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Protocol Title: Efficacy of doxycycline on metakaryote cell death in patients with resectable pancreatic cancer

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**Efficacy of doxycycline on metakaryote cell death in patients with resectable pancreatic cancer**


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# CLINICAL STUDY PROTOCOL

**Efficacy of doxycycline on metakaryote cell death in patients with resectable pancreatic cancer**

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| **Indication:** | Resectable Pancreatic Cancer |
| **Phase:** | Phase II |

Protocol Histoy

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| Original | Original / Version #1 /09/30/16 |
| | Version #2 Amendment v042817 |
| | Version #3 Amendment v1/12/18 |

This is an investigator-initiated study. The principal investigator Susan Tsai, MD, MHS (who may also be referred to as the sponsor-investigator), is conducting the study and acting as the sponsor. Therefore, the legal/ethical obligations of the principal investigator include both those of a sponsor and those of an investigator.

**PROTOCOL SUMMARY**

**Study Title:** Efficacy of doxycycline on metakaryote cell death in patients with resectable pancreatic cancer

**Phase:** Phase II

**Number of Patients:** Expected enrollment of 12 patients to for an evaluable primary endpoint in 10 patients.

**Study Objectives**

**Primary Objectives:**

To assess the efficacy of doxycycline on inducing metakaryotic cell death in primary pancreatic tumors from patients with resectable pancreatic cancer.

**Secondary Objectives:**

- To determine the plasma drug concentrations of the study drug at baseline and at days 1, 3, 5, 8, 15, 22, 29, and at restaging and at the time surgery.
- To assess the histopathologic treatment response of the primary tumors which have undergone neoadjuvant gemcitabine based chemoradiation and concurrent doxycycline therapy.
- To enumerate the number of observed dead/dying metakaryotes per 1 gram of resected pancreatic tissue.

**Overview of Study Design:**

This is a window of opportunity trial which will test the efficacy of doxycycline on metakaryotic cell death after 8-10 weeks of treatment.

**Study Population:**

**Inclusion criteria:**

1. Patients must have histologically or cytologically confirmed pancreatic adenocarcinoma
2. Patients will be receiving neoadjuvant therapy for resectable pancreatic cancer in anticipation of surgical resection. Resectable stage will be defined by radiographic criteria including; (a) no radiographic evidence of arterial abutment of the celiac, superior mesenteric, or hepatic arteries, and (b) < 50% tumor-induced narrowing of the superior mesenteric vein or portal vein.
3. Patients who will receive neoadjuvant therapy (chemoradiation) are eligible.
4. Age ≥18 years.
5. ECOG performance status ≤2 (Karnofsky ≥60%).
6. Life expectancy of greater than 6 months
7. Patients must have normal organ and marrow function as defined below:
    a. leukocytes ≥3,000/mcL
    b. absolute neutrophil count ≥1,500/mcL
    c. platelets ≥100,000/mcL
    d. Creatinine clearance ≥60mL/ min or cretinine ≤1.5mg/dL
    e. Bilirubin ≤2mg/dL: If the subject's serum bilirubin is greater than two at the time of enrollment but the patient has demonstrated a progressive decline and is anticipated to be ≤2mg/dL prior to start of therapy they are eligible, at the discretion of the trial investigators and their treatment assignment.
8. Have no active or chronic infection with HIV, Hepatitis B or Hepatitis C
9. Ability to understand and the willingness to sign a written informed consent document.

**Exclusion Criteria:**

1. Patients with more clinically advanced pancreatic cancer (borderline resectable, locally advanced, or metastatic)
2. Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements.
3. Pregnant women are excluded from this study because the effects of metakaryocidal agents have the potential for teratogenic or abortifacient effects.
4. Previous history of other malignancy (other than cured basal or squamous cell carcinoma of the skin or cured in-situ carcinoma of the cervix) within 2 years of study enrollment.
5. Active or chronic HIV, hepatitis B or hepatitis C
6. Patients who are receiving other investigational drugs or enrolled in other clinical trials

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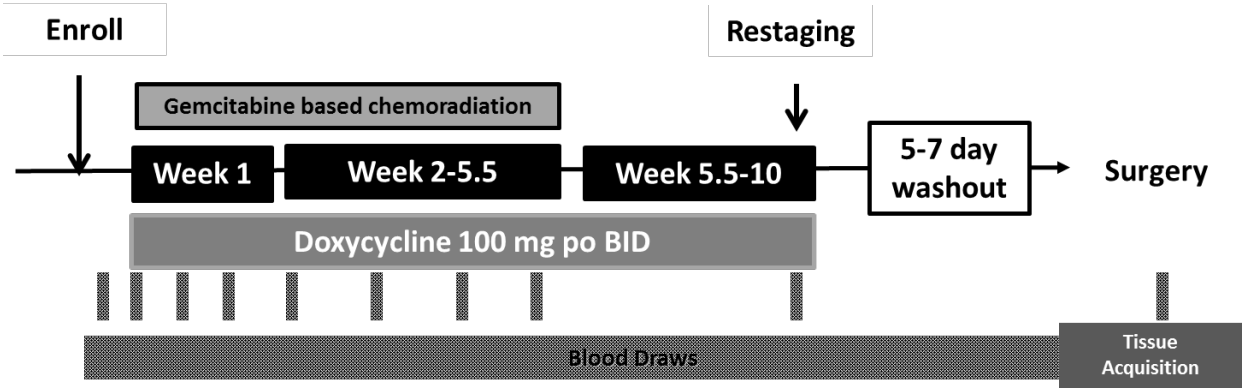
| 7. Inability to undergo scheduled blood acquisition per protocol.<br>8. Drug specific exclusion including history of allergic reactions to tetracyclines.<br>9. Prior treatment with doxycycline within a 7 day washout period prior to initiating treatment with alternate antimetakaryocidal medication. |
| **Duration of Study:** Accrual period: Approximately 12 months. |

**ABBREVIATIONS USED:**

| | |
|---|---|
| CT: | COMPUTED TOMOGRAPHY |
| MRI: | MAGNETIC RESONANCE IMAGING |
| AE: | ADVERSE EVENT |
| AESIS: | ADVERSE EVENTS OF SPECIAL INTEREST |
| DLT: | DOSE LIMITING TOXICITY |
| FDA: | FOOD AND DRUG ADMINISTRATION |
| MTD: | MAXIMUM TOLERATED DOSE |
| PK: | PHARMACOKINETIC |
| SAE: | SERIOUS ADVERSE EVENT |

**STUDY SCHEMA**



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## OBJECTIVES

### 1.1 Primary Objective

To assess the efficacy of doxycycline on inducing metakaryotic cell death in primary pancreatic tumors from patients with resectable pancreatic cancer using cytometric analysis.

### 1.2 Secondary Objective

1. To determine the plasma drug concentrations of the study drug at baseline and at days 1, 3, 5, 8, 15, 22, 29, and at restaging and at the time surgery.
2. To assess the histopathologic treatment response of the primary tumors which have undergone neoadjuvant gemcitabine based chemoradiation and concurrent doxycycline therapy.
3. To enumerate the number of observed dead/dying metakaryotes per 1 gram of resected pancreatic tissue.

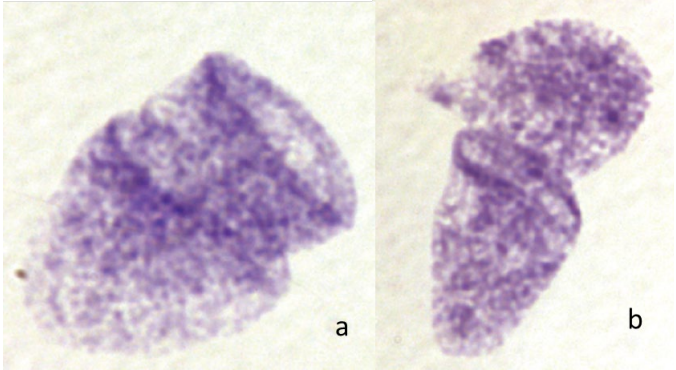
## 2. BACKGROUND

### 2.1 Metakaryotic Biology

Pancreatic cancer is a lethal disease which is distinguished by tumors which metastasize and cannot be cured by present day chemotherapy or radiotherapy. Even in the earliest stages of the disease, patients who undergo “curative” resection inevitably succumb to tumor recurrence and metastases. It is hypothesized that a small fraction of all cells in a human tumor are responsible for tumor regrowth after treatment. Such cells are called "cancer stem cells". Tumors are hypothesized to be composed of two distinct cell populations: eukaryotic tumor cells which often rapidly divide but have a finite number of cellular divisions, and a stem-cell population (possibly metakaryotic cells) which divide less frequently and may have an unlimited number of cellular divisions. Eukaryotic cells can be exquisitely sensitive to chemotherapeutic agents and radiotherapy which capitalize on the interruption of cellular mitotic division. In contrast, stem cells have previously been described as being relatively chemoresistant and radioresistant, and therefore are difficult to eradicate. Cell antigens such as CD44 and CD133 have been associated with cancer stem cells, but have variable specificity as a marker; not all cells that were labeled as $CD44^{+}/CD133^{+}$ behaved as stem cells.

Using an alternative approach, our colleagues at MIT have identified and reported a novel amitotic cell type, the metakaryote, using clear easily reproduced cellular morphological criteria. Before 2003, cancer stem cells could not be identified by microscopic examination. At the Massachusetts Institute of Technology, Dr. Elena Gostjeva in conjunction with Professor William Thilly, identified cells which had unusual large, hollow, bell-shaped nuclei on one end of the cell, which they termed “metakaryotic cells. [1-3] In particular, these cells are remarkable because unlike eukaryotic cells, when

**Figure 1: Metakaryotic division.** (a) Symmetric division similar to two paper cups separating (b) asymmetric division, yielding a bell-shaped nuclei and a close nuclei

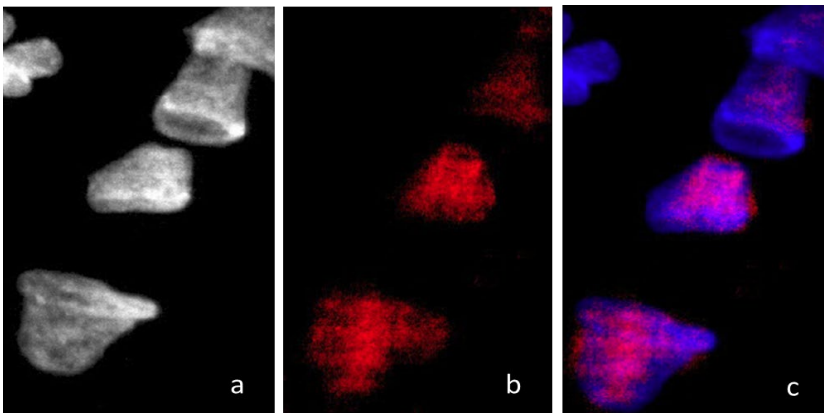


metakaryotic cells divide the chromosomes do not condense and separate in the process of mitosis. Instead their nuclei divide by an amitotic process resembling the separation of two paper cups making two metakaryotic cells. (Figure 1a) Other times they would give rise to a cell with a bell-shaped nucleus and one with a closed nucleus. (Figure 1b) Some of the new cells with closed nuclei then divided many times by mitoses; others did not further divide. This ability of metakaryotic cells to both grow in number and divide asymmetrically to create eukaryotic cells marked them as "stem cells". Importantly, since metakaryotic cells do not require mitosis for division, these cells were predicted to be resistant to chemotherapy and radiotherapy. In passing, Dr. V. Koledova of the MIT group has found the CD33 and CD144 antigens embedded in the cytoplasmic wall of mononuclear metakaryotes but not associated with the tubular syncytial metakaryotes. Further she found many eukaryotic cells that expressed these antigens in colonies that did not contain metakaryotic stem cells.

### 2.2 Amitotic division: replication and segregation of the metakaryotic nuclear genome via dsRNA/DNA intermediates

Quantitative Feulgen-based DNA image cytometry demonstrated that bell shaped nuclei of metakaryotic cells double their DNA content during and after symmetric and asymmetric amitotic fissions rather than in the separate, pre-mitotic S-phase of eukaryotic cells[1,2,5, 8]. The MIT group has observed that metakaryotic cells undergo division by first transforming their dsDNA genomes into two pangenomic dsRNA/DNA intermediates. The RNA/DNA intermediate is then segregated into sister nuclei in both symmetric and asymmetric amitotic divisions. Metakaryotic nuclei in amitosis stain strongly with an antibodies specific to dsRNA/DNA. In Figure 2a metakaryotic cells from human fetal spinal cord forming syncytia is shown illustrating this phenomenon. After segregation the dsRNA/DNA intermediate is retransformed to dsDNA form by combined action of RNAse H1 and a complex of other DNA polymerases, including DNA polymerases beta and zeta. Both DNA polymerases beta and zeta are expressed in large quantities (~300,000 copies per nucleus) in nuclei containing the dsRNA/DNA intermediate. Co-expression of these two DNA polymerases was discovered by Christopher Lawrence (U. Rochester) in S. cervesiae after ionizing irradiation; both polymerases are associated with high mutation rates. Mechanistically, it is believed that the two polymerases contribute to the highly error prone DNA repair process that by-passes otherwise lethal lesions in the DNA strand being copied. The phenomenon of using dsRNA/DNA replicative intermediates in conjunction with error prone DNA polymerases offers a mechanistic explanation of why the metakaryotic stem cells survive and demonstrate continued robust cell division after standard treatment regimens of radio- and chemo-therapy.

**Figure 2**: **Metakaryotic division via dsRNA/DNA intermediate.** (a) Metakaryote in syncytium. (b) immunofluorescent staining for dsRNA/DNA (red) duplex, (c) counterstain DAPI (blue).



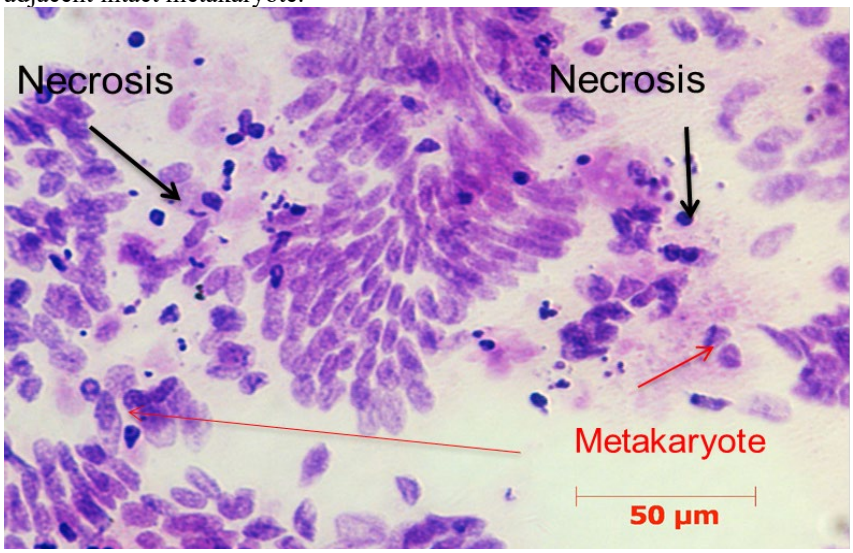

The research team at MIT has examined pancreatic, lung, and colon surgical specimens taken from patients who had undergone

preoperative chemotherapy or radiotherapy and observed that in such specimens most of the eukaryotic cells but not the metakaryotic tumor cells were killed. Furthermore, chemo-resistant and radio-resistant metakaryotic stem cells were also found in established colon and pancreatic cell lines. The research team further identified multiple FDA- approved medications which specifically kill metakaryotic cells in vitro when given at high doses over a prolonged period of time. Interestingly, such metakaryocidal doses of these drugs did not affect the growth of eukaryotic cells. One of these drugs is doxycycline, which has a tetracycline antibiotic with a long history of clinical use and a well described safety profile. Coincidentally, an independent report has recently been published in which antibiotics (erythromycins, tetracyclines, and chloramphenicol – but in particular doxycycline) were also used to selectively target cancer stem cells. [4]

### 2.3 Metakaryotes Are Present in Resected Pancreatic Cancers

Our primary collaborators, Dr. Elena Gostjeva and Professor William Thilly of MIT have published a series of peer-reviewed papers that have advanced and supported the hypothesis that the stem cells of organogenesis and carcinogenesis are non-mitotic metakaryotic cells that can be easily recognized by their pronounced hollow, bell shaped nuclei in mononuclear and tubular syncytial structures by ordinary light microscopy. The MCW Pancreatic Cancer Research Group has collaborated with Dr. Gostjeva over the past four years to supply additional pancreatic tissue from resected specimens for analysis. (Figure 3) Together they have demonstrated the presence of mononuclear metakaryotes in primary pancreatic tumors and both mononuclear and tubular syncytia (aka Indian file figures) in peritoneal metastases of pancreatic adenocarcinomas. Widespread pyknotic (dead) eukaryotic (non-stem) cells were observed in the presence of many rapidly dividing metakaryotic cells in primary pancreatic adenocarcinomas excised 3-6 weeks after the completion of neoadjuvant therapy. **Insofar as no dead or visibly disrupted metakaryotic nuclei were discovered in these specimens, they serve as a set of negative controls for the proposed trials of drugs that have shown specific metakaryocidal activity in cell culture assays at MIT.**

**Figure 3: Metakaryotic cells present in surgical specimens**. Post-resection analysis of pancreatectomy specimen demonstrates necrotic cells present with adjacent intact metakaryote.




### 2.4 Determining metakaryocidal activity

The MIT group grew and inspected a series of cell lines derived from human tumors, e.g. Capan-1 (pancreas) and HT-29 (colon) in which metakaryotic cells were required for continuous propagation. In a series of single cell experiments in microtitre dishes, they demonstrated that

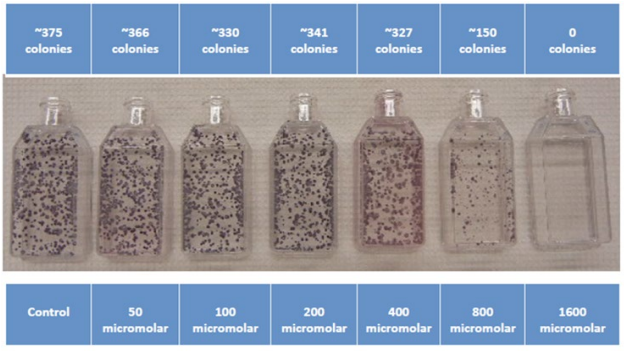
immortal colonies arose only from single cells that expressed the metakaryotic phenotype in the first few doublings. All single cells that did not express the metakaryotic phenotype gave rise to terminal colonies that did not grow further on passaging. Using these two cell lines they have devised means to measure metakaryocidal activity of test agents by either destruction of immortal colonies (clonal assays) or disruption of metakaryotic nuclei as observed by light microscopy (cytometric assays).

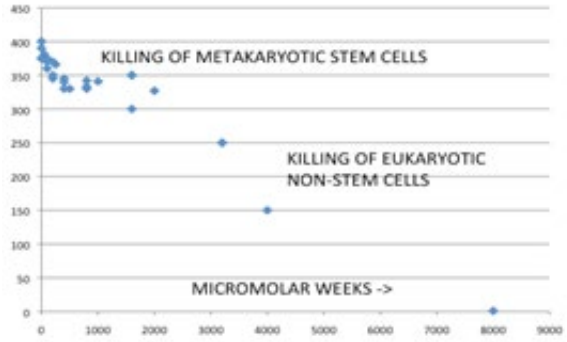
*Clonal assays*

Cells in this form of assay are treated for the desired period of exposure starting 24 hours after being seeded in T-flasks dispersed as single cells. Most single cells are non-stem, eukaryotic cells but under the conditions developed at MIT about 5-15% of single cells give rise to colonies containing metakaryotes that on continued passage are shown to be immortal. But large colonies, of ~200 to 2000 cells are formed from single non-stem eukaryotic cells which appear to be the earliest "transition" cells of a stable "turnover unit" of 8000 cells similar to the 8000 cell crypts observed in human colonic adenocarcinomas[2]. Colonies derived from metakaryotic stem cells, in contrast, exceed 4000 cells three weeks post-treatment and, on trypsinized transfer, continue to grow, "immortalized" by the presence of the metakaryotic stem cells. Thus large colonies obtained in this clonal assay contain a metakaryotic cell which allows for continued division of the cells beyond what would be possible for a colony derived from a eukaryotic cell.

Figure 4a shows decreasing large colonies observed/flask exposed to a range of metformin concentrations for 5 weeks. Varying both concentration and duration of exposure revealed conditions specifically toxic to metakaryotic stem cells between ~500-2000- microMolar weeks. Replating colonies after exposures between 500 and 2000 micromolar weeks showed that no immortal cells survived treatment. This is interpreted as a specific metakaryocidal effect. Above 2000 micromolar weeks the eukaryotic cells that form mortal colonies were also killed (Figure 4b). Some nine drugs have been identified in this way.

**Figure 4**: **Metakaryote clonal assay.** HT-29 cells after 5 week exposure to metformin. (a) Clonal assay - cells were initially seeded at ~800 total cells resulting in an average of ~375 total large colonies. (b) Five weeks at ~100 microM or two weeks at~250 microM reduced the total number of large colonies by ~ 10%

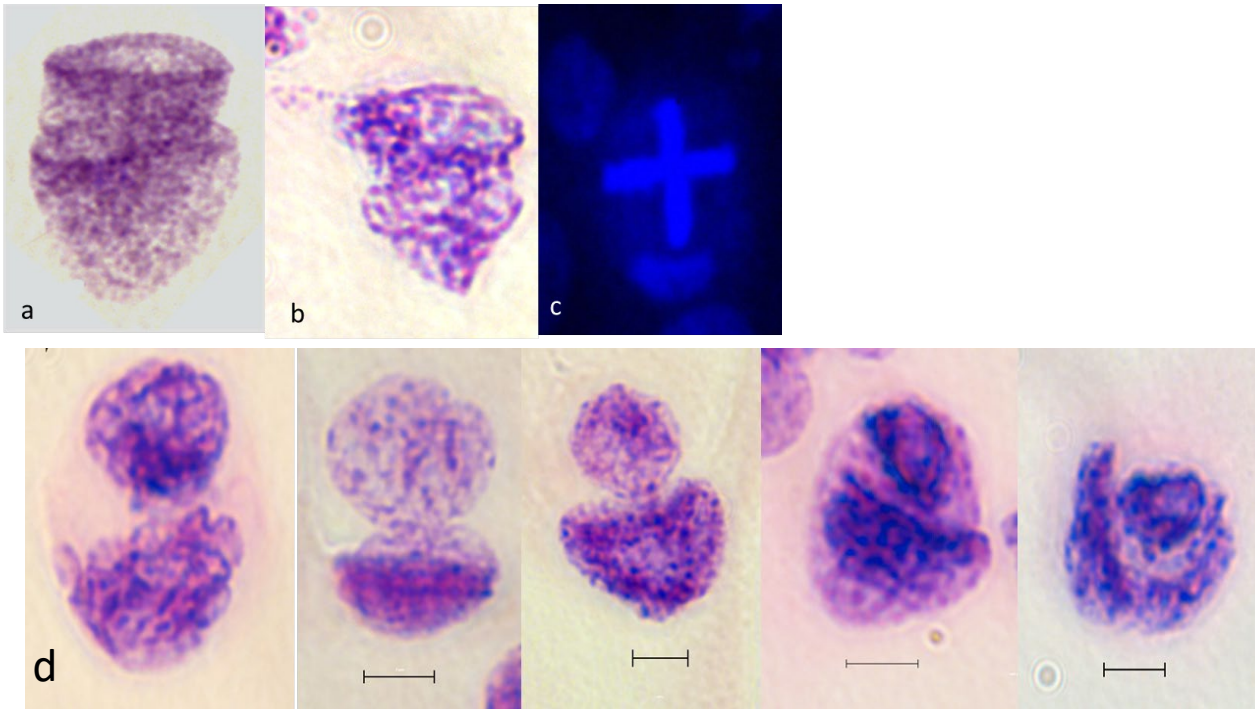


*Cytometric assays*

Following a prolonged (at least 2 week) period of time of drug exposure at concentrations well below plasma levels associated with frank toxicity, dead metakaryotic cells could be identified and enumerated by the appearance of the chromatin of the metakaryotic nuclei, well dispersed in healthy growing cells (Figure 5a) condensed into thick rope-like structures easily identified by cytometry (Figure 5b, 5d). With verapamil an additional marker of cytotoxicity was observed (Figure 5c) when treated cultures were stained with the dye Hoechst 33342. First a single bright blue bar then a cross (shown) and a three bar asterisk appeared in one to three weeks of verapamil treatment. For all metakaryocides found to date exposure of at least two weeks has been required to eliminate all immortal colony forming metakaryotic cells.

**Figure 5**: **Cytometric appearance of failed metakaryotic division.** (a) Untreated metakaryotic nuclei undergoing symmetrical amitosis (human fetal hindgut, (b) representative appearance of metakaryote after treatment with 200 micromolar metformin for two weeks (HT-29 cells). Cells were fixed and stained with Feulgen reagent that renders dsDNA purple. (c) Peculiar condensed form of Hoechst 33342 stained chromatin in the cytoplasm of a metakaryotic stem cell after two weeks treatment of HT-29 cells with verapamil. At this point metakaryotic cells do not further divide and bell shaped nuclei collapse. (d) Capan-1 cells treated with verapamil for two weeks. Feulgen staining demonstrates pyknotic nuclear forms. Further degradation of metakaryotic nuclei is observed.




## 2.5 Identification of metakaryocidal agents

The investigators at MIT began to test a variety of drugs on the cell cultures from pancreas (CAPAN-1) and colon (HT-29) cells. Initial investigation included several classes of chemotherapeutic agents, including antimetabolites, alkylating agents, and anti-tumor antibiotics. As expected, these agents had no effect on metakaryotic cells. The chemotherapeutic agent, gemcitabine, was effective killing eukaryotic but not metakaryotic

stem cells. However, nine drugs were identified that produced clearly specific metakaryocidal effects when treated for five or more weeks at concentrations in the range of FDA recommended plasma concentrations or somewhat higher plasma concentrations. In all cases the effective concentrations required to kill metakaryotic stem cells were well below the concentrations associated with clinical toxicity. Importantly, the eukaryotic cells were not immediately killed by conditions that killed metakaryotes, offering the possibility that they might be used in patients without the toxicities associated with chemotherapy and radiation therapy.

The concentrations of most metakaryocidal drugs required to eliminate metakaryotes after a five week exposure are well below the levels reported as toxic to humans but are above the FDA recommended level for ordinary use (acetaminophen, celecoxib, metformin, verapamil). Roughly 2 to 8 fold higher plasma levels might be required to create a potent metakaryocidal regimen for these drugs. One drug appears to be metakaryocidal within the approved plasma concentration range, doxycycline. (Figure 6 and 7)

**Figure 6. Doxycycline Clonal Assay.** Doxycycline maintained at a concentration as low as 3 - 10 micromolar for aduration of two weeks, reduced the fraction of large HT-29 cell colonies to about 90% indicative of a metakaryocidal effect. At concentrations at or above 40 micromolar colonies initiated by a eukaryotic cell were also killed. (Pink symbol indicates small surviving colonies.)

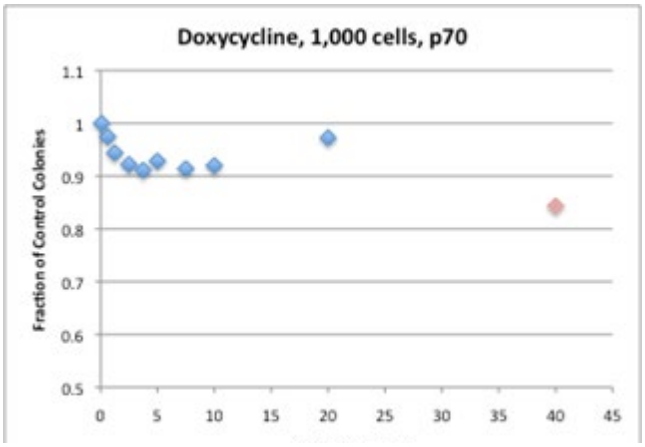
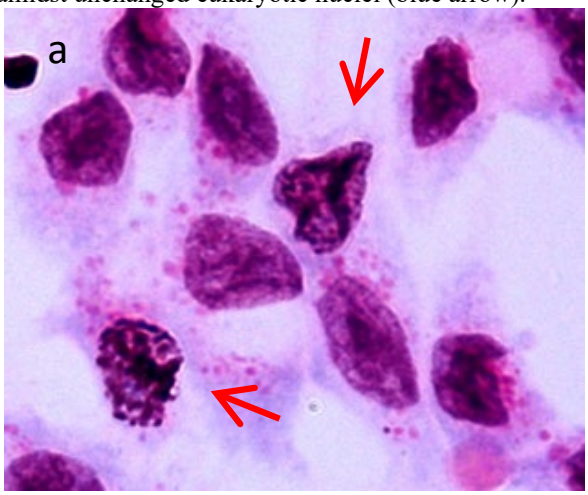
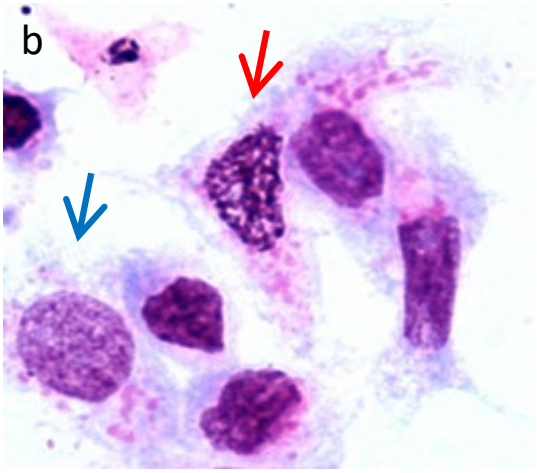



**Figure 7. Cytometric Response to Doxycycline.** HT-29 cells after 2 weeks in the presence of (a) 11.3 or (b) 22.6 micromolar doxycycline. Metakaryotic (bell shaped) show contracted, "pyknotic" metakaryotic nuclei (red arrow) amidst unchanged eukaryotic nuclei (blue arrow).



Dr. Elena Gostjeva (MIT) has received some ninety (90) deidentified samples of pancreatic adenocarcinomas and metastases from MCW colleagues in the past several years. Using her protocol for detecting and imaging metakaryotic cells and distinguishing between live and dead metakaryotic cells she has observed many hundreds of metakaryotic cells in samples from those patients who had received radiotherapy and or chemotherapy prior to surgery. In none of these samples has she detected a dead or dying metakaryotic cell, a result extending and confirming her original observations in human lung tumor samples after radiotherapy and chemotherapy.

In this proposed clinical trial, she and a cytopathologist (B. Hunt) will evaluate each patient sample until one hundred metakaryotic nuclei have been observed per sample. Each image of a live or dead metakaryotic cell will be electronically recorded so that (a.) other observers may confirm or dispute each reported observation and (b.) the images may be used in training new personnel.

The ultimate goal of the use of drugs found to kill human metakaryotic cancer stem cells in culture is to eradicate cancer stem cells in the patient to affect a cure. However the effect of doxycycline cannot be predicted based on its plasma concentration alone. There may be pharmacokinetic barriers to doxycycline penetrance into tumor or particular areas of tumors due to low vascularization, e.g. mesenchyme, or extracellular matrices that slow or prevent drug diffusion to the target stem cells.

The target level of 20% is one that would yield at least 10 dead metakaryotic nuclei in a tumor from which 100 total metakaryotic cells had been observed and imaged. As it is proposed to scan each tumor for 100 metakaryotic cells, no data of a sighting of one (1) or more dead metakaryotic nuclei will be lost to analysis.

It should be clearly noted that a clinical correlation such as significantly extended lifespan is possible but not expected at this stage of research. Here the experiment seeks to discover if the metakaryocidal drug doxycycline kills any significant fraction of the metakaryotic cells found in treated pancreatic tumors. Such a result would suggest the next steps to be taken including testing of increased levels and duration of doxycycline treatment, testing of other metakaryocidal drugs commonly used for other medical purposes and testing of methods to increase penetrance of metakaryocidal drugs to the target cancer stem cells in tumors and metastases.

In summary, our collaborators at MIT have demonstrated that metakaryotic cells are self-renewing, pluripotent, and resistant to chemotherapy and radiotherapy. Importantly, the metakaryotic process of amitotic division offers a logical explanation for the resistance of these cells to anti-mitotic therapies (chemotherapy and radiotherapy). Therefore targeting metakaryotic cells may decrease cancer relapse and metastases and the development of anti-metakaryotic is vital particularly for pancreatic cancer patients who are at risk for disease recurrence and cancer-related death. Development of a targeted cancer stem cell therapy potentially benefits patients at all stages of disease from operable to metastatic. By targeting the majority of tumor mass (eukaryotic cells) with the increasingly effective cytotoxic therapies in addition to targeting cancer stem cells (metakaryotic cells) we expect a resultant prolongation of survival and possibly durable eradication of disease.

In this proposal, we propose to test doxycycline for in vivo efficacy. We propose to discover if doxycycline will kill the metakaryotic stem cells in human pancreatic tumors. We hypothesize that doxycycline can be administered to pancreatic cancer patients in regimens that deplete and possibly eliminate their cancer stem cell populations and result in prolonged disease-free and overall survival. To do this, we propose to examine the efficacy of doxycycline to effect metakaryocidal death in a primary pancreatic cancer tumor. We propose to administer doxycycline during the 8 week period of neoadjuvant therapy for patients with resectable pancreatic cancer and analyze the resected pancreatic specimen for cytologic evidence of metakaryocidal death. A time frame of 8-10 weeks was selected because following neoadjuvant chemoradiation, surgery is usually performed at a minimum of 4 weeks from the end of radiation. To avoid a rebound effect of metakaryotic proliferation during this period, we plan to continue doxycycline during this period, allowing a 5-7 day washout period prior to surgery. Pancreatic cancer patients will receive oral doses of doxycycline for 8-10 weeks to maintain their plasma concentrations at the upper level of the FDA recommended plasma concentrations. Sections of tumors will be removed at the time of surgery and studied using the cytometric assays previously described by Dr. Gostjeva[2,5] to discover if the treatments have killed or disrupted the metakaryotic cells.

Because the metakaryotic stem cells of human organogenesis have been found to have very high mutation rates[6,7] it is anticipated that any pancreatic tumor will contain cancer stem cells will develop resistance to any single metakaryocidal drug. It is anticipated that treatment with a series or a combination of metakaryocidal drugs (e.g. 4, 5 or 6) would be required to kill all of the cancer stem cells in a pancreatic or other form of tumor. However, the effectiveness of each potentially metakaryocidal drug must be tested and authenticated before it could be expected to be useful in such anticipated multi-metakaryocide therapy. Thus in a first series of trials each drug found to be metakaryocidal to tumor stem cells in adenocarcinoma derived cell lines at levels expected to be well tolerated by patients will be individually tested in order to discover if they are in fact killing metakaryotic cancer stem cells in resected pancreatic tumors.

**2.6 Doxycycline Hyclate** Doxycycline hyclate is a member of the tetracycline class of antibiotics. It has a chemical designation of 4-(dimethlamino)-1,4,4a,5,5,6,11,12A-octahydro-3,5,10,12,12A-pentahydroxy-6-methy-1,11-dioxo-2-naphthacenecarboxamide monohydrochlorde. The empirical formula is $(C_{22}H_{24}N_2O_8HCl)_2C_2H_6OH_2O$ and the molecular weight is 1025.89. It is a yellow to light-yellow crystalline powder which is soluble in water.

**Pharmacology of Doxycycline**

Tetracyclines are readily absorbed and are bound to plasma proteins in varying degree. They are concentrated by the liver in the bile, and excreted in the urine and feces at high concentrations and in a biologically active form. Doxycycline is primarily a bacteriostatic antibiotic. The main mechanism of action of doxycycline is on protein synthesis. Doxycycline passes directly through the lipid bilayer of the bacterial cell wall and an energy dependent active transport system pumps the drug through the inner cytoplasmic membrane. Once inside the cell doxycycline inhibits protein synthesis by binding to 30S ribosomes and prevents the addition of amino acids to the growing peptide chain. Doxycycline will impair protein synthesis in the 50S ribosomes of mammalian cells at very high concentrations but these cells lack the active transport system

found in bacteria. However, the mammalian mitochondrial 30S ribosomes may preserve a doxycycline binding site and its action may be mediated by inhibition of mitochondrial protein synthesis in stem cells.

**Pharmacokinetics**

*Absorption and Bioavailability*

Doxycycline is virtually completely absorbed after oral administration and is not subject to presystemic metabolism. The mean bioavailability is approximately 93%. Following a 200 mg dose, normal adult volunteers averaged peak serum levels of 2.6 mcg/mL of doxycycline at 2 hours, decreasing to 1.45 mcg/mL at 24 hours.

*Distribution*

Tissue distribution is good and doxycycline has a strong affinity for renal and lung tissue. Plasma protein binding is in the range 82-93% and doxycycline is transferred into breast milk. The volume of distribution for doxycycline ranges from 0.9-1.8 $lkg^{-1}$ and the plasma half-life ranges from 18-22 hours.

*Metabolism and Elimination*

Excretion of doxycycline by the kidney is about 40% in 72 hours in individuals with normal function (creatinine clearance about 75 mL/min.). This percentage excretion may fall as low as 1–5% in 72 hours in individuals with severe renal insufficiency (creatinine clearance below 10 mL/min.). Studies have shown no significant difference in serum half-life of doxycycline (range 18–22 hours) in individuals with normal and severely impaired renal function.

**Safety Experience**

Doxycycline is used in the treatment of a variety of infections cause by susceptible strains of Gram-positive and Gram-negative bacteria and certain other microorganisms.

***Clinical experience with doxycycline 100 mg BID***

Review of treatment regimens using doxycycline shows that use of 200 mg/day or 2 x 100 mg/day in adults is a widely used therapeutic strategy that has been recommended by the US CDCP and international public health authorities for treatment of: anthrax, chlamydia, Lyme disease, malaria and tick borne rickettsial diseases for which direct citations from texts are provided here. No evidence of increased adverse effects were noted in treatment of Lyme disease patients for up to one month. Other than clear warnings regarding drug induced solar sensitivity, there appear to be no reports (Pub MED, ToxLine) of acute human toxicity associated with a prolonged exposure to doxycycline or doxycycline hyclate. This is notable as it has been in worldwide use since the 1940s. Cited immediately below is a report of an adult who was treated with 1000 mg/day of doxycycline for twelve years whose various systemic symptoms resolved after the drug was discontinued. However, the literature records several cases of doxycycline associated psychotropic responses including suicidal feelings, an example of which is cited below.

Chronic Doxycycline Intoxication (*J Intern Med. 1999 246:591-2*)

"We report the clinical case of a 12-years' intoxication by doxycycline. A patient with a depersonalization and derealization syndrome took 1 g doxycycline per day. In addition to hepatocellular necrosis with cholestasis, nephrotoxicity, leukopenia, anaemia and skin hyperpigmentation he suffered from hitherto unreported adverse cardiac events as intermittent supraventricular tachycardia and sporadic Wenckebach heart block. Despite a long period of self-medication these side-effects were reversible."

Anthrax **(**FDA Safety Alerts for Human Medical Products):
"The currently recommended dosage regimen of doxycycline for severe disease is 100 mg every 12 hours for adults and 1 mg per pound (2.2mg per kilogram) every 12 hours for children less than 100 pounds."

Chlamydial Infections (2010 CDCP Treatment Guidelines):
"Chlamydia treatment should be provided promptly for all persons testing positive for infection..." Recommended regimens: Doxycycline 100 mg orally twice a day for 7 days.

Lyme disease (J Am Acad Dermatol. 1995 32(2 Pt 1):223-7)
"Doxycycline is widely used to treat Lyme disease associated with erythema migrans. Whether it is comparable to tetracycline is unknown
We conducted a two-part retrospective analysis of (1) the safety and efficacy of doxycycline compared with tetracycline and (2) the safety and efficacy of a 14-day versus a 20-day course of doxycycline. Twenty-seven patients given tetracycline (500 mg four times a day for 14 days [group 1]) were compared retrospectively with 21 patients who received doxycycline (100 mg two or three times a day for 14 days [group 2]). The results for group 2 were also compared with that of 38 patients who received doxycycline for 20 days (100 mg three times daily) in a prospective treatment trial (group 3). There was no significant difference in the incidence of adverse drug effects or in efficacy at 1 month..."

Malaria **(**Am J Trop Med Hyg. 2011 84: 517–531):
"Doxycycline, a synthetically derived tetracycline, is a partially efficacious causal prophylactic (liver stage of *Plasmodium*) drug and a slow acting blood schizontocidal agent highly effective for the prevention of malaria. ... Although not recommended for pregnant women and children < 8 years of age, severe adverse events are rarely reported for doxycycline. This report examines the evidence behind current recommendations for the use of doxycycline for malaria and summarizes the available literature on its safety and tolerability." "Treatment: 100 mg twice a day for 7 days; must be used in conjunction with a fast acting schizontocide."

Polycystic Ovarian Syndrome (Completed randomized clinical trial: NCT01788215; University of Rochester). Assess the effectiveness of daily doxycycline use vs. placebo in the primary outcome of reduction of serum testosterone in women with PCOS at 12 weeks on study medication and upon conclusion of the study at week 24. Doxycycline dose 100 mg BID

***Clinical experience with doxycycline 200 mg BID***

Lyme Neuroborreliosis (*Antimicrob Agents Chemother, 1989; p1078-1080*) 10 patients treated with doxycycline 200 mg BID for 10 days. “Two patients complained of mild phototoxic reactions, none suffered adverse gastrointestinal reactions.”

Lyme Disease with Associated Facial Palsy and Meningitis (*Clin Infect Dis. 1999; p569-74*). Prospective clinical trial with 29 patients with Lyme disease and facial palsy. "Mean durations of treatment was 10.8 days (range: 9-17). Doxycycline dose was 200 mg BID for 28 of 29 patients and 100 mg BID for an 11 year old child."

Relapsed NHL (Open clinical trial: NCT02086591; University of Rochester). Assess the overall response rate to doxycycline monotherapy in patients with relapsed indolent and aggressive NHL at three months. Dose of doxycycline is 200 mg BID.

**Warnings and Precautions**

Consult the package insert for comprehensive toxicity information. Listed below are common drug associated toxicities.

*Photosensitivity*

Photosensitivity manifested by an exaggerated sunburn reaction has been observed in some individuals taking tetracyclines, including doxycycline. Patients likely to be exposed to direct sunlight or ultraviolet light should be advised that this reaction can occur with tetracycline drugs and treatment should be discontinued at the first evidence of skin erythema.

*Use in patients with impaired hepatic function*

Doxycycline should be administered with caution to patients with hepatic impairment or those receiving potentially hepatotoxic drugs. Abnormal hepatic function has been reported rarely and has been caused by both the oral and parenteral administration of tetracyclines, including doxycycline.

*Use in patients with renal impairment*

Excretion of doxycycline by the kidney is about 40%/72 hours in individuals with normal renal function. This percentage excretion may fall to a range as low as 1-5%/72 hours in individuals with severe renal insufficiency (creatinine clearance below 10ml/min). Studies have shown no significant difference in the serum half-life of doxycycline in individuals with normal and severely impaired renal function. Hemodialysis does not alter the serum half-life of doxycycline.

*Microbiological overgrowth*

The use of antibiotics may occasionally result in over-growth of non-susceptible organisms, including Candida. If a resistant organism appears, the antibiotic should be discontinued and appropriate therapy instituted. Pseudomembranous colitis has been reported with nearly all antibacterial agents, including doxycycline, and has ranged in severity from mild to life-threatening. It is important to consider this diagnosis in patients who present with diarrhea subsequent to the administration of antibacterial agents.

*Ionizing Radiation Sensitivity*

Of some 46,523 PubMed citations for doxycycline or tetracyclines, none were found to indicate any additive effects with x-rays or any other form of ionizing radiation. The UV sensitivity of patients treated with doxycycline appears to be mediated by a direct photochemical reaction of non-ionizing UV radiation with the drug that would not be expected with ionizing radiation such as x-rays.

**Drug Interactions**

1. Patients who are on anticoagulation therapy may require downward adjustment of their anticoagulant dose
2. Absorption of doxycyline may be impaired by antacids or other drugs containing aluminum, calcium, or magnesium, bismuth subsalicylate; iron-containing products and dairy projects. It is recommended that the doxycycline administration is separated by at least 2 hours before or after any of these products.
3. Concurrent use of doxycycline may render oral contraceptives less effective and a back-up method of birth control, such as a condom or a diaphragm with spermicide, or a contraceptive sponge is recommended.
4. Barbiturates, carbamazepine, and phenytoin decrease the half-life of doxycycline
5. Doxycycline may interfere with the bactericidal action of penicillin and co-administration should be avoided
6. Concurrent use of tetracyclines and methoxyflurane (Penthrane) has been reported to result in fatal renal toxicity
7. False elevations of urine catecholeamines may occur due to interference with the fluorescence test.
8. No drug interaction has been reported with gemcitabine.

## 3. PATIENT SELECTION

### 3.1 Eligibility Criteria

Patients must have histologically or cytologically confirmed pancreatic adenocarcinoma which may be acquired using a fine needle aspiration and who have not received any prior therapy. Patients must undergo either a dual phase pancreatic protocol CT scan or pancreatic protocol MRI to determine the clinical stage. Based on radiographic imaging, resectable pancreatic cancer will be defined by: (a) no radiographic evidence of arterial abutment of the celiac, superior mesenteric, or hepatic arteries, and (b) <50% tumor-induced narrowing of the superior mesenteric vein or portal vein. Patients with resectable pancreatic cancer will be eligible for the study.

Additional eligibility criteria include:

1. Patients will be receiving neoadjuvant therapy for resectable pancreatic cancer in anticipation of surgical resection. Resectable stage will be defined by radiographic criteria including; (a) no radiographic evidence of arterial abutment of the celiac, superior mesenteric, or hepatic arteries, and (b) <50% tumor-induced narrowing of the superior mesenteric vein or portal vein.
2. Patients who will receive neoadjuvant therapy (chemoradiation) are eligible.
3. Age ≥18 years.
4. ECOG performance status ≤2 (Karnofsky ≥60%).
5. Life expectancy of greater than 6 months

6. Patients must have normal organ and marrow function as defined below:
    a. leukocytes ≥3,000/mcL
    b. absolute neutrophil count ≥1,500/mcL
    c. platelets ≥100,000/mcL
    d. creatinine clearance ≥60 mL/min or creatinine ≤1.5 mg/dL
    e. Bilirubin ≤2mg/dL: If the subject's serum bilirubin is greater than two at the time of enrollment but the patient has demonstrated a progressive decline and is anticipated to be ≤2mg/dL prior to start of therapy they are eligible, at the discretion of the trial investigators and their treatment assignment.
7. Have no active or chronic infection with HIV, Hepatitis B or Hepatitis C
8. Ability to understand and the willingness to sign a written informed consent document.

### 3.2 Exclusion Criteria

1. Patients with more clinically advanced pancreatic cancer (borderline resectable, locally advanced, or metastatic)
2. Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements.
3. Pregnant women are excluded from this study because the effects of metakaryocidal agents have the potential for teratogenic or abortifacient effects.
4. Previous history of other malignancy (other than cured basal or squamous cell carcinoma of the skin or cured in-situ carcinoma of the cervix) within 2 years of study enrollment.
5. Active or chronic HIV, hepatitis B or hepatitis C
6. Patients who are receiving other investigational drugs or enrolled in other clinical trials
7. Inability to undergo scheduled blood acquisition per protocol.
8. Drug specific exclusion including history of allergic reactions to tetracyclines.
9. Prior treatment with doxycycline within a 7 day washout period prior to initiating treatment with alternate antimetakaryocidal medication.

### 3.3 Inclusion of Women and Minorities

Both men and women of all races and ethnic groups are eligible for this trial.

## 4. REGISTRATION PROCEDURES

### 4.1 General Guidelines

Eligible patients will be entered on study centrally at the Medical College of Wisconsin by the Study Coordinator. Patients must have a signed informed consent form prior to registration on study. Following registration, patients should begin protocol treatment within 28 days – allowing for coordination of radiation therapy. Issues that would cause treatment delays should be discussed with the Principal Investigator. If a patient does not receive protocol therapy following registration, the patient's registration on the study may be canceled. The Study Coordinator should be notified of cancellations as soon as possible.

### 4.2 Registration Process

Investigators or their appropriate designees will identify potentially eligible patients from their clinics, patient self-referrals, or referrals from other clinicians. Inclusion/exclusion criteria will be reviewed and patient eligibility will be confirmed after consent is signed.

If the patient (or LAR, legally authorized representative) agrees to study participation, first, he/she must provide signed informed consent for study screening. After signing a screening consent form, each patient will be assigned a unique subject number. The investigator will then review the patient's medical history, cardiac performance, and medication list to determine if there any contraindication to doxycycline therapy. If all criteria are met, the subject will undergo study treatment. Treatment must be initiated 0-4 weeks after registration.

To register a patient, the following documents should be completed by the study coordinator

- Copy of required laboratory tests, medication list
- Signed patient consent form
- Eligibility Screening Checklist

At the point of registration, the study staff will register the patient in the secure electronic database (OnCore), including on-study information. Any additional source documentation with personal health information will be stored on a secure folder maintained and accessed only by cancer center or study personnel with restricted access to the study.

## 5. STUDY DESIGN

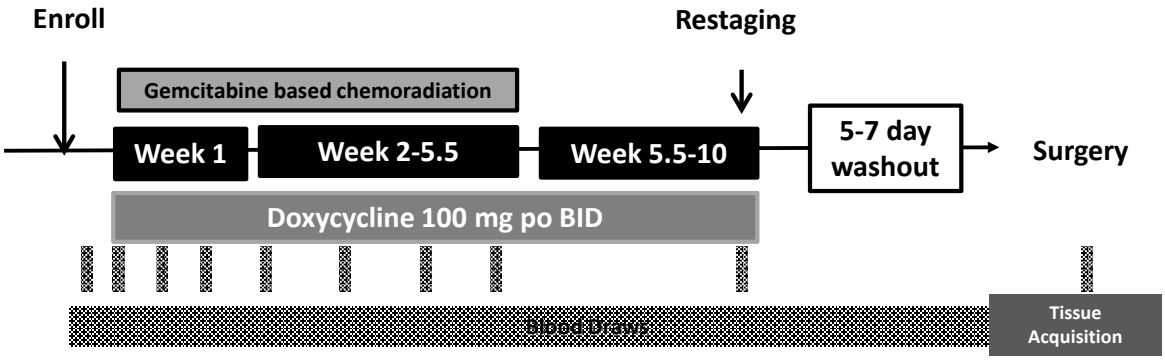
### 5.1 Treatment Plan

Treatment with doxycycline hyclate will be administered as an oral agent on an outpatient basis. Patients will receive doxycycline 100 mg twice daily for a period of 8-10 weeks (56-70 days). We plan to enroll 12 patients with anticipation of 10 patients completing all therapy and surgery. Following consent, patient will be referred to a radiation oncologist for consultation and treatment planning. A simulation CT scan will be performed and radiation planning will be performed at the discretion of the treating physician. Neoadjuvant therapy will consist of concurrent chemoradiation with gemcitabine 400 mg/m$^2$ given intravenously at a fixed dose rate, infused over 40 minutes on Day 1 (day-2 to +1), and then weekly x 6 during radiation. Radiation will be delivered using external-beam radiation. It will be delivered 5 days/week over 5.5 weeks with 6-18 meV photons. Using 3D conformal or IMRT techniques, patients will receive a total dose of 50.4Gy prescribed to the 95% isodose at 1.8 Gy/fraction (28 fractions). Doxycycline will be made available through the investigational pharmacy at MCW prior to the initiation of radiation therapy. Doxycycline hyclate 100 mg PO Q12 hours will be administered to the patient for a period of 8-10 weeks starting on the first day of radiation therapy.

Restaging imaging with either pancreatic protocol CT or MRI will be performed week 8 (+1) week to assess disease status. Patients will generally undergo surgery week 9-11. Doxycycline will be discontinued 5-7 days prior to surgery.

Upon registration (baseline) and at the first, third, and fifth days of doxycycline therapy, patients will be seen to obtain trough serum concentrations for pharmacokinetic studies. Additional serum levels will be checked on days 8, 15, 22, 29, and at restaging imaging to assess drug compliance and a final level will be checked at the time surgery, allowing for +/- 1 day window for any timepoint after the first week (days 7-9, 14-16, 21-23, 28-30, restaging +/- 1 day, surgery +/- 1 day). (Figure 9)The patient will be requested to maintain a medication diary of each dose of

**Figure 9: Study Schema**




medication to assess compliance. Reported adverse events and potential risks are described in Section 6. No investigational or commercial agents or therapies other than those described below may be administered with the intent to treat the patient's malignancy.

**5.2 Surgery**

Since surgery is usually performed 3-5 weeks after the completion of chemoradiation, there will be no delay in the timing for surgery for the patients who are enrolled. We anticipate that they will complete the study drug 2-4 weeks after the completion of chemoradiation and will undergo surgery 3-5 weeks after completion of chemoradiation.

Extent of surgical resection will be performed at the discretion of the treating surgeon. Pancreatectomy will be performed at the Medical College of Wisconsin by hepatobiliary surgeons (DBE, KKC, ST,) as previously described.[8] Approximately 75% of the tumor will be removed for evaluation of doxycycline treatment effect, the remaining 25% of the pancreatic tumor tissue except for margins and lymph nodes will be used for pathologic assessment and immediately fixed in Carnoy's solution. (See 10.4 Specimen Collection)

**5.3 Doxycycline pharmacokinetics**

Blood drawn for pharmacokinetics will be drawn in 8mL purple top tubes with EDTA and immediate processed within 2 hours of blood draw into plasma components. Plasma will be frozen at -80°C and shipped to an external contractor(s) for analysis using mass spectrometry (Wisconsin State Hygiene laboratory, Hepatochem). Twelve serial samples will be acquired for each patient. The data regarding serum concentrations of doxycycline will be reported to the data manager.

**5.4 Pathologic Assessment**

Tissues will be sent to either Dr. Tsai laboratory on the MCW campus for tissue processing as previously described by Gostjeva et al.(3) or assessment of metakaryote protocol will be performed by a cytogeneticist at MIT. Digital images will be made with annotation of the location and number of dead/dying metakaryotic nuclei. Images will be sent back to the MCW and an independent blinded pathologic examination will be performed by a board-certified cytopathologist. In addition, the histopathologic response of the primary tumor will be assessed using the College of American Pathology criteria for residual tumor response following neoadjuvant therapy for the exocrine pancreas. All slides will be stored at the Medical College of Wisconsin.

**5.5 General Concomitant Medication and Supportive Care Guidelines**

*Supportive care for doxycycline*

Anticipated toxicities with doxycycline include gastrointestinal discomfort and/or diarrhea and photosensitivity. If patient develops diarrhea, a stool specimen will be collected to evaluate for clostridium difficile, ova and parasite infection. In the absence of infection, anti-diarrheal agents may be initiated. Loperamide 4 mg may be taken after loose stools every 12 hours titrating up to 16 mg/24 hr as needed. In anticipation of photosensitivity, patients will be advised to avoid sun exposure and consistently use sun protection with protective clothing and broad-spectrum sunscreens with coverage in the UV-A and UV-B ranges. Topical corticosteroids and cool compresses may alleviate drug-induced photosensitivity. The use of systemic corticosteroids will be reserved for the most severe cases.

*Supportive care during radiation therapy*

1. Proton pump inhibitor BID throughout treatment up to 1 month post treatment.
2. Zofran 8 mg PO 1 hour prior to each radiation treatment. May take 8 mg TID if needed.
3. Intravenous fluids prn
4. Creon, simethicone, Reglan, Decadron on a per patient basis
5. Short or long acting analgesia for upper abdominal discomfort
6. Gas-X or Beno prn in addition to Creon for increased gas

If patient develops gastritis or duodenitis near end of treatment or within 2 weeks of completion, IV fluids, histamine receptor blockers, or antiemetics may be administered. Gaviscon liquid between meals or Carafate slurry may be added as needed.

Concomitant treatment with other tetracyclines should be avoided unless there is no appropriate alternative medication for the patient to use.

**5.6 Duration of Therapy**

In the absence of treatment delays due to adverse event(s), treatment with the study drug may continue for up to 10 weeks or until one of the following criteria applies:

Disease progression which precludes surgical exploration

Intercurrent illness that prevents further administration of treatment,

Unacceptable adverse event(s)

Patient decides to withdraw from the study

General or specific changes in the patient's condition render the patient unacceptable for further treatment in the judgment of the investigator.

**5.7 Duration of Follow Up**

Patients will be followed up to 60 days from the time of study termination using review of medical records or telephone contact to assess for adverse events. Adverse events up to 60 days after the last dose of investigational agent will only be recorded if deemed possibly, probably, or definitely related to the investigational agent. Patients removed from study for unacceptable adverse event(s) will be followed until resolution or stabilization of the adverse event.

**5.8 Patient Discontinuation**

Patients may discontinue treatment if any of the following occur: (1) recurrent or metastatic disease, (2) unacceptable toxicity, (3) patient withdrawal of consent, (4) investigator decision (see section 6; dose delays), or (5) non-compliance. The reason for study removal and the date the patient was removed must be documented in the Case Report Form if an investigator removes a patient from the study or if the patient declines further participation, a final assessment of the patient’s disease status should be performed prior to any therapeutic intervention. These results, along with a reason for study discontinuation will be recorded Patients will be monitored for up to 60 days after discontinuation for adverse events as long as

the patient agrees.The entire trial will be stopped if evidence emerges that makes the study continuation unnecessary or unethical, or when the stated objectives are achieved.

## 6. DOSE DELAYS/DOSE MODIFICATIONS

If toxicities occur, it may be difficult to identify whether this is related to the doxycycline or the radiation therapy. However, if the severity of the toxicity exceeds the level commonly observed during pancreatic cancer irradiation, the toxicity will be ascribed to doxycycline and the drug reductions or discontinuation will be managed as below.

| **Dose Level** | ***Doxycycline* Dose** |
|---|---|
| -1 | *50 mg po Q12H* |
| 0 | *100mg po Q12H* |

| **Diarrhea** | **Management/Next Dose for *Doxycycline*** |
|---|---|
| | |
| ≤ Grade 1 | No change in dose |
| Grade 2 | Hold until ≤ Grade 1. Resume at same dose level. |
| Grade 3 | Hold* until < Grade 2. Resume at one dose level lower, if indicated.** |
| Grade 4 | Off protocol therapy |
| *Patients requiring a delay of >2 weeks should go off protocol therapy.<br>**Patients requiring > two dose reductions should go off protocol therapy. | |
| Recommended management: Loperamide antidiarrheal therapy<br>Loperamide dosage schedule: 4 mg at first onset, followed by 2 mg with each loose motion until diarrhea-free for 12 hours (maximum dosage: 16 mg/24 hours)<br>Adjunct anti-diarrheal therapy is permitted and should be recorded when used. | |

| **Liver Function** | **Management/Next Dose for *Doxycycline*** |
|---|---|
| ≤ Grade 1 | No change in dose |
| Grade 2 | Hold until ≤ Grade 1. Resume at same dose level. |
| Grade 3 | Hold* until < Grade 2. Resume at one dose level lower, if indicated.** |
| Grade 4 | Off protocol therapy |
| *Patients requiring a delay of >2 weeks should go off protocol therapy.<br>**Patients requiring > two dose reductions should go off protocol therapy. | |

| **Phototoxicity** | **Management/Next Dose for *Doxycycline*** |
|---|---|
| ≤ Grade 1 | No change in dose |
| Grade 2 | Hold until ≤ Grade 1. Resume at same dose level. |
| Grade 3 | Hold* until < Grade 2. Resume at one dose level lower, if indicated.** |
| Grade 4 | Off protocol therapy |
| *Patients requiring a delay of >2 weeks should go off protocol therapy.<br>**Patients requiring > two dose reductions should go off protocol therapy. | |

## 7. ADVERSE EVENTS: LIST AND REPORTING REQUIREMENTS

### 7.1 Definitions

*Adverse Event*

Adverse event is defined as any undesirable sign, symptom or medical condition occurring after starting the investigational drug even if the event is not considered to be related to the study. Medical conditions/diseases present before starting the study treatment are only considered adverse events if they worsen after starting the study treatment. Adverse events occurring before the start of treatment but after the signing of consent will be recorded. Abnormal laboratory values or test results constitute adverse events only if they include clinical signs or symptoms or require therapy.

*Serious Adverse Events*

A serious adverse event is an undesirable sign, symptom, or medical condition which:

- Results in death
- Is life-threatening (an event in which the subject was at risk of death at the time of the event)
- Requires inpatient hospitalization or causes prolongation of existing hospitalization
- Is a congenital anomaly or birth defect
- An important medical event, e.g. intensive treatment in an emergency room
-

*Unexpected adverse event*

An adverse event, which varies in nature, intensity, or frequency from information on the investigational drug provided in the Investigator's brochure, package insert, or safety reports. Any adverse event that is not included in the informed consent is considered "unexpected".

*Expected adverse event*

An adverse event, which has been reported in the Investigator's Brochure. An adverse event is considered "expected" if it is included in the informed consent document as a risk.

### 7.2 Relationship

*Attribution of the AE:*

- Definite – The AE *is clearly related* to the study treatment.
- Probable – The AE *is likely related* to the study treatment.

- Possible – The AE *may be related* to the study treatment.
- Unlikely – The AE *is doubtfully related* to the study treatment.
- Unrelated – The AE *is clearly NOT related* to the study treatment.

**7.3 Reporting of Adverse Events**

All grade 3-4 adverse events (AE) occurring during the study protocol will be recorded. The National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE) version 4.0 will be used to grade adverse events. Grade 3-4 events must be recorded noting severity and causality. Any consequent changes to the dosage schedule or any corrective therapy should be recorded.

Adverse events will be recorded on electronic Case Report Forms (eCRFs) in OnCore, our clinical trial management system. SAEs requiring expedited reporting to the IR (unexpected and at least possibly related to the intervention) will be entered into OnCore and reported to the IRB within 5 calendar days of knowledge of the event. SAEs not requiring expedited reporting will be entered into OnCore for annual review by the IRB. SAEs will be followed until resolution and the eCRFs will be updated.

The trial will be stopped if more than 5 patients have to discontinue the therapy due to treatment related adverse events.

**7.4 Study Risks**

The known risk associated with participating in this study is related to the administration of doxycycline in conjunction with neoadjuvant chemoradiation. The administration of radiation therapy is per a standard protocol and not considered experimental. During the neoadjuvant treatment period, patients are monitored closely for treatment related toxicity, including a weekly radiation review. During this time, unanticipated toxicities related to the combination therapy of doxycycline and radiation therapy will be monitored. There is an additional risk of serial blood draws for pharmacokinetic monitoring which will involve obtaining up to 10 blood draws of 5-10 cc of extra blood at several time points during the study.

**7.5 Risk Discussion**

This study is assessing the efficacy of doxycycline as a potential selective cancer stem cell therapeutic. The risks with prolonged therapy with doxycycline include gastrointestinal discomfort (abdominal discomfort, cramping, diarrhea), infectious colitis, and photosensitivity. These risks will be reviewed with the subject at the time of consent and symptom monitoring will be performed on a weekly basis for the first 5weeks, then every other week until week 9, and at the first postoperative visit (30-60 days following discontinuation of investigational agent).

**7.6 Study Benefits**

This is a new treatment approach, and the exact degree of efficacy is unknown. The patient may benefit from participating in this study if doxycycline is effective at killing cancer stem cells, however, since it is anticipated that several such agents will be necessary to prevent future relapse, it is uncertain what benefit participation in the trial may bring. The knowledge gained from this study may benefit future patients with pancreatic adenocarcinoma.

**8. STUDY CALENDAR**

***Schedules shown in the Study Calendar below are provided as an example and should be***

***modified as appropriate.***

Pre-study evaluations are to be conducted within 6 weeks prior to start of protocol therapy. Scans and x-rays must be done ≤6 weeks prior to the start of therapy. Weekly monitoring will include medication review, physical examination, and laboratory studies as described below and will be performed in conjunction with the weekly radiation therapy review or at the time of the hematology oncology assessment within 7 days of the previous assessment. In the event that the patient's condition is changes and adverse events occur as described in section 7, laboratory evaluations should be repeated prior to the resumption of therapy.

| | Pre-Study | Wk 1 | Wk 2 | Wk 3 | Wk 4 | Wk 5 | Wk 6 | Wk 7 | Wk 8!! | Wk 9-11 Surgery | Routine postop Visit | Off Study |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Doxycycline 100 mg PO BID & Diary* | | X | X | X | X | X | X | X | X | X# | | |
| Radiation therapy | | X | X | X | X | X | X | | | | | |
| Informed consent | X | | | | | | | | | | | |
| Demographics | X | | | | | | | | | | | |
| Medical history | X | | | | | | | | | | | |
| Height | X | | | | | | | | | | | |
| Cross-sectional Imaging Study (CT or MRI) | X | | | | | | | | X | | | |
| Pathologic Assessment of Surgical Specimen | | | | | | | | | | X | | |
| Concurrent Meds* | X | X | X | X | X | X | X | X | X | | | |
| Physical exam* | X | Xx3† | X | X | X | X | X | X | X | | | |
| Vital signs* | X | X | X | X | X | X | X | X | X | | | |
| Weight* | X | X | X | X | X | X | X | X | X | | | |
| Performance status* | X | X | X | X | X | X | X | X | X | | | |
| CBC w/diff, plts* | X | X | X | X | X | X | X | X | X | | | |
| Basic Chemistry and LFT* | X | X-† | X | X | X | X | X | X | X | | | |
| Investigational Blood Draw§ | X | Xx3† | X | X | X | X | | | X | X | | |
| Adverse event evaluation* | | X | X | X | X | X | | X | | X | X | |

* With the exception of the first week, serial assessments of medications, PE, performance status, labs, and adverse event evaluation will coincide with weekly radiation therapy assessments and occur within a 7 day window of the previous assessment. Additional assessments may be performed as clinically indicated at the discretion of the treating physician. Final adverse event assessment will occur at the routine postop visit between (30-60 days from last dose of investigational agent).

†During the first week, serum concentrations of doxycycline will be measured every other day for the first 5 days.

§ Investigational blood draws must be performed at baseline and on days 1, 3, 5, 8, 15, 22, 29, at the time of restaging prior to surgery and at the time of surgery. A window of +/- 1 day will be allowed for assessment of serum concentrations of doxycycline after the first week.

# Doxycycline is tied to surgery and will be completed 5-7 days prior

!! Assessments due on week 8 will be allowed within +1 week window of week 8.

## 9. DRUG PROCUREMENT

### 9.1 Drug Dispensing

The study drug will be provided by the investigational pharmacy at the clinical cancer center of Froedtert Hospital. The study drug will be labeled and handled as open-lab material, and packaging labels will fulfill all requirements by governing regulations. Drugs will be dispensed in child-resistant containers. Upon receipt of the study drug, patients will be instructed to take the medication as directed. All study drug costs will be covered by the study (funding provided by the Batterman Foundation) and patients will not be responsible for the cost of the study drug.

## 10. MEASUREMENT OF EFFECT

Based on the previous assessments of resected surgical samples from patients treated with neoadjuvant radiation, no metakaryotic treatment has been previously observed. Assessment of the efficacy of doxycycline will be performed utilizing cytometric characteristics as outlined in section 2.4-2.5. Quantitation of the number of dead/dying metakaryotic cells per 100 metakaryotic cells in at least 1 gram of tissue will be performed. Digital image acquisition is mandatory for every dead/dying metakaryotic cell identified.

### 10.1 Definitions

*Evaluable for toxicity.*

All patients will be evaluable for toxicity from the time of their first treatment with the study drug.

*Evaluable for metakaryote cell death*:

Only patients who undergo surgical resection of the primary tumor will be considered evaluable for study drug effect. These patients will have their response classified according to the definitions below.

*Evaluable non-target disease response.*

Patients may be taken to the operating room without radiographic evidence of metastatic disease but at the time of diagnostic laparoscopy are found to have liver or peritoneal metastases. In such patients, resected metastatic lesions will be evaluable for non-target disease response per the definitions below.

A patient will be deemed unevaluable if: 1) patient has an insufficient amount of tissue removed for analysis or 2) patient experienced disease progression while on therapy and could not complete the designated therapy. Based on our experience, ~90% of patients are expected to complete all neoadjuvant therapy and undergo surgery.

### 10.2 Methods for Evaluation of Measurable Disease

Histologic confirmation of pancreatic adenocarcinoma of adjacent tissue will be confirmed by a histopathologist at the time of tissue acquisition. Only specimens with confirmed adenocarcinoma will be assessed for study drug response. Metakaryotes will be enumerated in serial sections of pancreatic parenchyma. The nuclei will be assess for changes consistent with cell death (pyknotic nuclei, nuclear fragmentation) and enumerated. The number of dead/dying metakaryotes per 100 metakaryotes will be assessed for each specimen.

## 10.3 Response Criteria

Evaluation of Metakaryote Cell Death

Cytometric criteria of dead/dying metakaryotic cells are based on assessment of the nuclear structure. Specifically, metakaryotic cells will be identified by previously described cellular morphologic criteria: (1) presence of an unusual large, hollow, bell-shaped nuclei, and (2) eccentric placed nuclei in relation to the cytoplasm. Characteristics of dead/dying metakaryotes include: (1) demonstration of pyknotic nuclear forms using Feulgen staining (figure 7a,b) or (2) failure of nuclear separation (figure 5b). Drug response will be assessed as the proportion of dead/dying metakaryotes out of 100 metakaryotic cells.

## 10.4 Specimen Collection

Blood collection and tissue collection is mandatory. The following must be provided in order for the case to be evaluable.

1. Serum
    a. One Red Top tube A at all investigational blood time points
        i. Within 1-2 hours of collection, spin in a standard clinical centrifuge at ~2500 RPM for 10 minutes at 4C.
        ii. Aliquot 1 mL serum into as many cyrovials as are necessary labeled with study ID, collection date, and clearly marked as serum
        iii. Freeze cyrovials at -70 to -90 C until ready to ship.
        iv. Ship specimens on dry ice to Wisconsin State Hygiene Lab and/or Hepatochem.
2. Tissue
    a. All pancreatic tumor tissue except for margins and lymph nodes for clinical evaluation will be removed from the pathologic specimen at the time of tissue accession
        i. One H&E stained slide will be provided from the adjacent specimen
        ii. Tissue will be placed in Carnoy's fixative (ethanol: acetic acid) for 24 hours
        iii. Tissue will then be placed in 70% methanol for storage
        iv. Specimens will be labeled with study ID, collection date, and clearly marked as pancreatic tumor.
        v. Specimens will be shipped on ice to the Thilly/Gostjeva Laboratory
3. Specimen Collection Summary

| Specimens Collection | | | |
|---|---|---|---|
| Specimens taken from patient | Collected when: | Submitted as: | Shipped: |
| Serum: ~ 3mL | Pre-treatment, Day 1, 3, 5, then week 2-5, restaging, and at surgery | Frozen serum samples containing 1mL per aliquot in 1 mL cryovials | Serum sent frozen on dry ice via overnight carrier |
| TISSUE: ~1gram | At the time of surgery | In 70% methanol following Carnoy's fixation for 24 hours with | Shipped on ice via overnight carrier |

| | | | |
|---|---|---|---|
| | | corresponding H&E slide | |

## 11. DATA REPORTING / REGULATORY REQUIREMENTS

Adverse event lists, guidelines, and instructions for AE reporting can be found in Section 7.0 (Adverse Events: List and Reporting Requirements).

### 11.1 Data Reporting

#### 11.1.1 Method

Study data will be recorded in OnCore, and study coordinators are responsible for keeping information in OnCore up to date. Every 6 months, the Data and Safety Monitoring Committee will download a summary report from OnCore and review the study for scheduled monitoring.

#### 11.1.2 Responsibility for Data Submission

Study coordinators are responsible for submitting CDUS data and/or data forms to DMSB on the study quarterly. When setting the dates, allow time for Coordinating Center compilation, Principal Investigator review, and timely submission to DSMB by the quarterly deadlines (see Section 12.1.1)

#### 11.1.3 Retention of Study Documentation

The Investigator must maintain adequate and accurate records to enable the conduct of the study to be fully documented and the study data to be subsequently verified. The patient's involvement in the study should be clearly documented in the clinical records. Details are to include the protocol number, the patient's identification number, the patient's consent(s) to take part in the study, the date of all study visits and the dates of treatment. Copies of CRFs must be retained by investigators, for as long as legally required after completion of the study. All study data including master files, CRFs and source data will be stored for at least 10 years according to MCW Office of Research SOP (see Appendix E).

### 11.2 Data Monitoring

This study will be reviewed by the Medical College of Wisconsin Cancer Center Data and Safety Monitoring Committee (MCWCC DSMC). A summary of the MCWCC DSMC activities are as follows:

1. Review the clinical trial for data integrity and safety.
2. Review all unexpected grade 3, and all grade 4, and 5 adverse events, as well as any others requiring expedited reporting as defined in this protocol. (Grades 4 & 5 events must be reported to the DSMC within 5 calendar days of study staff's knowledge.)
3. Review all DSM reports.
4. Submit a summary of any recommendations related to study conduct.
5. Terminate the study if deemed unsafe for patients.

A copy of the MCWCC Data and Safety Monitoring Plan and membership roster will be maintained in the study research file and updated as membership changes. The committee will review reports from the study PI twice annually (or more frequently if needed) and provide recommendations on trial continuation, suspension or termination as necessary.

Any available DSMC letters will be submitted to the IRB of record as required.

### 11.3 Collaborative Agreements Language

Agent(s) may not be used for any purpose outside the scope of this protocol, nor can Agent(s) be

transferred or licensed to any party not participating in the clinical study. Collaborator(s) data for Agent(s) are confidential and proprietary to Collaborator(s) and shall be maintained as such by the investigators. The protocol documents for studies utilizing Agents contain confidential information and should not be shared or distributed without the permission of the NCI. If a copy of this protocol is requested by a patient or patient's family member participating on the study, the individual should sign a confidentiality agreement. A suitable model agreement can be downloaded from: http://ctep.cancer.gov.

### 11.4 Ethics Consideration

The study must be approved by an appropriate institutional review committee as defined by Federal Regulatory Guidelines and the Office of Human Research Protection. The investigators will inform the IRB of subsequent protocol amendments.
The investigator must inform the patient about the background and present knowledge of treatment with doxycycline. The investigator must ensure the following items are discussed:

1. The patient must be provided the patient information and informed consent form consistent with the protocol version used and approved by the IRB. The patient must be informed that this is a new treatment approach and that the exact degree of efficacy is unknown, and that treating him/her will contribute to further knowledge.
2. The patient will be given time to discuss his/her participation with the investigator and members of their family. Before the patient is entered into the study, the patient's written consent must be obtained. A copy of the signed consent form will be provided to the patient and the original scanned into the electronic database created for this study.
3. The patient may refuse to provide a biopsy or may refuse treatment proposed in the context of the study before or at any time during the study. Refusal to participate will involve no penalty or loss of benefits to which the patient is otherwise entitled.
4. An explanation of whom to contact for answers to pertinent questions about the research and the patient's rights, and who to contact in the event of a research-related injury must be given to the patient.

### 11.5 Publication Policy

All the results are the property of the Principal investigators. Investigators may not submit for publication or presentation study results without allowing the PIs 30 working days to review and comment on the pre-publication manuscript. Co-authorship will be discussed and mutually agreed upon before submission of a manuscript to a publisher.

## 12. STATISTICAL CONSIDERATIONS

### 12.1 Study Design/Endpoints

The primary endpoint of the study is to evaluate for the presence of dead or dying metakaryotic cells as manifested by the cytometric characteristics.

Secondary objectives:

1. To determine the plasma drug concentrations of the study drug at days 1, 3, 5, 8, 15, 22, 29, and at restaging and at the time surgery.
2. To assess the histopathologic treatment response of the primary tumors which have undergone neoadjuvant gemcitabine based chemoradiation and concurrent doxycycline therapy.

3. To enumerate the number of observed dead/dying metakaryotes per 1 gram of resected pancreatic tissue.

## 12.2 Sample Size/Accrual Rate

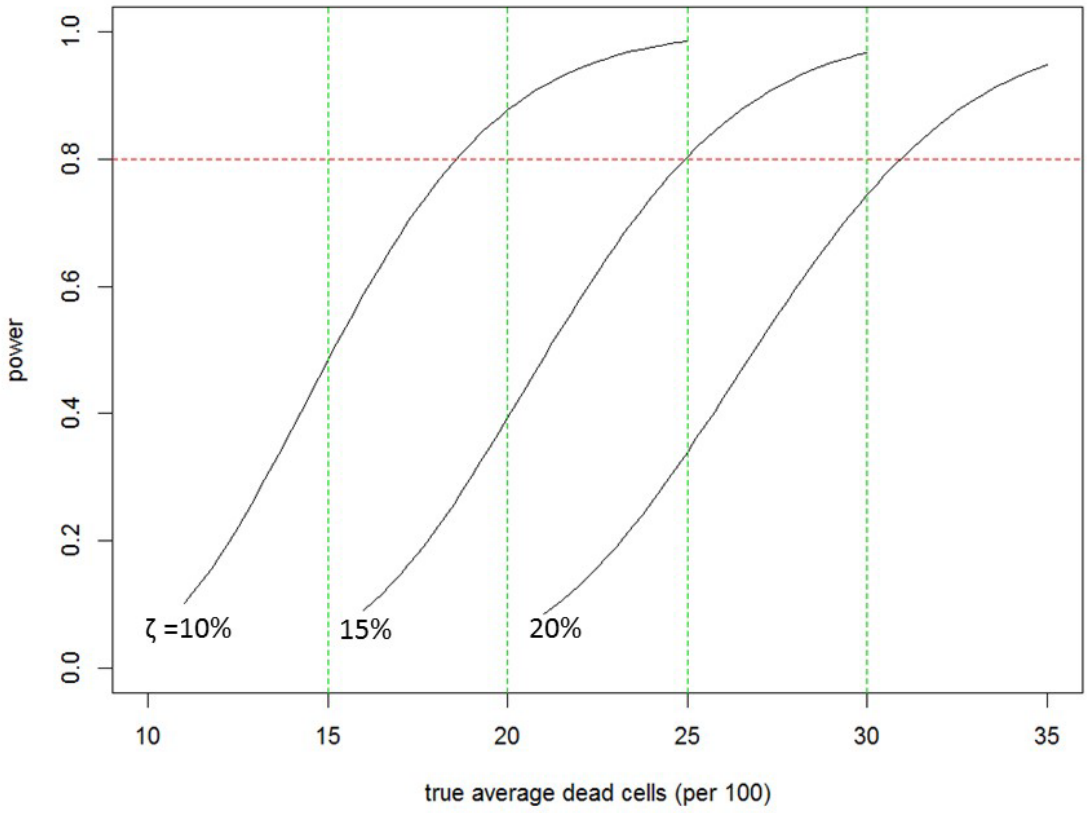
**Assessment of Primary Endpoint**

At the moment the number of dead metakaryotic cells observed by our MIT collaborator, Dr. E.V. Gostjeva in more than ten pancreatic and/or lung tumors is still zero (0) in about 1000 metakaryotic nuclei observed.

To be of clinical utility, we set up our hypothesis as follows:

$$H_0: \pi=\zeta \text{ vs } H_1: \pi>\zeta$$

where $\pi$ is the average proportion of cell death and $\zeta$ is determined a prior with clinical utility. Considering the heterogeneous response to treatment between individuals, we assume that the true number of cell death with doxycycline follows a beta-binomial distribution with shape parameters $(\alpha,\beta)=(k,100-k)$, where $k\geq 31$ (i.e, a mean of ≥31 per 100 cells and an over-dispersion parameter $\rho = 9.9$). For a one-sided test, a sample size of 10 will achieve 80% power to detect an average proportion of cell deaths > $\zeta$ =20% at a type I error 0.05 level. The following figure show the statistical power for three $\zeta$ values, (10%, 15%, and 20%) and the corresponding true proportion of dead cells.




For data analysis, the maximum likelihood estimation (by R package: VGAM) will be used to estimate the shape parameters $(\alpha,\beta)$ and calculate the mean and standard error based on a beta-binomial distribution. The normal approximation will be used to construct the 95% confidence

interval for the average proportion of cell death.

We plan a 1-year recruitment period with an anticipated attrition of 10% due to disease progression or drug toxicity. With an enrollment target of 12 patients, we anticipate that 10 patients will be able to complete the assigned therapy and surgery. Enrollment will be ongoing until the primary endpoint has been achieved.

**Assessment of Secondary Endpoints**

Plasma doxycycline concentrations and number of identified dead/dying metakaryotic cells will be reported as continuous variables. We will perform descriptive analysis of the continuous variables, including mean (SD) and median (IQR). We will further compare patients by the primary outcome (presence/absence of metakaryote death). We anticipate that our data will not be normally distributed and we will use nonparametric testing (Mann Whitney) to evaluate our results. The frequency of the number of dead/dying metakaryotic cells will provide a preliminary data on the expected distribution for the treatment dose.

Histologic treatment response is part of the standard pathologic reporting. Using the College of American Pathologists (CAP) criteria, which is a 4 tier grading system, chi-squared analysis will be used to compare observed histologic treatment response with reported treatment response from historical controls.

## 13. REFERENCES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