AWARD NUMBER: W81XWH-15-1-0509

TITLE: Autophagosomal Sequestration of Mitochondria as an Indicator of Antiandrogen Therapy Resistance of Prostate Cancer (PCa)

PRINCIPAL INVESTIGATOR: George Wilding, M.D.

CONTRACTING ORGANIZATION: University of Texas MD Anderson Cancer Center
Houston, TX 77030

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Fort Detrick, Maryland 21702-5012

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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.
Purpose: We have investigated if sequestration of metabolically dysfunctional mitochondria by the autophagosomes (mitophagy) imparts anti-androgen resistance.

Method: Effects of the anti-androgen enzalutamide on the autophagy and mitophagy of androgen-dependent LNCaP and -independent C4-2 cells are studied first. Autophagy is monitored by cellular fluorescence in cells treated with monodansylcadavarine (MDC) or stained with anti-LC3B antibody. Cellular fluorescence due to Mitosox dye oxidation is used to identify mitochondria producing high superoxide (O_2^-). Mitophagy is monitored using fluorescence resonance energy transfer (FRET) by visualization of FRET images and quantitation of FRET image intensities using a Nikon A1 or a Leica Di8 fluorescence confocal microscope and Image J software.

Results and Discussion: Our data show that the degree of mitophagy is more in androgen-dependent LNCaP cells than in -independent C4-2 cells, both growing in androgen-depleted media. Enzalutamide treatment induces mitophagy in both cell lines, but the increase in mitophagy is more pronounced in the enzalutamide-resistant C4-2 than in the _-sensitive LNCaP cells. Mitophagy in circulating tumor cells (CTCs) isolated from patient blood samples are currently being standardized.
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1. INTRODUCTION

Subject: Autophagy is activated in cells in response to stresses such as nutrient deficiency and/or oxidative stress for survival. Cancer cells with high metabolic activities require relatively higher levels of nutrients than do normal cells. They often use some of the autophagic signaling pathways such as mTOR or FoxO that are switched on by nutrient deficiency and oxidative stress for survival or self-destruction depending on the magnitude of the stress conditions. Androgens as well as anti-androgens such as bicalutamide, enzalutamide, etc. modulate oxidative stress in prostate cancer cells that affect mitochondrial functions. Our preliminary data suggest that autophagy of dysfunctional mitochondria (mitophagy) in response to anti-androgen therapy may play a critical role in Androgen Deprivation Therapy (ADT)-resistance in prostate cancer cells. We believe, this response can be observed in circulating tumor cells (CTCs) in patients undergoing ADT using a fluorescence resonance energy transfer (FRET) based high resolution confocal fluorescence microscopy. With the development of methodology for isolating CTCs from patient blood samples, this microscopic method can now be standardized to observe this response, which may identify non-responding patients and may also predict the onset of therapy-resistance in patients at an early-stage. The therapy can then be quickly switched to maximize efficacy as well as reduce unnecessary pain and suffering.

Purpose: We have focused on establishing the FRET confocal microscopy to analyze the degree of mitophagy of the high superoxide producing dysfunctional mitochondria in prostate cancer cells and CTCs isolated from patient blood samples. This information, once firmly established by a positive correlation with patient therapy outcome, could be used as an early-indicator of ADT-resistance. A successful establishment of the methodology and initial data on predictability of patient outcome should provide the basis of two new avenues of future investigations – i) a prospective clinical trial for validation and application of this method to identify therapy-resistant patients (this should help initiate personalized trial for patients, thus identified, with autophagy inhibitors such as metformin or chloroquine) and ii) a basic scientific research on some of the underlying mechanisms of the development of ADT-resistance in patients. CTCs isolated from patients using our standardized method are metabolically active and detailed genomic, proteomic and metabolomic mapping of these cells are possible.

Scope: A few of these methods such as LC3 and cytokeratin (CK) expression will be used in this proposal to confirm the autophagy and CTC identification, respectively. More detailed mechanistic studies such as initiation of autophagy due to ER-stress, Warbūrg effect, mTOR dependency, etc. and the role of glucose metabolism modulators and autophagy inhibitors fall beyond the scope of this proposal.

2. KEYWORDS

ADT = Androgen-Deprivation Therapy
AR = Androgen Receptor
CK = Cytokeratin
CRPC = Castrate-Resistant Prostate Cancer
3. ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1. Standardize confocal microscopy FRET analysis to quantitate mitophagy in androgen-dependent and castrate-resistant prostate cancer cells exposed to antiandrogens in presence or absence of androgen.

Major Task 1. Standardize confocal microscopy FRET assay and compare degree of mitophagy in anti-androgen –sensitive and –resistant prostate cancer cells.

Subtask 1: Use 96-well plate based FRET assay to determine effect of anti-androgens on androgen-dependent LNCaP and androgen-independent C4-2 cells. (1-6 months)

Subtask 2: Use confocal microscopy FRET assay to confirm results of 96-well plate based assay. (3-9 months)

Subtask 3: Verify 96-well plated based and confocal microscopy FRET assay results using other androgen-dependent (LAPC-4) and androgen-independent (CWR22Rv1) prostate cancer cells. (6-15 months)

Specific Aim 2: Apply the standardized method to live circulating tumor cells (CTCs) for prostate cancer patients undergoing ADT to determine correlation of degree of mitophagy in CTCs with response to ADT/ development of resistance to ADT.

Major Task 2: Optimization of CTC isolation device protocols for fixation, permeabilization and staining of CTCs.

Subtask 1: Submit documents for HRPO approval. (1-2 months)

Subtask 2: Utilize CTCs from estimated 15 patients to test variations in fixation buffer/time, permeabilization buffer/time, and stain concentration/time. (4-9 months)

Major Task 3: Perform confocal microscopy FRET assay of mitophagy on CTCs from patients pre- and post- Enzalutamide therapy.

Subtask 1: Quantitate mitophagy in CTCs from 30 patients’ pre-treatment and at the time of radiographic progression of the disease. (9-32 months)

Subtask 2: Analyze data for correlation with disease status. (33-36 months)
What was accomplished under these goals?

Major activities, objectives, and significant results/achievements

Standardization of a High throughput assay for Mitophagy: Prostate cancer cells are grown in DMEM with 1% FBS and 4% charcoal-stripped serum (F1C4) for at least 2 days to show strong androgen and/or anti-androgen response. Eighty microliters of cell suspension containing ~4,000 cells are then seeded in F1C4 in each well of a 96 well plate except blanks, which received only media. A day after seeding, cells are treated with 10 μL of graded concentrations of enzalutamide. After 96 h incubation at 37 °C in a 5% CO2/air incubator, cells are treated with calculated concentrations of 10 μL of MDC, Mito-O or Mito-S dyes following a published protocol. During this year, conditions have been standardized that yield observable fluorescence/FRET signal intensities. After the fluorescence/FRET readings, the cells are stained for DNA fluorescence using Hoechst 33258. All MDC and Mito-dye fluorescence and FRET data are normalized to DNA fluorescence in order to compensate for the effect of enzalutamide on cell growth. These data are shown in Figure 1.

Figure 1. Effect of enzalutamide on the (A) growth; (B) autophagy (C) O2•−−mitophagy in LNCa and C4-2 cells. The data and the error bars are the means and standard deviations of readings from 6 wells per enzalutamide concentration (in mM) run in duplicates.

Standardization of fluorescence imaging for co-localization of the cells in a confocal microscope: LNCaP and C4-2 cells are seeded separately in 300 μL F1C4 in each chamber of 8-chamber slides. Anti-androgen-sensitive LNCaP cells have been treated with 1 μM (IC50) and –resistance C4-2 cells have been treated with 10 μM (highest dose possible in DMSO formulation) enzalutamide for 96 h. Cells in different chambers are then treated with 10 μM MDC alone, Mito-O alone, Mito-S alone and a combination of MDC plus Mito-O or MDC plus Mito-S. Mito-S treated slides are incubated for 3 h at 37°C in a CO2/air incubator to allow for Mito-S oxidation before MDC addition. All slides are incubated for an additional 1 h in the same incubator. The slides are then washed and fixed with 95% EtOH. The co-localization of the mitochondria within autophagosomes is observed at 100x magnification in 0.5 mm optical sections using a Nikon C1 confocal microscope. The images are shown in Figure 2.

Figure 2. An optical section of confocal microscopic image (Mag. 100x) of MDC stained autophagosomes (blue) and Mitosox-red stained mitochondria (red). (A) LNCaP + 1 μM Enzalutamide; (B) C4-2 + 10 μM Enzalutamide.

Standardization of FRET imaging of the cells for co-localization in a confocal microscope: The FRET images are observed using a Nikon A1 confocal microscope at 90x magnification and 0.5
μm optical sections (note: mitochondrial dimension of human cells is ~1 μm) with MDC excitation and Mito-dye emission from the chambers treated with the dye combination as above (see Figure 2). During this year, the laser intensity and PM tube voltages of the FRET channel are standardized such that there is no detectable emission from chambers treated with either MDC or Mito-dye alone. The fluorescence intensities of the FRET signals from all optical sections have been quantified and integrated using Image J software with appropriate threshold to contour map each cell and are analyzed with the help of the Biostatistics Core of MD Anderson.

Representative FRET images of C4-2 cells treated with or without 10 μM enzalutamide are shown in Figure 3. Similar FRET signal intensities from MDC and Mito-O dye combination are also obtained for normalization (data not shown). The mean FRET intensities calculated from enzalutamide-treated and untreated LNCaP and C4-2 cells are shown in Figure 4. In another set of experiment cells after staining with Mito-S dye are fixed and ICC stained with LC3B antibody that have been tagged with Alexafluor 488 dye that shows FRET with Mito-S or Mito-O. FRET data for Mitosox and LC3B fluorescence are shown in Figure 5. Where there is no appreciable change in FRET intensities for Mito-S and LC3B and there is a decrease of that for Mito-S and MDC in LNCaP cells, in C4-2 cells, an appreciable increase in FRET intensity has been observed in Enzalutamide treated cells as compared to that in the control vehicle treated cells for MDC and LC3B stains.

Standardizing a method for Circulating Tumor Cell (CTC) isolation from patient blood samples: We received IRB and HRPO approval for the protocol that allows longitudinal collection and analysis of CTCs from 45 patients for mitophagy and correlating the data with patient disease outcome (PA15-0956). During this year, methods have been standardized to isolate CTCs from patient blood using a size exclusion microfluidic device that has been standardized and employed routinely for CTC and epithelial cell isolation in our collaborator Dr. Reuben’s laboratory. These cells are viable for at least 24 hours after sorting.

Standardization of FRET imaging of the isolated CTCs: The cells are plated immediately after isolation in 3 chambers of a lysine coated 8 chamber slide in RPMI1640 with 10% FBS. The cells
are allowed to attach overnight. The next day, the cells in 2 chambers are treated with Mito-S for 3 hours. All cells are then fixed with paraformaldehyde and the cells in the third chamber are subject to immunocytochemical staining (ICC) with anti-CK, anti-AR, anti-EpCam and DAPI to identify nucleated epithelial cells of prostatic origin, which we have designate as CTCs as proposed in our application. A representative CTC is shown in Figure 6 as identified by the ICC stains. The cells in the other 2 chambers are stained for anti-AR (Alexa 647, not shown) and anti-LC3 (Alexa 488) antibodies. A representative FRET signal between LC-3 (Ex 488/Em 535) and Mito-S (Ex 540/Em 595) is shown in Figure 7. The FRET signals from the CTCs isolated from two patients thus far normalized to the Mito-S signal of corresponding CTCs are shown in Figure 8. There is a clear difference in FRET intensities between the two patients. These data for a total of 45 patients will be collected and correlated with patient outcome to determine the threshold of intensity value that can predict the therapy outcome.

![Figure 6](image6.jpg)  Prostate cancer patient CTC as identified by ICC staining with 3 antibodies and a nuclear stain – anti-EpCam (purple), anti-CK (red), DAPI (blue) and anti-AR (green) (100x).

![Figure 7](image7.jpg)  Representative FRET image of prostate cancer patient CTC stained with Mito-S and LC3B antibody with Alexa488 ICC at 160x magnification in a Leica confocal microscope (160x).

![Figure 8](image8.jpg)  FRET intensities of CTCs isolated from two prostate cancer patients stained with Mito-S and then with LC3B antibody (Alexafluor-488). N represents the number of CTCs obtained from 7 ml of the collected blood samples.

**What opportunities for training and professional development has the project provided?**

Nothing to report

**How were the results disseminated to communities of interest?**

A poster summarizing the FRET data for prostate cancer cell lines shown above has been presented at the annual meeting of American Association of Cancer Research, Philadelphia, PA (See Appendix).

**What do you plan to do during the next reporting period to accomplish the goals?**

The FRET data will be collected longitudinally from advanced prostate cancer patients undergoing enzalutamide treatment (a total of 45 patients in the course of next two years) and will be correlated to their disease and therapy outcome during the course of this grant proposal. We anticipate to determine a threshold FRET signal intensity, above which the patient do not respond to enzalutamide therapy, which is the ultimate goal of this proposal.
4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

The FRET method for detecting enzalutamide resistant in prostate cancer cell line has thus far been established. Same data from prostate cancer patient CTCs are currently being collected. The actual impact of the study will come in next 2 years, when this method is translated to patient CTC analysis and the determination of the threshold to identify the development of enzalutamide therapy resistance has been delineated.

During the course of this year, the FRET technology has also been extended to live primary prostate cancer cells isolated from patient prostatectomy tissues under a separate NIH grant proposal (R01 CA185251). One of the PIs of that proposal Hirak Basu, who was a key personnel in the original application submitted from Wisconsin, has recently joined MD Anderson and will be a participant in our research team for this proposal. This new application of the technology should extend the impact even farther by elucidating a role of mitochondria in cell invasion and metastasis and thus, establishing a role of mitophagy in prostate cancer progression and drug resistance.

What was the impact on other disciplines?

We believe these studies will open up a new avenue of research in the field of mitochondrial function (or dysfunction) in cancer progression, in general and may lead to application of this technology in the prognosis and prediction of drug resistance in other types of cancers in addition to prostate cancer.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

No significant change of technology and/or goal has thus far been necessary.

Actual or anticipated problems or delays and actions or plans to resolve them

This award experienced an initial delay when Dr. George Wilding transferred from the University of Wisconsin to the University of Texas MD Anderson Cancer Center on 09/01/2015. The award was transferred to MD Anderson and a revised agreement was provided to extend the effective period to 10/31/2019.
We have also encountered some delay in patient accrual and cell line loss during the hurricane Harvey. We are glad to report that we are recovering from the damage and loss of some perishable reagents and are moving smoothly after the short stoppage of lab function.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

None

Significant changes in use or care of vertebrate animals

Not applicable. No vertebrate animal work in this project.

Significant changes in use of biohazards and/or select agents

No use of biohazard or select agent in this study.

6. PRODUCTS

Publications, conference papers, and presentations


Journal publications

Nothing to report

Books or other non-periodical, one-time publications

Nothing to report

Other publications, conference papers, and presentations

Nothing to report

Website(s) or other Internet site(s)

Nothing to report
Technologies or techniques

FRET assay using high resolution confocal fluorescence microscopy for prostate cancer cell and patient CTC has been standardized. The technology has been described in detail in the AACR poster and will be published in a peer reviewed journal when all the data are collected.

Inventions, patent applications, and/or licenses

Nothing to report

Other products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

The following individuals worked on this project during the current reporting period.

The University of Texas MD Anderson Cancer Center

<table>
<thead>
<tr>
<th>Name</th>
<th>George Wilding</th>
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<tr>
<td>Project Role:</td>
<td>Principal Investigator</td>
</tr>
<tr>
<td>Person months worked:</td>
<td>1.20 calendar months</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Wilding directs the research and supervise personnel in his laboratory. He is primarily responsible for data analysis and interpretation, troubleshooting, writing and editing all reports and publications, and overall completion of the project.</td>
</tr>
<tr>
<td>Funding Support</td>
<td>Dr. Wilding was supported by this DOD award.</td>
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<tr>
<th>Name</th>
<th>Hirak Basu</th>
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<tr>
<td>Project Role:</td>
<td>Collaborator</td>
</tr>
<tr>
<td>Person months worked:</td>
<td>0 calendar months, 0.60 calendar effective 11/1/2017</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>His expertise in autophagy and metabolism is considered invaluable for the success of this project. He will assist the PI in troubleshooting and paper and report writing.</td>
</tr>
<tr>
<td>Funding Support</td>
<td>Dr. Basu’s contribution for the current funding period has been compensated from departmental funding. He will receive salary support from this grant effective 11/1/2017. The approval request was sent to DOD. See appendix.</td>
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<tr>
<th>Name</th>
<th>James Reuben</th>
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<tr>
<td>Project Role:</td>
<td>Collaborator</td>
</tr>
<tr>
<td>Research Identifier:</td>
<td>0000-0001-8972-2103</td>
</tr>
<tr>
<td>Person months worked:</td>
<td>0.12 calendar months</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Reuben is collaborating with Dr. Zurita for the last several years in isolating and detecting CTCs in prostate cancer patients under an IRB approved protocol. His laboratory has a standardized protocol</td>
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for prostate cancer CTC identification and isolation that has been adopted for the studies proposed in this project. He is assisting Dr. Wilding with CTC isolation and identification and with troubleshooting and writing reports and publications.

**Funding Support:**
Dr. Reuben started receiving salaried support from this DOD award effective 7/1/2017. A formal approval request was sent to DOD. See appendix.

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<thead>
<tr>
<th>Name:</th>
<th>Amado Zurita</th>
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<td>Project Role:</td>
<td>Collaborator</td>
</tr>
<tr>
<td>Person months worked:</td>
<td>0.12 calendar months starting 9/1/2017</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Our collaboration with Dr. Zurita began on 09/01/2017. Dr. Zurita and his clinical team are involved in patient identification, consenting and blood sample collection for CTC isolation. His clinical team will de-identify the samples and store the link for protected patient information to be used for outcome correlation at the end of the project in compliance with the IRB approved protocol.</td>
</tr>
<tr>
<td>Funding Support</td>
<td>Dr. Zurita contribution for the month of September and October has been compensated from departmental funding. He will receive salary support from this grant effective 11/1/2017. The approval request was sent to DOD. See appendix.</td>
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<tr>
<th>Name:</th>
<th>Grace T. Wu</th>
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<tr>
<td>Project Role:</td>
<td>Lab Manager</td>
</tr>
<tr>
<td>Person months worked:</td>
<td>Ms. Wu’s worked 3.60 calendar months from 11/1/2016-3/30/2017. She worked 1.20 calendar effort 4/1/2017-10/31/2017. And she will dedicate 0.60 calendar effective 11/1/2017.</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Ms. Wu is directly responsible for maintenance of cell lines and assists in cell culture studies in Aim 1 and microscopy in Aim 2. Additionally, she is primarily responsible for maintenance of laboratory supplies and solutions for this project and assisting the PI in ensuring compliance, coordinating data collection, data analysis, and data and financial management for the entire project.</td>
</tr>
<tr>
<td>Funding Support:</td>
<td>Ms. Wu was supported by this DOD award.</td>
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**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Yes, see below.

**WILDING, George**

**ACTIVE**
(Transferred to MD Anderson)
5 R01 CA185251-02 Basu/Beebe (PIs) 09/02/2015-05/31/2018 0.12 calendar NIH/NCI-University of Wisconsin $157,967 annual direct

[PQC-3] A Metabolic Pathway Activation Marker for Prostate Cancer Prognosis
The overall goal of this project is to develop an integrated prognostic test based on growing primary tumor cells obtained from routine prostate biopsies in micro-scale in vitro models of tumor microenvironments, perform quantitative imaging of FAD fluorescence to determine
specific metabolic activities that lead to prostate cancer progression, and correlate the data with patient clinical outcome to validate the pathway biomarkers as predictors of disease progression.

Aims: 1. Quantitate the difference between fluorescence properties of wild-type and genetically engineered prostate cancer cell lines with varying levels of activation of the ROS generating pathway in a microfluidic mimetic of the basement membrane and endothelium capable of determining their relative invasive properties. 2. Establish FAD fluorescence intensity and lifetime data collected from multiple prostate TMAs as prognostic markers by correlating the data with patient outcome in a retrospective clinical validation study.

Role: Co-Investigator

Contract specialist: Kathleen Sielaff, Managing Officer Resch & Sponsored Programs, University of Wisconsin

(This Grant)

W81XWH-15-1-0509 Wilding (PI) 09/01/2015-10/31/2019 1.20 calendar

DOD $116,460 annual direct

Autophagosomal Sequestration of Mitochondria as an Indicator of Anti-Androgen Therapy Resistance of Prostate Cancer

The goal of this project is to standardize a confocal fluorescence microscopic method using FRET to quantitate extent of mitophagy in androgen-dependent and castrate-resistant prostate cancer cells exposed to anti-androgens, and apply the method to live circulating tumor cells (CTCs) obtained from prostate cancer patients undergoing hormonal therapy to correlate degree of mitophagy as a marker for development of resistance in patients.

Aims: 1. Standardize confocal microscopy FRET analysis to quantify mitophagy in androgen-dependent and castrate-resistant prostate cancer cells exposed to antiandrogens in presence or absence of androgen. 2. Apply standardized method to live CTCs for prostate cancer patients undergoing ADT to determine correlation of degree of mitophagy in CTCs with response to ADT/development of resistance to ADT.

Role: Principal Investigator

Contract specialist: Kimberly Carter, kimberly.m.carter47.civ@mail.mil, Phone: 301-619-2249.

BASU, Hirak

ACTIVE

(Transferred to MD Anderson)

5 R01 CA185251-02 Basu/Beebe (PIs) 09/02/2015-05/31/2018 1.80 calendar

NIH/NCI-University of Wisconsin $157,967 annual direct

[PQC-3] A Metabolic Pathway Activation Marker for Prostate Cancer Prognosis

The goal of this project is to develop an integrated prognostic test based on growing primary tumor cells obtained from routine prostate biopsies in micro-scale in vitro models of tumor microenvironments, perform quantitative imaging of FAD fluorescence to determine specific metabolic activities that lead to prostate cancer progression, and correlate the data with patient clinical outcome to validate the pathway biomarkers as predictors of disease progression.

Aims: 1. Quantitate the difference between fluorescence properties of wild-type and genetically engineered prostate cancer cell lines with varying levels of activation of the ROS generating pathway in a microfluidic mimetic of the basement membrane and endothelium capable of determining their relative invasive properties. 2. Establish FAD fluorescence intensity and
lifetime data collected from multiple prostate TMAs as prognostic markers by correlating the data with patient outcome in a retrospective clinical validation study.

Role: Currently, Subcontract PI

Contract specialist: Kathleen Sielaff, Managing Officer Research & Sponsored Programs, University of Wisconsin

(NEW)

Prostate Moon Shot Logothetis/Giancotti (PIs) 09/01/2017-08/31/2018 1.20 calendar
MD Anderson Moon Shot Program $303,670 annual direct
Flagship 3: Targeting Non-Immune Tumor-Associated Microenvironment in Prostate Cancer
The overall goals of this project are to: 1. Identify biomarkers in accessible tissue (“liquid biopsy”) within the bone secretome predictive for earlier clinical intervention with radium-223 in patients with metastatic prostate cancer to the bone and in combination with other targeted therapies. 2. Determine whether combining radium-223 with cabozantinib is likely to prolong patient survival. 3. Assess whether radium-223 antitumor efficacy clinically and co-clinically in bone metastasis, synergize with cabozantinib and/or FGF signaling blockade. 4. Study the interactions between FGF signaling and MET/VEGFR2 and to determine whether there is antitumor synergy between FGF axis and MET/VEGFR2 blockade in m-CRPC co-clinically and clinically.

Aims: 1. Identify biomarkers within the secretome predictive of responsiveness to cabozantinib, radium-223. 2. Identify biomarkers within the bone secretome predictive for earlier clinical intervention in patients with metastatic prostate cancer to the bone using radium-223 and/or checkpoint inhibitors in combination with other targeted therapies. 3. Rationally integrate immune Novel Microenvironment targeted therapy (Wnt/bcatenin, FGF with cabozantinib and radium-223). 4. Determine functional properties of prostate cancer and bone cell-derived exosomes that contribute to prostate cancer metastasis.

Role: Investigator

Contract specialist: Carrie C. Feighl, Director, Research Finance, cfeighl@mdanderson.org, Phone: 713-792-3477

(NEW)

REUBEN, James

ACTIVE

Reuben (PI) 02/01/2014-03/31/2018 0.12 calendar
Hitachi Chemical Company, Ltd. $790,992 direct
Evaluation of a Technology and CTC System
Role: Principal Investigator

Hitachi Reuben (PI) 04/19/2016-04/18/2020 5.88 calendar
Hitachi Chemical Company, Ltd. $711,198 direct
Evaluation of a Microcavity Array (MCA) System Integrated with a Microfluidic Device for the Isolation of Circulating Tumor Cells (CTCs)
The overall goal of this study is to develop a CTC platform for the isolation and molecular characterization of CTCs in solid tumors
Role: Principal Investigator
ZURITA, Amado

ACTIVE

Zurita (PI)  03/28/2017-03/27/2019  0.12 calendar
Infinity Pharmaceuticals Inc. $37,500 annual direct
PI3K Gamma Inhibition in Combination with Anti-Angiogenics and/or Immunotherapy in Renal Cell and Lung Carcinoma Models
The overall goal of this study is to evaluate new combinations for renal cell carcinoma (RCC) treatment in preclinical mouse models.
Aims: 1. Assess the combinatorial effect of PI3Kgamma blockade upon anti-angiogenic treatment resistance in experimental models of RCC. 2. Evaluate the synergizing effect of PI3Kgamma with PD1 checkpoint blockade to enhance anti-tumor immune response in RCC.
Role: Principal Investigator
Contract contact: Legal Department, 784 Memorial Drive, Cambridge, Massachusetts, 02139, Phone: 617-453-1000

Prostate Moon Shot Logothetis/Giancotti (PIs) 09/01/2017-08/31/2018 0.60 calendar
MD Anderson Moon Shot Program $724,810 annual direct
Flagship 1: Optimizing Androgen Signaling Inhibition to Transition from a Treatment to Curative Paradigm
Flagship 1 aims to optimize androgen receptor (AR) signaling inhibition and address mechanisms of resistance to complete blockade of the AR.
Aims: 1. Design and implement innovative clinical trials based on an increased understanding of the tumor cell-intrinsic mechanisms driving tumor progression and resistance to AR-targeted agents. 2. Identify mechanisms and biomarkers of response and resistance to agents that target oncogenic signaling pathways and/or non-oncogene dependencies, including lineage-dependent transcription factors and synthetic essential genes. 3) Develop a personalized management of prostate cancer based on the evolving repertoire of genetic and epigenetic lesions driving disease progression on therapy.
Role: Investigator
Contract specialist: Carrie C. Feighl, Director, Research Finance, Phone: 713-792-3477, cfeighl@mdanderson.org

What other organizations were involved as partners?

Not applicable

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS

Not applicable

QUAD CHARTS

Not applicable
Mitophagy Impacts Enzalutamide Resistance in Prostate Cancer

Hirak S Basu, Cynthia Schreiber, Jamie Spenger, MaryAnne Naundorf, Ashley Weichmann, Farideh Mehran-Ghomi, Dawn Church, Joshua Lang* and George Wiling
University of Wisconsin Carbone Cancer Center, Madison, WI

Abstract #: 2899/3

Mitophagy

Co-localization of O₂-producing mitochondria with autophagosomes

Fluorescence Resonance Energy Transfer (FRET)

MDC-Mitosox FRET

Figure 1. An optical section of confocal microscopic image (a) of LNCaP cells stained with anti-LC3 (b), LNCaP and control cells stained with anti-LC3 and MDC (c) and MDC-Mitosox FRET (d).

Figure 2. Spatiotemporal regulation of mITGAB and mITGAM.

Figure 3A. An optical section of confocal microscopic FRET image (a) of LNCaP and Enzalutamide treated cells (b).

Figure 3B. A series of the three experiments: determining the median FRET intensity of control and Enzalutamide treated cells after 50 h of the experiment. All data are normalized to FRET intensity of control cells with 100% and Enzalutamide treated cells with 0% in the y-axis.

Figure 3C. The median FRET intensity of control and Enzalutamide treated cells.

Figure 4. A.Mitochondria and autophagosomes are staining with C2A and LC3 respectively. Enzalutamide treated cells (a) and control cells (b). Enzalutamide treated cells are stained with C2A (a), LC3 (b) and MDC (c).

Figure 5. Fluorescence microscopy of CTCs in vivo with the fluorescence microscope. (a) CTCs stained with Alexa Fluor 647-conjugated anti-EpCAM antibody and (b) CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 6. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 7. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 8. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 9. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 10. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 11. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 12. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 13. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 14. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 15. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 16. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 17. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 18. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 19. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 20. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 21. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 22. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 23. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.

Figure 24. Representative images of FRET signal from CTCs stained with Alexa Fluor 488-conjugated anti-EpCAM antibody.
Appendix 2. Addition of Key Personnel Approval Request sent to DOD

September 14, 2017

Ms. Kimberly Carter
Grants Management Specialist
US Army Medical Research Acquisition Activity (USAMRAA)
820 Chandler Street
Fort Detrick, MD 21702-5014
Kimberly.m.carter47.civ@mail.mil

Re: W81XWH-15-1-0509, PCI140850, Addition of Key Personnel

Dear Ms. Carter:

This is in continuation of our institutional communication with the DOD for approval of our proposal to replace Dr. Joshua Lang, University of Wisconsin (UW), with the following Physicians and Scientists at The University of Texas MD Anderson Cancer Center for my DOD grant entitled “Autophagosomal sequestration of mitochondria as an indicator of antiandrogen therapy resistance of prostate cancer” This substitution became necessary because of my move from UW to MD Anderson on September 1, 2015.

I am writing this to formalize our request to add the following key personnel to the proposal effective November 1, 2017, with the exception of Dr. James Reuben, who had to be added effective July 1, 2017, for the immediate need for live cell analysis of patient samples collected at MD Anderson.

Amado Zurita-Saavedra, MD, is an associate professor in the Department of Genitourinary Medical Oncology and a molecular pathologist. He is a key member of the MD Anderson team who has developed liquid biopsy methods to monitor prostate cancer progression by analyzing blood samples. On the basis of this technology, individual circulating tumor cells (CTCs) are being analyzed for exome-wide/targeted panel sequencing and genome-wide copy number profiling at high resolution. Dr. Zurita-Saavedra and his clinical team will be involved in patient identification, consenting, and blood sample collection for CTC isolation. His team will de-identify the samples and store the link for protected patient information to be used for outcome correlation at the end of the project, in compliance with the IRB-approved protocol.

James Reuben, MD, a professor in the Department of Hematopathology, is the deputy director of MD Anderson’s Morgan Welch Inflammatory Breast Cancer Research Program and Clinic. He is one of the pioneers of detecting, isolating, and characterizing CTCs from patient blood samples. He and his collaborators were the first to demonstrate the prognostic clinical utility of CTCs in metastatic breast cancer with respect to progression-free and overall survival. He has collaborated with Dr. Zurita-Saavedra for the past several years in isolating and detecting CTCs in prostate cancer patients, under an IRB-approved protocol. His laboratory has a standardized protocol for prostate cancer CTC isolation and identification that has been adopted for the studies proposed in our project. He and his research team will help the PI in CTC isolation and identification and will also help in troubleshooting and writing reports and publications.
Hirak Basu, PhD, from UW, has joined MD Anderson during the course of the past year as an associate professor in the Department of Genitourinary Medical Oncology Research. For the past 20+ years, Dr. Basu has collaborated with the PI in several grants and projects and has contributed substantially to this research, from developing the concept to collecting preliminary data. Dr. Basu was one of the key personnel named in the original proposal submitted from UW. His research interest mainly focuses on diagnostic, prognostic, and new drug development for prostate cancer. He has worked in the field of prostate cancer and oxidative stress for the past 15+ years with numerous funded federal grants, and he is an active member and chair of several NIH and DOD Scientific Review Committees. Dr. Basu also has nearly 30 years of experience in the field of cancer research, in both academic and industrial settings. We are fortunate to get Dr. Basu back on our team. His expertise in autophagy and metabolism is considered invaluable for the success of this project.

Drs. Basu, Reuben, and Zurita-Saavedra together will assist in carrying out all of the aims of this proposal. We are including their biosketches and an updated budget for your review.

Please feel free to contact me if you have any questions or concerns.

Sincerely,

Georgé Wilding, MD
Vice President of Clinical and Interdisciplinary Research

Claudia Delgado
Executive Director
Grants and Contracts Accounting
# BUDGET SUMMARY FORM

Cost Estimate for MD Anderson’s Year 2

Name of Principal Investigator (last, first, middle): Wilding, George  
eBRAP log # PC140850, Award # W81XWH-15-1-0509

<table>
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<th>PERSONNEL</th>
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<th>TITLE/POSITION</th>
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<th>% EFFORT ON PROJECT</th>
<th>DOLLAR AMOUNT REQUESTED (OMIT CENTS)</th>
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<td>Res Asst II</td>
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| SUBTOTALS | 73,951 | 20,706 | $94,657 |

- **CONSULTANT COSTS**
- **MAJOR EQUIPMENT (IT+M/RF)**
- **MATERIALS, SUPPLIES, AND CONSUMABLES (ITEMIZE BY CATEGORY)** 23,167
- **RESEARCH-RELATED SUBJECT COSTS**
- **TRAVEL** 1,800
- **OTHER DIRECT COSTS (ITEMIZE BY CATEGORY)**

**SUBTOTAL DIRECT COSTS FOR THIS BUDGET PERIOD** $119,624

**SUBCONTRACT COSTS**
- **DIRECT COST**
- **INDIRECT COST**

**TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD** $119,624

**TOTAL INDIRECT COSTS FOR INITIAL BUDGET PERIOD** $71,774

**TOTAL DIRECT AND INDIRECT COSTS FOR THIS BUDGET PERIOD** $191,398
BUDGET JUSTIFICATION
The University of Texas MD Anderson Cancer Center

PERSONNEL: $94,657
Federally approved fringe rate: 28%

George Wilding, M.D., Principal Investigator (1.20 calendar, 10% effort)
Dr. Wilding is Professor and Vice President and Deputy CAO for Clinical and Interdisciplinary Research at MD Anderson. He is Emeritus Director of the University of Wisconsin Carbone Cancer Center at the University of Wisconsin-Madison. He is on the National Cancer Institute (NCI) Board of Scientific Counselors. Dr. Wilding has worked in the field of prostate cancer for over 25 years. He is a well-known clinical oncologist specializing in the field of prostate cancer and is considered one of the world's experts in the field of oxidative stress in prostate cancer, with his laboratory research on oxidative stress and prostate carcinogenesis and cancer progression. The proposed study focuses on metabolic oxidative stress generation in prostate cancer leading to prostate cancer progression and drug resistance. Dr. Wilding will direct the research and supervise personnel in his laboratory. He will be primarily responsible for data analysis and interpretation, troubleshooting, writing and editing all reports and publications, and overall completion of the project.

Hirak Basu, Ph.D., Co-Investigator (0.60 calendar, 5% effort)
Dr. Basu, from the University of Wisconsin, has joined us as an Associate Professor in the Genitourinary Medical Oncology Research department at MD Anderson. At MD Anderson, he shares his research space with his long-term collaborator George Wilding, PI, where Dr. Basu mainly focuses on prostate cancer diagnostic, prognostic, and new drug development research. Dr. Basu also has nearly 30 years of experience in the field of cancer research both in the academic as well as in the industrial settings and has developed 3 agents from computer-aided design to Phase I/II clinical trials, including standardizing liquid-chromatography mass-spectroscopy (LC-MS) methods for GLP analysis of several lead agents in preclinical and clinical samples. He has 10 new drug patents and raised over $15M from various venture and investor funds in addition to multiple NIH SBIR awards. He has worked in the field of prostate cancer and oxidative stress for the last 15+ years with numerous funded federal grants and is an active member and also chair of several NIH and DOD Scientific Review Committees. His expertise in autophagy and metabolism is considered invaluable for the success of this project.

Amado Zurita, M.D., Co-Investigator (0.12 calendar, 1% effort)
Dr. Zurita is an Associate Professor in the Department of Genitourinary Medical Oncology and a molecular pathologist. He is a key member of the MD Anderson team that has developed liquid biopsy methods to monitor prostate cancer progression by analyzing blood samples. Based on this technology, individual tumor cells in blood (CTCs) are being analyzed for exome-wide/targeted panel sequencing and genome-wide copy number profiling at high resolution. He and his clinical team will be involved in patient identification, consenting and blood sample collection for CTC isolation. His team will de-identify the samples and store the link for protected patient information to be used for outcome correlation at the end of the project in compliance with the IRB approved protocol.

James Reuben, M.D., Co-Investigator (0.12 calendar, 1% effort)
Dr. Reuben is a Professor in the Department of Hematopathology at and a deputy director of the MD Anderson’s Morgan Welch Inflammatory Breast Cancer Research Program and Clinic. He is one of the pioneers of detecting, isolating and characterizing circulating tumor cells (CTCs) from patient blood samples. He and his collaborators were the first to demonstrate the clinical utility of CTCs in
metastatic breast cancer as prognostic with respect to progression free survival and overall survival of metastatic breast cancer patients. He is collaborating with Dr. Zurita for last several years in isolating and detecting CTCs in prostate cancer patients under an IRB approved protocol. His laboratory has a standardized protocol for prostate cancer CTC identification and isolation that has been adopted for the studies proposed in this project. He and his research team will help the PI in CTC isolation and identification and will also help in troubleshooting and writing reports and publications.

Grace Wu, Research Laboratory Manager (0.60 calendar, 5% effort)
This person will be directly responsible for maintenance of cell lines, and will assist in cell culture studies in Aim 1 and microscopy in Aim 2. Additionally, she will be primarily responsible for maintenance of laboratory supplies and solutions for this project and assisting the PI in ensuring regulatory compliance, coordinating data collection, data analysis, and data and financial management for the entire project.

Justin Weldon, Research Data Coordinator (2.40 calendar, 20% effort)
Mr. Weldon is a research data coordinator in the Department of Genitourinary Medical Oncology. He is trained in coordinating GU clinical trials. He will assist in identifying and consenting eligible prostate cancer patients, managing clinical data, and ensuring maintaining patient information and privacy according to the IRB approved protocol.

Jessica Liebling, Research Assistant II, (1.20 calendar, 10% effort)
Jessica has a Master’s degree in biomedical sciences with expertise in cell culture and tissue analysis. She will work under the direction of Dr. Basu and the Lab manager Grace Wu. She will be primarily responsible for performing cell culture and microscopy studies related to image analysis of culture cells and human samples. Maintaining all relevant cell cultures, preparing buffers and cataloguing data will also be in her job description.

TRAVEL: $1,800 per year

The travel fund will mainly pay for the PI to attend the DOD impact meeting. Any surplus will be used to defray the cost of attending annual AACR meeting where the data generated under this award.

MATERIALS AND SUPPLIES: $23,167

*Labware, Reagents, and Chemicals* ($11,167): Anticipated costs include dyes and antibodies needed for staining cells and mitochondria and autophagosomes, general consumables such as pipettes, tubes, gloves and kimwipes used in the laboratories for sample processing and analyses; labware such as cell culture plates, slides and reservoirs; reagents for cell culture maintenance and experiments such as medium, serum, antibiotics, trypsin, enzalutamide, bicalutamide, and ethanol.

*CTC isolation* ($12,000): Anticipated costs of special Angle Parsortix system for CTC isolation, all disposable microfluidic devices, personnel time, buffers and consumables.
Appendix 3.  A-18869.1 Continuing Review HRPO Approval

From: Fordham, Allison Lorraine (Allie) CTR USARMY MEDCOM USAMRMC (US) [mailto:allison.l.fordham.ctr@mail.mil]
Sent: Thursday, November 02, 2017 8:06 AM
To: Logothetis,Christopher J <clogote@mdanderson.org>
Cc: Delgado,Claudia Y <cydelgad@mdanderson.org>; AwardNotice <AwardNotice@mdanderson.org>; Bennett, Jodi H CIV USARMY MEDCOM USAMRMC (US) <jodi.h.bennett.civ@mail.mil>; Wilding,George <GWilding@mdanderson.org>; Perezouz,Elsa M <eperezou@mdanderson.org>; Carter, Kimberly M CIV USARMY MEDCOM USAMRAA (US) <kimberly.m.carter47.civ@mail.mil>; Arudchandran, Ramachandran CIV USARMY MEDCOM CDMRP (US) <ramachandran.arudchandran.civ@mail.mil>; Stubbs, Susie Carolyn CTR USARMY MEDCOM USAMRMC (US) <susie.c.stubbs.ctr@mail.mil>; Researchadministration <Researchadministration@mdanderson.org>; Brosch, Laura R CIV USARMY MEDCOM USAMRMC (US) <laura.r.brosch.civ@mail.mil>; Odam, Kimberly L CIV USARMY MEDCOM USAMRMC (US) <kimberly.l.odam.civ@mail.mil>

Subject: A-18869.1, Continuing Review Acknowledgment Memorandum (Proposal Log Number PC140850, Award Number W81XWH-15-1-0509) (UNCLASSIFIED)

Classification: UNCLASSIFIED
Caveats: NONE

SUBJECT: Acknowledgement of the Continuing Review documents for the protocol, "Evaluation of Circulating Tumor Cells in Solid Tumor Malignancies," Submitted by Christopher J. Logothetis, MD, University of Texas, MD Anderson Cancer Center, Houston, Texas, in Support of the Proposal, "Autophagosomal Sequestration of Mitochondria as an Indicator of Antiandrogen Therapy Resistance of Prostate Cancer (PCa)," Submitted by George Wilding, MD, University of Texas, MD Anderson Cancer Center, Houston, Texas, Proposal Log Number PC140850, Award Number W81XWH-15-1-0509, HRPO Log Number A-18869.1


2. The USAMRMC ORP HRPO received the MD Anderson Cancer Center (MDACC) Institutional Review Board (IRB) approval on 1 November 2017. The MDACC IRB approved full continuation of the subject protocol on 27 October 2017; this approval will expire on 12 October 2018.

3. This correspondence serves to acknowledge HRPO receipt of the continuing review documents for the protocol. No further action related to this continuing review is needed. The documents in support of this continuing review will be placed in the HRPO file.

4. The Principal Investigator must provide the following post-approval submissions to the HRPO via email to Usarmy.detrick.medcom-usamrmc.other.hrpo-cr-documents@mail.mil. Failure to comply could result in suspension of funding.

a. Substantive modifications to the research protocol and any modifications that could potentially increase risk to subjects must be submitted to the HRPO for approval prior to implementation. The USAMRMC ORP HRPO defines a substantive modification as a change in Principal Investigator, change or
addition of an institution, elimination or alteration of the consent process, change in the IRB of Record, change to the study population that has regulatory implications (e.g. adding children, adding active duty population, etc.), significant change in study design (i.e. would prompt additional scientific review), or a change that could potentially increase risks to subjects.

b. A copy of the IRB continuing review approval letter must be submitted to the HRPO as soon as possible after receipt of approval. According to our records, it appears the next continuing review by the IRB is due no later than 12 October 2018. Please note that the HRPO conducts random audits at the time of continuing review and additional information and documentation may be requested at that time.

c. The final study report submitted to the IRB, including a copy of any acknowledgement documentation and any supporting documents, must be submitted to the HRPO as soon as all documents become available.

d. The following study events must be promptly reported to the HRPO by telephone (301-619-2165), by email (usarmy.detrick.medcom-usamrmc.other.hrpo@mail.mil), or by facsimile (301-619-7803) or mail to the US Army Medical Research and Materiel Command, ATTN: MCMR-RP, 810 Schreider Street, Fort Detrick, Maryland 21702-5000.

(1) All unanticipated problems involving risk to subjects or others.

(2) Suspensions, clinical holds (voluntary or involuntary), or terminations of this research by the IRB, the institution, the sponsor, or regulatory agencies.

(3) Any instances of serious or continuing noncompliance with the federal regulations or IRB requirements.

(4) The knowledge of any pending compliance inspection/visit by the Food and Drug Administration (FDA), Office for Human Research Protections, or other government agency concerning this clinical investigation or research.

(5) The issuance of inspection reports, FDA Form 483, warning letters, or actions taken by any government regulatory agencies.

(6) Change in subject status when a previously enrolled human subject becomes a prisoner must be promptly reported to the USAMRMC ORP HRPO. The report must include actions taken by the institution and the IRB.

e. Events or protocol reports received by the HRPO that do not meet reporting requirements identified within this memorandum will be included in the HRPO study file but will not be acknowledged.

5. Please note: The USAMRMC ORP HRPO conducts site visits as part of its responsibility for compliance oversight. Accurate and complete study records must be maintained and made available to representatives of the USAMRMC as a part of their responsibility to protect human subjects in research. Research records must be stored in a confidential manner so as to protect the confidentiality of subject information.
6. Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer/Grants Officer can authorize expenditure of funds. It is recommended that you contact the appropriate contract specialist or contracting officer regarding the expenditure of funds for your project.

7. The HRPO point of contact for this study is Allie Fordham, MA, Human Subjects Protection Scientist, at 301-619-6657, DSN 343-6657, or allison.l.fordham.ctr@mail.mil.

Regards,

Allison Fordham, M.A.
Human Subjects Protection Scientist
General Dynamics Health Solutions (GDHS) Human Research Protection Office (HRPO) Office of Research Protections (ORP) United States Army Medical Research and Materiel Command (USAMRMC) Fort Detrick, Maryland
Phone: 301-619-6657
Email: allison.l.fordham.ctr@mail.mil

Classification: UNCLASSIFIED
Caveats: NONE