticles suppressed T helper 1 cell-mediated immunity in a murine model of delayed-type hypersensitivity. Int J Nanomedicine. 2012;7:2729-37

Training and Professional Development: Nothing to report

Dissemination: Nothing to date to report – two manuscripts are in the writing phase

Next period – what do we plan to do: The aconitase knockdown cells are almost ready for study so the goal will be to measure doubling time of the cells (compared to wild type cells) and cell migration, and measure the effect in vivo and compare to the effect of DFP. We will continue the iron measurements of the TRAMP C2 tumors in vivo, which we view as comparable to the original metabolic measurements proposed since they are 1) metabolism oriented and 2) most importantly are viewed as a potential non-invasive surrogate, which was the goal fo the metabolism studies.

4. Impact on principal discipline: This is premature since our goal is to show that DFP has an effect on prostate cancer outcome and we need to finish our in vivo studies

Impact on other disciplines: Nothing to report

Technology transfer: Nothing to report

Impact on society beyond science and technology: Nothing to report

5. Changes/Problems

Changes in approach and reasons for changes: In our quest for a potential non-invasive surrogate, we have changed to a more sensitive, direct method of measuring iron metabolism,

rather than the proposed indirect, less sensitive method of measuring the effect of DFP on glucose metabolism. The original technique was less sensitive, required the administration of an exogenous agent, and was not feasible in orthotopic models.

<u>Actual or anticipated problems or delays:</u> As noted above, the development of knockdown cell lines has been difficult but we think the problem is resolved.

<u>Changes in expenditures:</u> The above delay has led us to decrease some of our expenditures and we anticipate these funds will go for a possible request for a no-cost extension due to the delay cited above.

<u>Significant Changes in Human subject, vertebrate animals, biohazards, and or select agents</u>. We no longer require administration of 1,6-13C glucose as proposed.

<u>6. Products:</u>

We are writing 2 manuscripts at present outlining the in vitro and in vivo studies.

<u>7. Participants and other Collaborating Organizations:</u>

Name:	Koutcher, Jason
Project role:	PI
Research Identifier	Nothing to support
Nearest Person month	0.6 months
Contribution to Project:	Planned projects and experiments, reviewed data
Funding Support:	Nothing to report

Name:	Ackerstaff, Ellen
Project role:	Co-investigator
Research Identifier	Nothing to support
Nearest Person month	2.0 months
Contribution to Project	Directs postdoctoral fellow, reviews methods to be used, reviews data, teaches new methods
Funding Support:	Nothing to report

Name:	Thaler, Howard
Project role:	Co-investigator
Research Identifier	Nothing to support
Nearest Person month	0.6 months
Contribution to Project	Statistical analysis
Funding Support:	Nothing to report

Name:	Serganova , Inna
Project role:	Co investigator
Research Identifier	Nothing to report
Nearest Person month	1 month

Contribution to Project   Derives aconitase knockdown cells, provides biological expertise
for project
Funding Support: Nothing to report

Name: Veeraperumal, Suresh
Project role: Postdoctoral fellow
Research Identifier   Nothing to report
Nearest Person month   6 months
Contribution to Project   Performs most of the experiments of the project
Funding Support: Nothing to report

No other organizations are involved in this project.

8. Special Reporting Requirements: None

9. Appendices: None - one paper has a draft but is premature to send.