

*Abbreviated Title: Mithramycin for Cancer Stem Cells*  
*Version Date: 09/14/2018*

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**CC Protocol #:** 12-C-0151 L  
**Version Date:** 09/14/18  
**NCT Number:** NCT01624090

**Phase II Evaluation of Mithramycin, an Inhibitor of Cancer Stem Cell Signaling, in Patients with Malignancies Involving Lungs, Esophagus, Pleura, or Mediastinum**

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**IND Information:**

Drug Name:	Mithramycin
IND Number:	115272
Sponsor:	NCI, Center for Cancer Research
Authorized Representative	William Dahut, MD
Manufacturer:	IriSys LLC

**Commercial Agents:** None

## **PRÉCIS**

### **Background:**

Increasing evidence indicates that activation of stem cell gene expression is a common mechanism by which environmental carcinogens mediate initiation and progression of thoracic malignancies. Similar mechanisms appear to contribute to extra-thoracic malignancies that metastasize to the chest. Utilization of pharmacologic agents, which target gene regulatory networks mediating “stemness” may be novel strategies for treatment of these neoplasms. Recent studies performed in the Thoracic Epigenetics Laboratory, TOSB/NCI, demonstrate that under exposure conditions potentially achievable in clinical settings, mithramycin diminishes stem cell gene expression and markedly inhibits growth of lung and esophageal cancer and MPM cells *in vitro* and *in vivo*. These findings add to other recent preclinical studies demonstrating impressive anti-tumor activity of mithramycin in epithelial malignancies and sarcomas that frequently metastasize to the thorax.

### **Primary Objective:**

- To assess clinical response rates of mithramycin administered as 6 hour intravenous infusions in patients with malignancies involving lungs, esophagus, pleura, or mediastinum.

### **Eligibility:**

- Patients with measurable inoperable, histologically confirmed primary lung and esophageal carcinomas, thymic neoplasms, germ cell tumors, malignant pleural mesotheliomas or chest wall sarcomas, as well as patients with gastric, colorectal or renal cancers and sarcomas metastatic to the thorax are eligible.
- Patients with germline SNPs in ABCB4, ABCB11, RALBP or CYP8B1 that are associated with resistance to mithramycin-induced hepatotoxicity.
- Patients must have had or refused first-line standard therapy for their malignancies.
- Patients must be 18 years or older with an ECOG performance status of 0 – 2, without evidence of unstable or decompensated myocardial disease. Patients must have adequate pulmonary reserve evidenced by FEV1 and DLCO equal to or greater than 30% predicted; Oxygen saturation  $\geq 92\%$  on room air. ABG will be drawn if clinically indicated.
- Patients must have a platelet count greater than 100,000, an ANC equal to or greater than 1500 without transfusion or cytokine support, a normal PT/PTT, and adequate hepatic function as evidenced by a total bilirubin of  $<1.5 \times$  upper limits of normal (ULN) and AST/ALT  $\leq 3 \times$  ULN. Serum creatinine less than or equal to 1.6 mg/ml, or creatinine clearance greater than 70 ml/min/1.73m<sup>2</sup>.

### **Design:**

- Simon Optimal Two Stage Design for Phase II Clinical Trials targeting an objective response rate (RECIST) of 30%.
- Patients will be stratified based on location of primary disease (thoracic vs. extra-thoracic).
- Patients will receive 6 hour infusions of mithramycin at 30 -50 mcg/kg every day for 7 days, every 21 days (1 cycle). Three cycles will constitute one course of therapy. Those patients tolerating 30 mcg/kg infusions during the first cycle will receive subsequent cycles of mithramycin at a dose of 50 mcg/kg using the same infusion schedule.

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- Following each course of therapy, patients will undergo restaging studies. Patients exhibiting objective response to therapy or stable disease by RECIST criteria will be offered an additional course of therapy.
- Patients exhibiting disease progression will be removed from study.
- Biopsies of index lesions will be obtained at baseline and on day 8 of the second cycle of therapy for analysis of molecular end-points.
- Pharmacokinetics will be assessed during cycle 1 and cycle 2 of the first course of therapy.

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## **1 INTRODUCTION**

### **1.1 STUDY OBJECTIVES**

#### **1.1.1 Primary Objective**

- To determine objective response rates (CR and PR) in patients with malignancies involving lungs, esophagus, pleura or mediastinum following mithramycin infusions

#### **1.1.2 Secondary Objective(s)**

- To determine pharmacokinetics and toxicities of mithramycin administered as 6 hour IV infusions
- To ascertain if mithramycin inhibits stem cell gene expression in patients with thoracic malignancies
- To evaluate gene expression, DNA methylation and micro-RNA profiles in pre- and post-treatment tumor biopsies.
- To compare gene expression, DNA methylation, and microRNA profiles in patient tumor biopsies with treatment response profiles in pre-clinical studies.
- To examine if mithramycin decreases pluripotent cancer stem cells (side population)
- To develop methodologies for assessing effects of mithramycin on cancer stem cells, hematopoietic stem cells, mesenchymal stem cells, and circulating tumor cells (CTC).

### **1.2 BACKGROUND AND RATIONALE:**

Lung and esophageal cancers, and malignant pleural mesothelioma (MPM) are leading causes of cancer-related deaths worldwide <sup>1</sup>. In 2012, these malignancies accounted for an estimated 1.8 million deaths globally; in the United States, approximately 160,000 deaths were attributed to lung cancer, 15,000 deaths were due to esophageal carcinoma and approximately 2,500 deaths were attributed to MPM <sup>2</sup>. Presently, 80% of lung cancers, and 50% of esophageal carcinomas are directly attributable to cigarette smoke <sup>3,4</sup>. Interestingly, whereas tobacco abuse has not been linked to the pathogenesis of MPM, approximately 70% of patients developing this malignancy are active or former smokers <sup>5</sup>. Currently, more than 1.3 billion people smoke; as such, the global burden of tobacco-associated thoracic malignancies will continue to increase, with particularly devastating consequences in developing countries <sup>6</sup>.

In addition to being a significant risk factor for major morbidity and mortality in individuals undergoing potentially curative resections <sup>7,8</sup>, cigarette smoking diminishes responses to chemo- and radiation therapy, enhances systemic metastases, and decreases survival of patients with locally advanced or disseminated lung and esophageal cancers and MPM <sup>9-12</sup>; the mechanisms underlying these phenomena have not been fully established. Previously, we reported that under clinically relevant exposure conditions, cigarette smoke enhances tumorigenicity of lung cancer cells via polycomb-mediated repression of Dickkopf-1 (Dkk1), which encodes an antagonist of Wnt signaling <sup>13</sup>. In unpublished studies, we observed a similar phenomenon in esophageal adenocarcinoma cells following cigarette smoke exposure. Additionally, we have observed that cigarette smoke activates miR-31, targeting Dkk1 as well as several other Wnt antagonists in lung cancer cells; constitutive expression of this microRNA significantly enhances proliferation of lung cancer cells *in vitro* and *in vivo* <sup>14</sup>. In more recent studies <sup>15</sup>, we observed that cigarette

smoke mediates epigenetic repression of miR-487b in lung cancer cells, resulting in over-expression of polycomb group proteins BMI1 and SUZ12, as Wnt5A, k-ras and C-myc, all of which have been implicated in modulating stem cell pluripotency <sup>16-19</sup>; consistent with these observations, knock-down of miR-487b increases proliferation and tumorigenicity of lung cancer cells <sup>15</sup>. Collectively, these studies suggest that activation of stem cell gene expression may be a common mechanism by which tobacco components mediate initiation and progression of thoracic malignancies. As such, utilization of pharmacologic agents, which target gene regulatory networks mediating “stemness” may be novel strategies for treatment of thoracic malignancies. Our recent studies, which are summarized below, suggest that under exposure conditions potentially achievable in clinical settings, mithramycin diminishes stem cell gene expression and markedly inhibits growth of lung and esophageal cancer and MPM cells *in vitro* and *in vivo*.

*CSC induces ABCG2 expression in cultured lung and esophageal cancers and MPM cells:* A series of experiments have been performed in the Thoracic Epigenetics Laboratory, TGIB/NCI to further examine mechanisms by which cigarette smoke enhances “stemness” of thoracic neoplasms <sup>20</sup>. Briefly, Affymetrix microarrays were used to identify gene expression profiles in cultured lung and esophageal cancer as well as MPM cells mediated by CSC under clinically-relevant exposure conditions. Interestingly, *ABCG2* [also known as breast cancer resistance protein (BCRP)] was the third to seventh most commonly up-regulated gene in Calu-6, A549, EsC1, EsC2, and SB-MES1 and SB-MES2 cells exposed to CSC. Subsequent qRT-PCR experiments (**Figure 1A** upper panel) demonstrated that A549 and EsC2 had relatively high basal expression of *ABCG2*, which was increased 2-4 fold and ~8 fold, respectively, by CSC treatment. In contrast, Calu-6 and EsC1 exhibited relatively low level basal expression of *ABCG2*, which was augmented approximately 25-30 fold and 6 fold, respectively, by CSC. Interestingly, normal aerodigestive tract epithelial cells (SAEC and HET1A) exhibited very low basal levels of *ABCG2*, and minimal induction of *ABCG2* by CSC. A similar phenomenon was observed for cultured normal mesothelial cells (data not shown). Immunofluorescence experiments confirmed that CSC exposure increased *ABCG2* protein levels in cancer cells (**Figure 1A** lower panel). CSC-mediated induction of *ABCG2* was observed across numerous additional lung cancer and MPM lines (data not shown). The lack of validated lines precluded further induction experiments using esophageal cancer cells.

*Clinical Relevance of ABCG2 Activation in Thoracic Malignancies:* *ABCG2* is a member of the ATP binding cassette (ABC) transporters, which functions as a xenobiotic pump in many normal tissues <sup>21</sup>. Its substrates include numerous environmental toxins as well as chemotherapeutic agents. Several recent studies suggest that *ABCG2* is a critical mediator of stem cell homeostasis. For example, *ABCG2* is highly expressed in pluripotent cells, and is an essential upstream mediator of sonic-hedgehog signaling, which has been implicated in maintenance of stemness <sup>22</sup>. Furthermore, *ABCG2* binds to heme, thereby diminishing intracellular porphyrin levels, rendering stem cells resistant to hypoxia <sup>23</sup>. Constitutive expression of *ABCG2* protects cardiac stem cells from oxidative stress <sup>24</sup>, and enhances expansion, while impairing maturation of hematopoietic progenitor cells <sup>25</sup>. Of particular relevance regarding thoracic malignancies are recent reports demonstrating that increased expression of *ABCG2* correlates with chemoresistance and stem-like phenotype of lung and esophageal carcinomas <sup>26-30</sup> as well as pleural mesotheliomas <sup>31-33</sup>, and decreased survival of patients with these neoplasms <sup>34-36</sup>.

*CSC increases side population of cultured cancer cells:* Because *ABCG2* is highly expressed in stem cells, flow cytometry experiments were performed to examine if CSC exposure increased

side population (SP), which is believed to be enriched with cancer stem cells <sup>27,29</sup>. Representative results pertaining to A549 and Calu-6 cells (high and low *ABCG2* expressors, respectively) are depicted in **Figure 1B**. Hoechst staining with and without verapamil revealed a SP fraction of 0.23 % in untreated A549 cells, compared to .95% (a 4-fold increase) in A549 cells exposed to CSC for 5 days (**Figure 1B**). The SP fraction in untreated Calu-6 cells (0.06%) was much lower than A549 cells and increased to 0.98% (a 16 fold increase) following CSC exposure (**Figure 1B**). Subsequent qRT-PCR analysis demonstrated significant increases in *ABCG2* expression in SP fractions relative to non-SP fractions in A549 and Calu-6 cells (**Figure 1C**), suggesting that CSC-mediated induction of *ABCG2* coincides with enhanced pluripotency of cancer cells. This phenomenon was less evident in A549 cells, possibly due to the relative levels of *ABCG2* induction in these cells following CSC exposure.

*Role of Aryl Hydrocarbon Receptor and Sp1 in ABCG2 up-regulation by CSC:* Purified carcinogens as well as HDAC inhibitors such as rhomidepsin induce *ABCG2* expression in cancer cells by aryl hydrocarbon receptor (AhR) signaling <sup>37,38</sup>. As such, additional experiments were performed to examine the mechanisms by which CSC induces *ABCG2* expression in lung and esophageal cancer cells. qRT-PCR experiments utilizing Calu-6 and EsC1 cells demonstrated dose-dependent induction of *ABCG2* by benzopyrene, 3-MC as well as TCDD, the magnitude of which varied somewhat between the cell lines (**Figure 1D**; left panel). Interestingly, the AhR antagonist, Resveratrol, only partially abrogated CSC-mediated induction of *ABCG2* in these cells (**Figure 1D**; right panel). Furthermore, knock-down of *HDAC6*, which is required for activation and nuclear transport of AhR in response to tobacco carcinogens <sup>39</sup>, only modestly diminished CSC-mediated induction of *ABCG2* in lung and esophageal cancer cells (**Figure 2B**; upper and lower panels). These findings suggested that induction of *ABCG2* by CSC was not mediated solely by AhR signaling.

In addition to xenobiotic response elements (XRE) that are binding sites for AhR, the *ABCG2* promoter contains a number of Specificity protein 1 (Sp1) recognition sites <sup>37</sup> that could mediate activation of this gene in response to cigarette smoke. As such, transient transfection experiments using *ABCG2* promoter-reporter constructs were performed to examine potential roles of AhR and Sp1 in mediating *ABCG2* activation by CSC. As shown in **Figure 2A**, serial deletion of XRE as well as Sp1 sites markedly diminished *ABCG2* promoter activity. Collectively, these data indicated that Sp1 contributes significantly to CSC-mediated activation of *ABCG2* in cancer cells.

*Effects of Mithramycin on CSC-mediated Induction of ABCG2:* Additional experiments were performed to ascertain if mithramycin, which inhibits binding of Sp1 to GC-rich DNA <sup>40</sup> could repress *ABCG2* expression in cancer cells. Briefly, cancer cells were cultured in normal medium (NM) with or without CSC in the presence or absence of escalating doses of mithramycin. qRT-PCR analysis revealed that 24h treatment with mithramycin decreased basal levels of *ABCG2* in lung and esophageal cancer and MPM cells; furthermore, mithramycin markedly attenuated CSC-mediated induction of *ABCG2* in these cells (representative results pertaining to A549 and Calu-6 are depicted in **Figure 2B**). Down-regulation of these genes persisted for at least 16 hours following removal of mithramycin from culture media (data not shown). Additional analysis revealed that mithramycin inhibited basal levels as well as CSC-mediated up-regulation of *Sp1* and *AhR* in these cells. Interestingly, mithramycin also decreased basal as well as CSC-mediated expression of *Nuclear Factor Erythroid Related Factor 2 (Nrf2)*, which has been shown recently to modulate *ABCG2* expression <sup>41</sup>. Immunofluorescence experiments demonstrated that mithramycin decreased *ABGC2* expression in cultured cancer cells (**Figure 2C**; left panel).



Additional immunoblot experiments revealed that mithramycin mediated dose-dependent decreases in Sp1, AhR, and Nrf2 expression, and partially abrogated CSC-mediated increases in levels of these transcription factors in A549 and Calu-6 cells (**Figure 2C**; right panel). Quantitative chromatin immunoprecipitation (ChIP) experiments (**Figure 2D**) demonstrated that CSC induced recruitment of Sp1, AhR, and Nrf2 to the *ABCG2* promoter; these results were most dramatic for Sp1, and were consistent with aforementioned promoter-reporter experiments. Additional ChIP analysis revealed that mithramycin diminished CSC-mediated occupancy of these transcription factors within the *ABCG2* promoter; these effects coincided with appropriate alterations in RNA polymerase II (pol II), as well as H3K9Ac and H3K9Me3 (histone activation and repression marks, respectively).

*Effects of Mithramycin on Proliferation and Tumorigenicity of Cancer Cells:* Additional experiments were undertaken to examine the effects of mithramycin on proliferation and tumorigenicity of cultured thoracic malignancies. Results of this analysis are shown in **Figure 3**. MTS experiments demonstrated that 24h mithramycin exposure dramatically inhibited proliferation of lung and esophageal cancer as well as MPM cells (**Figure 3A**). Flow cytometry experiments suggested that the growth inhibitory effects of mithramycin were due to cell cycle arrest rather than apoptosis (data not shown). Subsequent experiments demonstrated that mithramycin administered IP qMWF x 3 weeks mediated significant dose-dependent growth inhibition of established subcutaneous tumor xenografts in athymic nude mice (**Figure 3B**); the in-vivo antitumor effects were not associated with appreciable systemic toxicities such as decreased activity, skin changes, or significant weight loss. Histopathologic analysis revealed that tumors from mithramycin treated mice were less glandular in appearance with somewhat less stroma. Furthermore, tumors from mice treated with 2mg/kg mithramycin had 50% fewer mitoses relative to control tumors (data not shown). Immunofluorescence experiments confirmed that mithramycin decreased ABCG2 expression in tumor xenografts (**Figure 3C**).

*Mechanisms of Mithramycin-Mediated Cytotoxicity:* Affymetrix micro-array experiments were performed to examine global gene expression profiles in A549 and Calu-6 lung cancer cells ( wt p53 and p53 null, respectively) cultured in NM with or without mithramycin for 24 hours. Mithramycin mediated dramatic dose-dependent alterations in gene expression in these lung cancer cells. Highly reproducible results were noted among cell lines and within treatment groups (**Figure 4A**, upper left panel). Using highly stringent criteria of fold change >3 and adjusted  $p < 0.01$  for drug treatment vs. control, 1582 and 3771 genes were simultaneously modulated in A549 and Calu-6 cells following 50 nM and 200 nM mithramycin exposures, respectively (**Figure 4A**; lower left panel). 1258 genes were commonly regulated by mithramycin across two cell lines and two drug concentrations; the majority of differentially regulated genes were down-regulated in both cell lines (**Figure 4A** right panel). Interestingly, 8 canonical pathways related to stem cell signaling were down-regulated by mithramycin in cultured lung cancer cells (**Table 1A**). Consistent with these results, mithramycin decreased SP fraction in A549 cells (**Figure 4B**).

Additional micro-array experiments were performed to examine effects of mithramycin in A549 xenografts (9 each from drug-treated or control mice). Similar to what was observed following in-vitro drug treatment, mithramycin mediated highly reproducible, dose-dependent alterations in gene expression in A549 tumor xenografts (**Figure 4C**; left panel, and upper right panel). Using criteria of fold change >2 and  $p < 0.05$  for drug treatment vs. control, 351 and 1896 genes were differentially expressed in xenografts from mice receiving mithramycin at 1 mg/kg and 2 mg/kg, respectively, relative to control tumors (**Figure 4C**; lower right panel). 299 genes were modulated

by mithramycin under both doses. *ABCG2* was down-regulated > 2 fold in xenografts from mice receiving 2mg/kg, but not 1mg/kg mithramycin. All eight of the stem-cell related pathways modulated *in-vitro* by mithramycin, were also targeted in tumor xenografts by systemic drug treatment, albeit to a somewhat lesser degree (**Table 1B**). A similar phenomenon was observed regarding the remaining 8 canonical pathways listed in **Table 1A**. A variety of additional networks regulating intracellular signaling, DNA damage response, chromatin remodeling, and chromosomal replication were down-regulated in A549 tumor xenografts following mithramycin treatment (**Figure 5**).

### 1.3 MITHRAMYCIN

#### 1.3.1 Preclinical Studies

Mithramycin, a polyureic acid isolated from streptomyces, was initially evaluated as a chemotherapeutic agent in cancer patients during the 1960's and 70's<sup>42</sup>, but was discontinued due to excessive systemic toxicities<sup>43</sup>. Recently there has been renewed interest in clinical development of mithramycin and its derivatives because of their ability to specifically inhibit binding of Sp1 to GC-rich DNA<sup>40</sup>, thereby down-regulating numerous genes mediating stemness, chemo resistance, invasion and metastasis of cancer cells<sup>20,44-46</sup>. Of particular interest in this regard are recent studies indicating that currently approved agents such as cyclo-oxygenase inhibitors markedly enhance mithramycin mediated-inhibition of Sp1 expression/ activity in cancer cells<sup>47</sup>. Such combinational strategies could enable reduction of mithramycin doses, and possibly decrease systemic toxicities in clinical settings.

Recent experiments have been performed to correlate *in vivo* effects of mithramycin with *in vitro* drug exposures in A549 and Calu-6 lung cancer cells. A progressive dose-dependent increase in genes commonly regulated *in vitro* and *in vivo* by mithramycin was observed (**Figure 5**). Two to ten percent (average 5%) of genes modulated *in vitro* overlapped with 13-24% (average 18%) of genes altered by *in vivo* mithramycin across various treatment comparisons. 337 genes were simultaneously modulated in cultured A549 and Calu-6 cells following 200 nM mithramycin and A549 xenografts from mice receiving 2 mg/kg mithramycin IP. Top molecular and cellular functions mediated by these 337 genes included stem cell pluripotency, cell cycle progression, gene expression, cellular morphology, and death. In all likelihood, the pleiotropic antitumor effects of mithramycin are mediated by direct inhibition of Sp1 binding to promoters of master genes regulating diverse cellular functions, with subsequent repression of down-stream targets by direct as well as indirect mechanisms<sup>48-51</sup>.

Our recent experiments have direct translational implications regarding evaluation of mithramycin in patients with thoracic malignancies. Extrapolation of data from previous animal studies<sup>52,53</sup> suggests that tissue mithramycin levels achieved in our xenograft experiments were in the 50-200 nM range over 24h (**Table 2**); these exposure conditions, which closely approximated those used for our *in vitro* experiments, are potentially achievable using previous mithramycin dosing schedules in humans<sup>42</sup>. As such, gene expression signatures corresponding with treatment response in our preclinical studies may be relevant surrogate endpoints in patients receiving mithramycin infusions.

Although preclinical data presented thus far have focused primarily on lung and esophageal cancers, and malignant pleural mesotheliomas, considerable laboratory efforts have been undertaken to evaluate the effects of mithramycin in a variety of other human cancer histologies.

For example, Sp1 is over-expressed in gastric, colon, breast, pancreas and prostate cancers [54-58](#)) that frequently metastasize to the thorax. Sp1 over-expression upregulates VEGF and other genes encoding angiogenic factors in gastric, pancreas, colon and breast cancer cells [48,49,58-63](#); over-expression of Sp1 correlates significantly with advanced stage of disease and decreased survival in patients with gastric or pancreatic carcinomas [54,57,59](#). Targeted inhibition of Sp1 activity by mithramycin potently decreases angiogenesis as well as growth of gastric and pancreatic cancer xenografts [60,64](#). Collectively these findings, together with data pertaining to the antitumor effects of mithramycin in sarcomas [65](#), support evaluation of mithramycin in GI, breast, urologic, and sarcomatous malignancies that have metastasized to the thorax.

### **1.3.2 Preclinical toxicology**

In mice, the LD<sub>50</sub> was determined to be about 2000 mcg/kg (6000 mcg/m<sup>2</sup>) of body weight, which is higher than the 100 mcg/kg of body weight that was found to be lethal in some dogs (2000 mcg/m<sup>2</sup>) and monkeys (1200 mcg/m<sup>2</sup>) [66](#). However, mithramycin was found to be essentially non-toxic in dogs and monkeys when administered at a dose of 24-50 mcg/kg/day (480-1000 mcg/m<sup>2</sup> dog, 288-600 µcg/m<sup>2</sup> monkey) intravenously for 24 consecutive days [66](#)). The most frequently encountered toxicities at higher doses were bleeding, anorexia, vomiting, elevated liver function tests, electrolyte abnormalities, bone marrow suppression and azotemia [66](#).

Our preclinical data as well as those reported by Grohar et al [65](#), indicate that IP doses of 1-2 mg/kg/day three times a week for 3 weeks are well tolerated in mice. In the Grohar study [65](#), necropsy demonstrated that mice treated at this dose and schedule had mild hypoalbuminemia, slightly increased ALT/AST levels with some evidence of liver toxicity described as mild to moderate, and minimal thrombocytopenia.

#### **1.3.2.1 Pharmacokinetics**

Administration of tritium labeled mithramycin to monkeys showed a terminal half-life of 5 hours. The peak cerebrospinal fluid concentration: plasma ratio was 0.12 and reached after 1 ½ hours [67](#). Preliminary pharmacokinetic parameters of <sup>125</sup>I-plicamycin (mithramycin) in human plasma using a radioimmunoassay were described [68](#). Three patients were studied and received mithramycin at a dose ranging from 0.85 to 1.0 mg/m<sup>2</sup> IV over 2 hours. Drug elimination was biphasic, with a mean elimination half-life of 10.6 hours (±1.7), and a clearance rate of 11.1± 0.4 ml/min/m<sup>2</sup>.

Kennedy et al [52](#) administered titrated mithramycin by IV or IP routes in mice at doses closely approximating 2 mg/kg. Blood, liver, and kidney mithramycin concentrations following IP administration were similar if not higher than these observed following IV dosing. Tissue levels two hours after administration ranged from ~335 – 425 nM compared to 90 nM in blood (**Table 2**). Assuming an elimination half-life of ~10h, tissue levels most likely exceeded 50-100nM for >24h.

### **1.3.3 Clinical studies in adults**

Mithramycin underwent broad clinical evaluation in solid tumors and leukemias in the 1960's and was found to have some activity against leukemias, lymphomas and carcinomas [68-72](#); however, patient numbers and standard methods of evaluating responses were limited (**Appendix 1**). In particular, mithramycin was found to have activity against testicular cancers. In one series of 305 patients with stage III testicular cancer, 10% of patients achieved a complete response, and an additional 25% showed some evidence of tumor regression [66](#); many of these cases were

durable responses in patients with widely metastatic disease. In addition, mithramycin exhibited activity against Ewing sarcoma; two of five patients with EWFT exhibited widespread regression of metastatic disease following mithramycin treatment, one of whom had a durable complete response <sup>73</sup>. This impressive response rate may be due to the ability of mithramycin to inhibit expression of EWS-FLI fusion transcript <sup>65</sup>. Whereas more than 1,500 patients were treated with mithramycin, very few (~5) patients with lung cancers and no patients with esophageal carcinomas or MPM received this drug. Furthermore, only a small number of patients with breast, ovarian, or GI malignancies were treated with mithramycin. No conclusions can be drawn regarding clinical activity of mithramycin in these malignancies due to the variability of infusion schedules and timing of treatment response assessments.

Initial clinical trials suggested that 50 mcg/kg/dose, administered daily times 5 was an optimal treatment regimen <sup>68</sup>. However, Spear et al <sup>74</sup> observed that 70% of 58 patients receiving this dose and schedule of mithramycin experienced significant toxicity including anorexia, nausea and vomiting. As a result, the dose of drug used in most subsequent studies was 25 mcg/kg/day on various schedules. The recommended dose in the Pfizer package insert for adults is 25-30 mcg/kg administered intravenously over 6 hours every 7-10 days of a 28 day cycle. Systemic toxicities appeared to be dose-related, tending to occur at doses above 30 mcg/kg/dose <sup>66</sup>.

In general, the most frequent toxicities observed were nausea, vomiting, elevated liver function tests, infusional fever, mucositis, bleeding tendencies, thrombocytopenia, electrolyte abnormalities, proteinuria and elevated BUN/creatinine (summarized in <sup>66</sup>). Unfortunately, there was no systematic investigation of these toxicities, and it is unclear what levels of supportive care these patients received. For example, nausea and vomiting occurred quite frequently; however, in at least two reports, nausea was controlled with phenothiazines or other anti-emetics <sup>43,69,75</sup>.

Hemorrhage was the most profound toxicity observed in patients receiving mithramycin. In early reports hemorrhage was attributed to thrombocytopenia, and accounted for 3 deaths in 84 patients <sup>42</sup>. Subsequent studies revealed that bleeding was dose-related, occurring in 5.4% of 1150 patients treated at 30 mcg/kg/day or more <sup>66</sup>. Bleeding tended to occur around day 4 of treatment, which is atypical of chemotherapy-induced thrombocytopenia. In addition, it appears that platelet counts remained >20,000/mcL in most of the patients experiencing hemorrhage, suggesting that bleeding was due to underlying clotting factor deficiencies or dysregulation of coagulation pathways. In all the cases of hemorrhage reported in the literature, it is not clear if the patients were taking any anti-coagulation medications, what type of blood product support was provided, how effective the blood product support was in that era, or what specific clinical and laboratory abnormalities were seen prior to the hemorrhage. Therefore, the frequency and severity of hemorrhage from mithramycin administered in the current era are unknown. Nevertheless, hemorrhage must be considered a rare, but serious potential toxicity of mithramycin therapy.

Additional rare but significant side effects attributable mithramycin include toxic epidermal necrolysis <sup>76,77</sup>, and possible potentiation of anthracycline-mediated cardiotoxicity <sup>78</sup>. The true incidences of these toxicities are unknown and are not discussed in the package insert from Pfizer, or the previously filed IND for mithramycin.

Mithramycin has also been used for the treatment of malignant hypercalcemia, with 45% of patients responding to a single dose of mithramycin in one study <sup>79</sup>. However, the bisphosphonate, pamidronate, has replaced mithramycin in this setting. For similar reasons, mithramycin was investigated for the treatment of Paget's disease of bone and was found to have good activity in

the management of this disease. In one report, 10/10 patients with Paget's disease reported improvements in pain, and an increase in overall activity with treatment <sup>75</sup>.

#### **1.3.4 Recent Experience with Mithramycin in Adult Oncology Patients at the NCI**

Twelve patients (10 males; 2 females; median age 51y; range 40-72y) with refractory thoracic malignancies (six MPMs, 2 esophageal cancers, two lung cancers, 2 synovial sarcomas) received 6h mithramycin infusions q day x 7, every 28 days. Response rates following two cycles, pharmacokinetics, and systemic toxicities (cycle 1) were evaluated. A total of 20 cycles were administered to 12 patients; all were evaluable for toxicities. No objective responses were observed in 7 patients who were evaluable for treatment responses. No myelosuppression, uncontrollable nausea or vomiting was observed. No patients experienced bleeding. The lack of these toxicities, which have been associated with mithramycin treatment <sup>66</sup> may be attributable at least in part to higher purity of the current drug relative to what had been used previously.

Eight patients experienced transient, asymptomatic dose-limiting transaminitis following 25mcg/kg/infusions. This toxicity always occurred on day 4 of the infusion. Percutaneous biopsies from three individuals revealed apoptotic hepatocellular death (**Figure 6A**). Four patients (two with MPM) had no hepatotoxicity and tolerated dose escalation to 30 mcg/kg/infusion. Peak and steady state mithramycin levels were 20 and 8nM, respectively, which were not different in patients with or without hepatotoxicity (**Figure 6B**). DMET genotyping revealed that two SNPs (rs2302387 and rs4668115) in genes encoding two related bile transporters (ABCB4 and ABCB11, respectively) were significantly associated with LFT elevations in the 12 patients treated with mithramycin (**Figure 7A** and **Figure 7B**).

Following this observation, we amended the clinical trial to require *a priori* genotyping of *ABCB4* and *ABCB11* in patients with thoracic malignancies. Three additional patients with wild-type genotypes at rs2302387 (CC) and rs4668115 (GG) were treated with mithramycin. The first patient with stage IV esophageal cancer had a Grade 3 LFT elevation (ALT=347 U/L, AST=314 U/L), but this patient also had significant liver disease. We therefore decided to not recruit additional patients with liver metastases. The next patient (also with stage IV esophageal cancer) appeared to respond to mithramycin without transaminitis (ALT=81 U/L, AST=62 U/L); however, this individual died several days later from a spontaneous esophageal perforation that might have been secondary to tumor response in a previously radiated field. The final patient experienced LFT elevations (ALT=3178 U/L, AST=3695 U/L) that was not explained by genotype.

To address additional genetic variants that may have contributed to these LFT elevations, we conducted additional genotyping analysis using the DMET Plus array. We excluded the patient who had liver involvement to avoid confounding the analysis. *ABCB4* and *ABCB11* genotypes retained their significant association with LFT elevation ( $P<0.040$ ) in spite of an individual with grade 4 LFT elevations who was included in this group (**Figure 8A-B**). Variants in three other genes were found to be associated with LFT elevations: rs1143670 in *PEPT2* (**Figure 8C-D**), rs12680 in *RALBP* (**Figure 8E-F**), and rs6774801 in *CYP8B1* (**Figure 8G-H**). Since *PEPT2* genotypes did not demonstrate a genotype dose effect (i.e., the patient with GG had greater ALT and AST than those carrying GA), association between LFT elevations and this genotype may be confounded by other factors. No relationship between *PEPT2* and mithramycin outcomes was apparent in the literature. *RALBP* rs12680 was strongly associated with LFT elevations ( $P=0.0040$ ), which are related to hepatocellular necrosis (<https://livertox.nlm.nih.gov/Plicamycin.htm>). *RALBP* has previously been associated with

promoting hepatic inflammation and LFT elevations in mice<sup>80</sup> and regulates cellular accumulation of doxorubicin by a poorly-characterized non-ABC-transport mechanism<sup>81</sup>. Mithramycin has a conjugated quinone- and semiquinone-rich structure that is similar in structure to doxorubicin, suggesting that RALBP may influence cellular accumulation of mithramycin. CYP8B1 is involved in the synthesis of cholic acid and thereby influences the composition of the bile pool<sup>82</sup>. Since ABCB4 and ABCB11 are both involved in bile efflux, it is likely that CYP8B1 and these transporters are involved in hepatocellular bile accumulation. Accordingly, we suggest that the enrollment criteria of the clinical trial be amended to include the following genotypes: *RALBP* rs12680 (GG), *CYP8B1* rs6774801 (CC or CA), *ABCB4* rs2302387 (CC), and *ABCB11* rs4668115 (GG) (**Figure 8I-J**). Since *RALBP* and *CYP8B1* variant genotypes are relatively rare (variant allele frequency = 13% and 36% respectively), the present genotyping criteria should not preclude accrual of patients with thoracic malignancies.

## **2 ELIGIBILITY ASSESSMENT AND ENROLLMENT**

### **2.1 ELIGIBILITY CRITERIA**

#### **2.1.1 Inclusion Criteria**

- 2.1.1.1** Patients with measurable (per RECIST see Section **6.5.1.1**), inoperable, histologically confirmed primary lung and esophageal carcinomas, thymic neoplasms, germ cell tumors, malignant pleural mesotheliomas or chest wall sarcomas, as well as patients with gastric, colorectal or renal cancers and sarcomas metastatic to the thorax.
- 2.1.1.2** Histologic confirmation of disease in the Laboratory of Pathology, CCR, NCI, NIH.
- 2.1.1.3** Disease amenable to biopsy via percutaneous approach or other minimally invasive procedures such as thoracoscopy, bronchoscopy, laparoscopy, or GI endoscopy
- 2.1.1.4** Age  $\geq 18$ .
- 2.1.1.5** ECOG status 0-2.
- 2.1.1.6** Patients must have had or refused first-line standard chemotherapy for their inoperable malignancies.
- 2.1.1.7** Patients must have had no chemotherapy, biologic therapy, or radiation therapy for their malignancy for at least 30 days prior to treatment. Patients may have received localized radiation therapy to non-target lesions provided that the radiotherapy is completed 14 days prior to commencing therapy, and the patient has recovered from any toxicity. At least 3 half-lives must have elapsed since monoclonal antibody treatment. At least six weeks must have elapsed between mitomycin C or nitrosourea treatment.
- 2.1.1.8** Patients must have adequate organ and marrow function as defined below:
  - a) Hematologic and Coagulation Parameters:
    - i. Peripheral ANC  $\geq 1500/\text{mm}^3$
    - ii. Platelets  $\geq 100,000/\text{mm}^3$  (transfusion independent)
    - iii. Hemoglobin  $\geq 8 \text{ g/dL}$  (PRBC transfusions permitted)

- iv. PT/PTT within normal limits (patient may be eligible for trial if abnormality is deemed clinically insignificant and cleared for protocol therapy by Hematology Consult service)
  - b) Hepatic Function
    - i. Bilirubin (total) < 1.5 X upper limit of normal (ULN)
    - ii. ALT (SGPT)  $\leq$  3.0 X ULN
    - iii. Albumin > 2 g/dL
  - c) Renal Function
    - i. Creatinine within normal institutional limits or creatinine clearance  $\geq$  60 mL/min/1.73 m<sup>2</sup> for patients with creatinine levels above institutional normal.
    - ii. Normal ionized calcium, magnesium and phosphorus (can be on oral supplementation)
- 2.1.1.9** Cardiac Function: Left ventricular ejection fraction (EF) >40% by Echocardiogram, MUGA, or cardiac MR.
- 2.1.1.10** Ability of subject to understand, and be willing to sign informed consent
- 2.1.1.11** Female and male patients (and when relevant their partners) must be willing to practice birth control (including abstinence) during and for two months after treatment, if of childbearing potential during sexual contact with a female of childbearing potential.
- 2.1.1.12** Patients must be willing to undergo 2 tumor biopsies
- 2.1.2 Exclusion Criteria**
- 2.1.2.1** Patients with ABCB4, ABCB11, RALBP or CYP8B1 genotypes associated with mithramycin-mediated hepatotoxicity.
- 2.1.2.2** Clinically significant systemic illness (e.g. serious active infections or significant cardiac, pulmonary, hepatic or other organ dysfunction), that in the judgment of the PI would compromise the patient's ability to tolerate protocol therapy or significantly increase the risk of complications
- 2.1.2.3** Patients with cerebral metastases
- 2.1.2.4** Patients with any of the following pulmonary function abnormalities will be excluded: FEV<sub>1</sub>, < 30% predicted; DLCO, < 30% predicted (post-bronchodilator); Oxygen saturation  $\geq$  92% on room air. Arterial blood gas will be drawn if clinically indicated.
- 2.1.2.5** Patients with evidence of active bleeding, intratumoral hemorrhage or history of bleeding diatheses, unless specifically occurring as an isolated incident during reversible chemotherapy-induced thrombocytopenia
- 2.1.2.6** Patients on therapeutic anticoagulation. Note: prophylactic anticoagulation (i.e. intraluminal heparin) for venous or arterial access devices is allowed.

**2.1.2.7** Patients who are concurrently receiving or requiring any of the following agents, which may increase the risk for mithramycin related toxicities, such as hemorrhage:

- Thrombolytic agents
- Aspirin or salicylate-containing products, which may increase risk of hemorrhage
- Dextran
- Dipyridamole
- Sulfinpyrazone
- Valproic acid
- Clopidogrel

**2.1.2.8** Lactating or pregnant females (due to risk to fetus or newborn, and lack of testing for excretion in breast milk).

**2.1.2.9** Patients with history of HIV, HBV or HCV due to potentially increased risk of mithramycin toxicity in this population.

**2.1.2.10** Hypersensitivity to mithramycin

**2.1.2.11** Patients who in the opinion of the investigator may not be able to comply with the safety monitoring requirements of the study.

## **2.2 RESEARCH ELIGIBILITY EVALUATION**

Pre-treatment blood tests should be performed within two weeks and imaging studies **within 4 weeks prior to enrollment** on the trial unless otherwise stated. The evaluations required prior to starting treatment are listed in table form in **Appendix 2: Study Calendar**.

- Complete history and physical examination including assessment of vital signs and ECOG status
- Laboratory
  - ROUTINE LABS: complete blood count, differential, LDH, SGPT (ALT), SGOT (AST), alkaline phosphatase, bilirubin (total and direct), BUN, creatinine, amylase, lipase, electrolytes, ionized calcium, magnesium, phosphorus, uric acid, albumin and thyroid panel.
    - DMET genotyping of PBMC for ABCB4/ABCB11/RALBP/ CYP8B1 SNPs (to be performed by the CLIA Molecular Diagnostics Laboratory (CLIA #21D0947274) through Dr. William D Figg's lab under study 06C0014). (**May be performed at any time prior to enrollment**)
  - Urinalysis.
  - Women of child-bearing potential will have a urine or serum  $\beta$ hCG pregnancy test. *Note: this test is to be done within 2 days prior to start of treatment.*



- Coagulation Studies: PT/PTT/Thrombin Time/Fibrinogen, PFA-100, VWF antigen, VWF activity, Factor VIII activity, D-dimer, Fibrin degradation products and FXIII activity, lupus anticoagulant (LA) test
- Radiographic Evaluation
  - Assessment of disease sites by appropriate radiological evaluation. This should include a CT scan of chest, abdomen and pelvis, brain MR, PET-CT, and other standard-of-care imaging specific for each patient’s malignancy.
- Cardiac Function Studies
  - Assessment of cardiac function (ejection fraction/shortening fraction) must be performed within 4 weeks of enrollment by MUGA, Echocardiogram, or cardiac MR to rule out cardiomyopathy.
  - A 12-Lead electrocardiogram (EKG) will also be performed.
- Pathologic/Tissue Evaluation
  - Histologic confirmation of tumor by the NCI Laboratory of Clinical Pathology, NIH (may be performed at any time prior to registration).

### 2.3 REGISTRATION PROCEDURES

Authorized staff must register an eligible candidate with NCI Central Registration Office (CRO) within 24 hours of signing consent. A registration Eligibility Checklist from the web site (<http://home.ccr.cancer.gov/intra/eligibility/welcome.htm>) must be completed and send via encrypted email to: NCI Central Registration Office [ncicentralregistration-l@mail.nih.gov](mailto:ncicentralregistration-l@mail.nih.gov). After confirmation of eligibility at Central Registration Office, CRO staff will call pharmacy to advise them of the acceptance of the patient on the protocol prior to the release of any investigational agents. Verification of Registration will be forwarded electronically via e-mail to the research team. A recorder is available during non-working hours.

#### 2.3.1 Treatment Assignment Procedures (For registration purposes only)

##### Cohorts

Number	Name	Description
1	Primary thoracic malignancy	Patients with primary thoracic malignancy with favorable ABCB4/ABCB11/RALBP/CYP8B1 genotypes
2	Metastatic extra-thoracic malignancy	Patients with extra-thoracic malignancy metastatic to lungs, pleura or mediastinum with favorable ABCB4/ABCB11/RALBP, CYP8B1 genotypes
3	Thoracic malignancy, primary or metastatic -no genotyping (Closed)	Subjects enrolled without regard to ABCB4/ABCB11 genotypes

##### Arms

Number	Name	Description
1	Mithramycin	Single agent IV mithramycin.

## **Arm Assignment**

Patients in cohorts 1 and 2 will be directly assigned to arm 1.

The 12 patients in cohort 3 were directly assigned to arm 1. Cohort 3 is now closed.

## **3 STUDY IMPLEMENTATION**

### **3.1 STUDY DESIGN**

This study will be conducted as a single site, phase II trial utilizing a Simon Optimal Design. Patients will be analyzed in two cohorts on the basis of primary thoracic malignancy vs. extra-thoracic malignancy metastatic to lungs, pleura or mediastinum. The goal of the study is to determine response rates, pharmacokinetics and molecular responses following 6h mithramycin infusions as a prelude to combinatorial studies as well as protocols utilizing 24h continuous infusions targeting pharmacodynamic endpoints in tumor tissues.

Patients will undergo eligibility evaluation in the Thoracic Surgery Clinic (OP-3). Patients deemed eligible for therapy will sign an informed consent and receive mithramycin as described in Section 3.2. Patients may continue to receive mithramycin as long as they tolerate drug and do not have progressive disease (Section 6.4).

Patients on study will be closely monitored for the development of toxicities using regular physical examinations, blood pressure monitoring, and laboratory evaluations with particular attention to the development of any signs of bleeding. Patients will be admitted to the hospital for the first treatment cycle to allow for close monitoring of toxicities. For subsequent cycles, local patients may receive infusions in the day hospital; patients traveling from out-of-town will be admitted to the hospital. Two weeks (plus 12 or minus 3 days) following each course of therapy (three 21-day cycles) patients will undergo appropriate staging studies to determine response to therapy using RECIST criteria.

### **3.2 DRUG ADMINISTRATION**

Given our recent findings pertaining to ABCB4, ABCB11, RALBP and CYP8B1 SNPs that distinguish patients who did and did not experience mithramycin-mediated hepatotoxicity on this trial, the protocol has been amended to accrue only those patients with favorable ABCB4/ABCB11/RALBP/CYP8B1 genotypes. Fourteen of these patients (7 with primary thoracic malignancies and 7 with extrathoracic malignancies metastatic to the chest) will receive mithramycin infusions during cycle 1 at 30mcg/kg per day. Those patients tolerating 30 mcg/kg infusions during the first cycle will receive subsequent cycles of mithramycin at a dose of 50 mcg/kg using the same infusion schedule. Based on the PK data obtained on this trial to date, this dose of mithramycin would be expected to produce C<sub>max</sub> of ~40nM and steady state levels of ~20-25nM, concentrations approximating lowest therapeutic doses in preclinical tissue culture experiments. Patients exhibiting dose-limiting toxicities during cycle 1 will receive subsequent infusions at a dose of 25mcg/kg, with dose reduction guidelines listed in section 3.3. If one or more patients in each cohort exhibit response to therapy, an additional 14 patients will be accrued to the respective cohort(s) to further define response rates Simon Second Stage). Drug administration in the Second Stage will be identical to that in the First stage.

Mithramycin will be administered intravenously via central line over 6 hours once daily for 7 days to be repeated every 21 days (+7 days/-3 days). Three cycles will constitute one course. Intravenous hydration will be provided as clinically indicated.

**Note:** At least 23 hours should elapse between the beginning of one infusion and the beginning of the next.

### 3.3 DOSING DELAYS AND MODIFICATIONS

#### General guidelines for treatment modifications:

Adverse Events: All adverse events in this trial will be graded using the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 - a complete listing is available at the CTEP website: <http://ctep.cancer.gov/forms/CTCAEv4.pdf>.

Dose adjustments will be made according to the guidelines below, with dose levels defined as follows:

Dose Level	Mithramycin Dose
2	50 mcg/kg
1	30 mcg/kg
-1	25 mcg/kg
-2	18 mcg/kg

Summary of dose holding/interruptions and dose de-escalation recommendations for mithramycin in case of mithramycin -related adverse events (graded according to NCI-CTCAEv4.0)

General Adverse Events	Action
Non-hematological, Grade 1 or 2 (excluding hemorrhage)	<ul style="list-style-type: none"> <li>Continue mithramycin therapy at full dose prescribed.</li> <li>Apply maximum supportive care recommendations.</li> <li>If prolonged duration of Grade 2 adverse event (<math>\geq 7</math> days) is affecting quality of life, start the next treatment cycle at DL -1.</li> <li>If event persists and continues to affect quality of life for <math>\geq 7</math> more days following initial dose reduction, start the next treatment cycle at DL -2.</li> <li>If events continue to persist, discontinue drug.</li> </ul>
Grade 3 ALT and AST	<ul style="list-style-type: none"> <li>Hold mithramycin therapy until recovery to Grade <math>\leq 1</math> (up to 21 days of start of cycle).</li> <li>If ALT and AST resolve to Grade <math>\leq 1</math> within 21 days, maintain current dose level.</li> <li>If ALT and AST do NOT resolve to Grade <math>\leq 1</math> within 21 days, dose reduce 1 level.</li> <li>If grade 4 ALT or AST occur in a subsequent cycle, dose reduce 1 level independent of the date of resolution.</li> </ul>
Grade 4 ALT and AST	<ul style="list-style-type: none"> <li>Hold mithramycin therapy until recovery to Grade <math>\leq 1</math> (up to 21 days of start of cycle).</li> <li>If ALT and AST resolve to Grade <math>\leq 1</math> within 21 days,</li> </ul>

General Adverse Events	Action
	<p>reduce 1 level.</p> <ul style="list-style-type: none"> <li>• If grade 4 ALT or AST occur in a subsequent cycle, dose reduce 1 level independent of the date of resolution.</li> </ul>
<p>Non-hematological, Grade 3 or 4 (excluding ALT, AST, hemorrhage and QT prolongation)</p>	<ul style="list-style-type: none"> <li>• Apply maximum supportive care recommendations. Hold mithramycin therapy until recovery to Grade <math>\leq 1</math> (up to 21 days of start of cycle) and start the next treatment cycle at the next lower dose level. <i>Note: if patient's toxicity recovers to Grade <math>\leq 1</math> prior to day 7 of treatment cycle, dosing for that cycle may resume at one dose level lower. Dosing will stop on day 7 (example: grade 3 event occurs on day 2 of dosing and resolves by day 5; patient will be allowed to receive mithramycin on days 6 and 7 at one dose level lower.)</i></li> <li>• If recurrence of adverse event after drug hold/interruptions is observed at the reduced dose, and maximum supportive care measures applied, hold drug once again until recovery to Grade <math>\leq 1</math> (up to 21 days) and start the next treatment cycle <i>one dose level lower</i>. <i>Note: if patient's toxicity recovers to Grade <math>\leq 1</math> prior to day 7 of treatment cycle, dosing for that cycle may resume one dose level lower.</i></li> <li>• If symptoms continue to persist at grade 3 or 4, following maximal dose reduction, discontinue drug.</li> </ul>
<p>QT prolongation, Grade 3 or 4</p>	<ul style="list-style-type: none"> <li>• Cardiology consultation will be obtained to determine whether any cardiac functional assessment is warranted or if therapy should be delayed or discontinued. When warranted, ECGs will subsequently be reviewed by the NCI consulting cardiologist.</li> <li>• If therapy is continued, dose reduce 1 level for grade 4 events</li> </ul>
<p>Bleeding Events (hemorrhage)</p>	
<p>Grade 1 events</p>	<ul style="list-style-type: none"> <li>• Continue mithramycin therapy at full dose prescribed.</li> <li>• Apply maximum supportive care recommendations.</li> </ul>
<p>Grade 2 events</p>	<ul style="list-style-type: none"> <li>• Apply maximum supportive care recommendations. Hold mithramycin therapy until recovery to Grade <math>\leq 1</math> (up to 28 days from start of treatment cycle) and start the next treatment cycle at DL -1.</li> <li>• If recurrence of adverse event after drug hold/interruptions is observed, and maximum supportive care measures applied, hold drug once again until recovery to Grade <math>\leq 1</math> (up to 21 days from start of treatment cycle) and start the next treatment cycle at DL -2.</li> <li>• If symptoms continue to persist at any grade, discontinue drug.</li> </ul>
<p>Grade 3 events</p>	<ul style="list-style-type: none"> <li>• Discontinue mithramycin</li> </ul>

General Adverse Events	Action
Grade 4 events	<ul style="list-style-type: none"> <li>Discontinue mithramycin</li> </ul>
<i>All grade 2-4 hemorrhage events will initiate full hematologic evaluations as indicated in Section 3.5.1- Coagulation Studies, as well as formal Hematology consult by Dr. Jay Lozier or one of his designates</i>	
Hematological Adverse Events	
Grades 1 and 2 events	<ul style="list-style-type: none"> <li>Continue mithramycin therapy at full dose prescribed. Apply maximum supportive care recommendations.</li> </ul>
Grade 3 events	<ul style="list-style-type: none"> <li>Apply supportive care</li> <li>If toxicities resolved to allow retreatment by day 14, resume at current dose.</li> <li>If toxicities resolve between days 14-21 to allow retreatment (See section 3.4), resume at DL-1.</li> <li>Following dose reduction, if toxicities persist and do not allow treatment at day 14, but are sufficiently resolved to allow retreatment by day 21, resume at DL-2.</li> </ul>
Grade 4 events	<ul style="list-style-type: none"> <li>Apply maximum supportive care recommendations.</li> <li>Hold mithramycin therapy until patient meets hematologic criteria for retreatment (See section 3.4) (must be within 21 days of start of treatment cycle) and start the next treatment cycle at DL -1.</li> <li>If recurrence of hematological adverse event after drug hold/ interruptions is observed with treatment at DL-1, in the setting of maximum supportive care measures applied, hold drug once again until patient meets hematologic criteria for retreatment (See section 3.4) (must be within 28 days of start of treatment cycle) and start the next treatment cycle at DL -2.</li> <li>If symptoms continue to persist at any grade, discontinue drug.</li> </ul>

### 3.4 CRITERIA FOR ADDITIONAL TREATMENT

Patients will be eligible to continue to receive mithramycin for as long as they do not experience clinical or radiographic disease progression (See section 6.4) and tolerate mithramycin (as defined in Section 3.3).

Patients may receive the next course of mithramycin at the same dose level (or reduced level as above) provided the following criteria are met by day 21.

- patient has stable or responding disease;
- has a platelet count  $\geq 75,000/\mu\text{L}$  (transfusion independent), and an ANC  $\geq 1000/\mu\text{L}$ , and meets other laboratory parameters defined in the eligibility section; and
- has not met any criteria for removal from treatment or off study (Section 3.6)

### **3.5 ON STUDY PROTOCOL EVALUATION (APPENDIX 2: STUDY CALENDAR)**

#### **3.5.1 Baseline Evaluations**

- Total and fractionated serum bile acid levels within 2 weeks prior to treatment. *Note: results need not be back prior to commencing treatment.*
- Liver elastography assessment by ultrasound within 2 weeks prior to treatment

#### **3.5.2 Monitoring During Treatment:**

- Physical exam, to include vital signs, weight, toxicity evaluation and ECOG status within 4 days of each treatment cycle.
- Toxicity assessment during cycles 1 and 2: daily during treatment and then weekly. During cycle 3 and subsequent cycles, twice weekly during treatment and then weekly.
- Laboratory evaluations:
  - CBC with differential count within 2 days prior to treatment and daily during drug administration (days 1-7 prior to each infusion, active treatment week); then twice weekly on off-treatment weeks of cycles 1-2. During subsequent cycles: CBC with differential count within 2 days prior to commencing treatment and twice weekly during administration prior to infusion, then weekly.
  - Chemistry: sodium, potassium, chloride, CO<sub>2</sub>, creatinine, glucose, BUN, albumin, ionized calcium\*, magnesium, alkaline phosphatase, ALT/GPT, AST/GOT, total bilirubin, total protein daily within 2 days of commencing treatment and during drug administration (days 1-7 prior to each infusion, active treatment week); then twice weekly on off-treatment weeks of cycles 1-2. During subsequent cycles chemistries are performed weekly.
  - Coagulation Studies: PT/PTT/Thrombin Time, Fibrinogen prior to day 1 and on days +4 and +8 of every cycle.
  - Total and fractionated bile acids within 2 days prior to and on day +7 of every treatment cycle and at time of treatment evaluation at the end of the 3<sup>rd</sup> cycle of therapy.

**Note:** The patient may have evaluations and lab work obtained through his or her local physician between cycles and have the results faxed to the Research Nurse. The Research RN will contact the patient as noted above to discuss toxicity.

\* Ionized calcium is not part of a standard lab panel and could be omitted if not available or not covered by insurance when performed outside NIH.

- 12-Lead EKG:
  - Cycles 1 and 2: Days 1, 4, and 7, before infusion within 1 hour of pre infusion PK sample to evaluate for QT prolongation potential

**Note:** although it is preferable to have the EKGs done within 1 hour of PK sample collection, EKGs may be done outside of the specified times if unexpected events in clinic or floor prevent staff from conducting the study within the specified timeframe. EKGs performed outside of this window will NOT be considered protocol deviations.

- Cycle 3 and subsequent cycles: Within 1 hour of start of infusion on day 1 and day 7.
- Tumor Measurements: CT scan of the chest, abdomen and pelvis, brain MR scan as clinically indicated, as well as FDG-PET and other standard-of-practice scans relevant to each patient's malignancy, as well as liver elastography by ultrasound will be performed two weeks (plus 12 or minus 3 days) after every course of therapy (three cycles).

### **3.5.3 Research Evaluations**

**Note:** all samples are to be placed on wet ice and transported to the Thoracic Epigenetics Lab (TEL) within one hour of draw

- Cycles 1 and 2 day 1, prior to infusion: 1 mL of plasma will be obtained, filtered through a 0.22 micron filter (Kit available in TEL) to be saved for future clinical reference as needed
- Pharmacokinetic (PK) studies - See [Appendix 3](#) for details - PK samples will be processed and stored in the TEL and later transported to the NCI, POB, Pharmacology and Experimental Therapeutics Section (PETS). Samples will be drawn on days 1, 2, 4, 7, 8, and 9. (**Note:** Patients who do not live locally will not be required to remain in the area for the day 9 PK sample.)
- Baseline (once) and then day 8 of **every cycle** - Two 10 mL red top tubes and 4 10 mL lavender top tubes for the TEL (Note: these are the only specimens collected at each cycle.)
- Tumor biopsies will be collected once at baseline and again on day 8 of cycle 2 as described in Section [5.2](#).
  - Within two days prior to biopsies: Normal PT/PTT with exception of lupus anticoagulant, platelets  $\geq 75,000/\mu\text{L}$ , peripheral ANC  $\geq 750/\mu\text{L}$

### **3.5.4 Follow-up Evaluations:**

After completion the last treatment cycle (See section [3.4](#) for criteria for retreatment), patients will be monitored weekly until all clinically significant toxicities resolve to less than or equal to grade 1 or baseline as follows:

- Toxicity Evaluation
- Laboratory evaluation: CBC, Chem20 equivalent including ionized calcium\*, PT/PTT
- For patients with stable disease or responding to therapy, the following will be done every 2 months until off study criteria are met:
  - History and physical exam
  - Laboratory evaluation: CBC, Chem20 equivalent including ionized calcium\*, PT/PTT
  - Tumor Measurements: CT scan of the chest, abdomen and pelvis as well as PET/CT and brain MR or CT scan.

Note: The patient may have evaluations and lab work obtained through his or her local physician during follow up.

\* Ionized calcium is not part of a standard lab panel and could be omitted if not available or not covered by insurance when performed outside NIH.

### **3.6 CRITERIA FOR REMOVAL FROM PROTOCOL THERAPY AND OFF STUDY CRITERIA**

#### **3.6.1 Criteria for removal from protocol therapy**

- Patient refusal of further treatments
- It is deemed in the best interest of the patient.
- A patient who develops a concurrent serious medical condition that might preclude or contraindicate the further administration of mithramycin will be removed from treatment.
- A patient who becomes pregnant will be immediately taken off therapy.
- Excessive toxicity

**Note:** Patient will be followed until toxicity resolves to baseline or grade 1 or less.

#### **3.6.2 Off-Study criteria**

- Voluntary withdrawal for any reason, or noncompliance with protocol requirements
- Completion of the follow up period (See section 3.5.4)
- Progressive disease, unless the patient is being followed for a serious adverse event related to the research. The event must resolve to less than or equal to grade 1 or baseline prior to removing from the study.
- If the investigator determines that it is in the best interest of the patient to discontinue follow up
- Death

#### **3.6.3 Off Protocol Therapy and Off Study Procedure**

Authorized staff must notify Central Registration Office (CRO) when a subject is taken off protocol therapy and when a subject is taken off-study. A Participant Status Update Form from the web site (<http://home.ccr.cancer.gov/intra/eligibility/welcome.htm>) main page must be completed and sent via encrypted email to: NCI Central Registration Office [ncicentralregistration-1@mail.nih.gov](mailto:ncicentralregistration-1@mail.nih.gov).

## **4 CONCOMITANT MEDICATIONS/MEASURES**

### **4.1 HEMATOLOGIC AND BLOOD PRODUCT SUPPORT**

Blood product support should be provided to maintain platelets > 20,000 cells/mcl, Hgb > 8.0 gm/dl and as clinically indicated. Growth factor support is not permitted with exception of administration of filgrastim. Filgrastim will not be administered prophylactically but may be administered during cycle 1 if a patient experiences neutropenic sepsis, or during subsequent cycles if clinically indicated.

### **4.2 ANTIEMETIC THERAPY AND TRANSAMINITIS PROPHYLAXIS**

Ondansetron (or other drugs from this same class of anti-emetics, as per investigator preference) prior to and after mithramycin as needed. Additional antiemetic therapy may be provided by either intravenous or oral route as needed. Patients that experience significant nausea during cycle one may be offered aprepitant for subsequent cycles. Patients will receive either dexamethasone 8 mg



PO or dexamethasone 12mg IV the evening prior to the first dose of mithramycin and then will receive dexamethasone 12 mg IV daily through at least the first course of therapy.

### **4.3 ANTI-DIARRHEAL**

Antidiarrheal agents will be prescribed using standard clinical practice guidelines at the preference of the investigator.

### **4.4 ELECTROLYTE REPLACEMENT**

Electrolyte replacement will be provided to maintain serum levels within normal limits.

## **5 CORRELATIVE STUDIES / BIOSPECIMEN COLLECTION**

### **5.1 PHARMACOKINETIC (PK) SAMPLING**

Detailed plasma pharmacokinetic (PK) sampling of mithramycin will be performed prior to and at the completion of the first dose of mithramycin during cycle 1 and cycle 2 of course 1 (up to 11 tubes, Refer to details in [Appendix 3](#)). In addition, through and end of infusion samples will be obtained with the day 2, 4 and 7 doses to assess mithramycin accumulation, and samples will be obtained 24 and 48 hours after the day 7 dose of mithramycin on days 8 and 9, respectively. PK analysis will be performed at the NCI, Genitourinary Malignancies Branch (GMB). Samples will be stored in the TEL and batched and later transferred to the Figg Lab on dry ice for analysis. Please refer to section [5.3.2](#) for instructions on sample processing.

### **5.2 STUDIES ON TUMOR TISSUE**

Tissue biopsies will be obtained by minimally invasive methods such as CT guided percutaneous, endoscopic, laparoscopic or video-assisted thoracoscopic techniques at baseline and on day 8 ( $\pm 2$  days) of the second cycle. If insufficient tissue was obtained or if there was harm related to the first biopsy, a second biopsy will not be obtained. Should a patient experience a dramatic clinical response to treatment, additional tumor tissue biopsies may be requested, but are not required. At the time of tissue acquisition, immediate on-site cytopathologic analysis will be performed to confirm that malignant cells are present in the biopsies, to enhance potential yield and improve subsequent evaluation of molecular endpoints. Samples will be placed in saline in an orange top tube on wet ice and transported to the Thoracic Epigenetics Lab for further processing. In the Thoracic Epigenetics Lab, a portion will be separated and frozen on dry ice for RNA collection for microarray and/or gene signature evaluation, using a customized panel of < 100 genes, microRNA and DNA methylation sites noted to be modulated in human cancer xenografts in mice treated intraperitoneally with mithramycin. If sufficient tissue is available, another portion will be imbedded in paraffin for subsequent immunostaining experiments, focusing on expression of genes correlating with treatment response in our preclinical experiments. Additional tissue will be used to establish cell lines to examine effects of mithramycin on the epigenome and stem cell signaling *in vitro*, in an attempt to correlate these findings with results obtained from analysis of tumor biopsies, or to examine SP. All of the analyses, which are predicated on acquisition of sufficient biopsy materials, will be performed in the Thoracic Epigenetics Lab/TGIB under direction of the PI.

*Note:* The patient will consent to the optional biopsy at the time of the procedure. If the patient refuses the optional biopsy at that time, the refusal will be documented in the medical record and in the research record.

### **5.3 SAMPLE STORAGE, TRACKING AND DISPOSITION**

#### **5.3.1 Thoracic Epigenetics Section, TOSB/NCI**

This study will be conducted within the Thoracic Epigenetics Section, TGIB/NCI. Sample collection and initial processing will be performed in the Thoracic Epigenetics Laboratory. Samples will be stored in designated monitored freezers (at least -20°C). All samples obtained on this study will be tracked using LabMatrix. Pharmacokinetic samples will be analyzed at the NCI PETS. Samples will be identified and tracked using unique identifiers linked to each subject's unique patient number (study number). Codes linking personal identifiable information to the unique identifier will be stored in secure, computer servers with limited coded access or locked file cabinets in Thoracic Epigenetics Section, TOSB/NCI with access limited to the PI or study coordinator. Focused gene expression, microRNA, and DNA methylation analysis will be performed by nano-string and pyrosequencing techniques in Dr. David Schrupp's laboratory. The study will remain open and status reported to the NCI IRB until all samples have been analyzed, reported or destroyed. Unintentional loss or destruction of any samples will be reported to the NCI IRB as part of annual continuing reviews. Any use of samples not outlined in Section 5 will require prospective NCI IRB review and approval.

At the conclusion of this study, those samples from patients who have not authorized the continued use of their specimens for research purposes will be destroyed in accordance with the environmental protection laws, regulations and guidelines of the Federal Government and the State of Maryland. Any unintentional destruction or loss of the specimens will be reported to the IRB. Patients who agree to allow continued use of their specimens for research purposes will be enrolled on 06-C-0014.

#### **5.3.2 Blood Processing Core (BPC)**

##### **Blood Collection**

Please e-mail Julie Barnes at [Julie.barnes@nih.gov](mailto:Julie.barnes@nih.gov) and Paula Carter [pcartera@mail.nih.gov](mailto:pcartera@mail.nih.gov) at least 24 hours before transporting samples (the Friday before is preferred).

For sample pickup, page 102-11964.

For immediate help, call 240-760-6180 (main blood processing core number) or, if no answer, 240-760-6190 (main clinical pharmacology lab number).

For questions regarding sample processing, contact Julie Barnes by e-mail or at 240-760-6044.

The samples will be processed, barcoded, and stored in Dr. Figg's lab until requested by the investigator.

##### **Sample Data Collection**

All samples sent to the Blood Processing Core (BPC) will be barcoded, with data entered and stored in the LABrador (aka LabSamples) utilized by the BPC. This is a secure program, with access to LABrador limited to defined Figg lab personnel, who are issued individual user accounts. Installation of LABrador is limited to computers specified by Dr. Figg. These computers all have a password restricted login screen. All Figg lab personnel with access to patient information annually complete the NIH online Protection of Human Subjects course.

LABrador creates a unique barcode ID for every sample and sample box, which cannot be traced back to patients without LABrador access. The data recorded for each sample includes the patient

ID, name, trial name/protocol number, time drawn, cycle time point, dose, material type, as well as box and freezer location. Patient demographics associated with the clinical center patient number are provided in the system. For each sample, there are notes associated with the processing method (delay in sample processing, storage conditions on the ward, etc.).

Sample bar-codes are linked to patient demographics and limited clinical information. This information will only be provided to investigators listed on this protocol, via registered use of the LABrador. It is critical that the sample remains linked to patient information such as race, age, dates of diagnosis and death, and histological information about the tumor, in order to correlate genotype with these variables

### **Sample Storage and Destruction**

Barcoded samples are stored in barcoded boxes in a locked freezer at either -20 or -80°C according to stability requirements. These freezers are located onsite in the BPC and offsite at NCI Frederick Central Repository Services in Frederick, MD. Visitors to the laboratory are required to be accompanied by laboratory staff at all times.

Access to stored clinical samples is restricted. Samples will be stored until requested by a researcher named on the protocol. All requests are monitored and tracked in LABrador. All researchers are required to sign a form stating that the samples are only to be used for research purposes associated with this trial (as per the IRB approved protocol) and that any unused samples must be returned to the BPC. It is the responsibility of the NCI Principal Investigator to ensure that the samples requested are being used in a manner consistent with IRB approval.

Following completion of this study, samples will remain in storage as detailed above. Access to these samples will only be granted following IRB approval of an additional protocol, granting the rights to use the material.

If, at any time, a patient withdraws from the study and does not wish for their existing samples to be utilized, the individual must provide a written request. Following receipt of this request, the samples will be destroyed (or returned to the patient, if so requested), and reported as such to the IRB. Any samples lost (in transit or by a researcher) or destroyed due to unknown sample integrity (i.e. broken freezer allows for extensive sample thawing, etc.) will be reported as such to the IRB.

## **6 DATA COLLECTION AND EVALUATION**

### **6.1 DATA COLLECTION**

The Principal Investigator will be responsible for the collection, maintenance, and quality control of the study data. All clinical data collected for each study subject will be entered into the C3D database. Pharmacokinetic and pharmacodynamic research specimens will be entered into Labmatrix. The Principal Investigator is responsible for maintaining any source documentation related to the study, including any films, tracings, computer discs or tapes. The principal investigator, associate investigators/research nurses and/or a contracted data manager will assist with the data management efforts. All data obtained during the conduct of the protocol will be kept in secure network drives or in approved alternative sites that comply with NIH security standards. Primary and final analyzed data will have identifiers so that research data can be attributed to an individual human subject participant. The Sponsor and its monitoring agency will have access to C3D and Labmatrix for data monitoring and verification. The investigators will allow study monitors and/or the Food and Drug Administration (FDA) to inspect study documents (e.g. consent

forms, drug distribution forms, IRB approval) and pertinent hospital or clinical records for confirmation of data throughout the study period.

**End of study procedures:** Data will be stored according to HHS, FDA regulations, and NIH Intramural Records Retention Schedule as applicable.

**Loss or destruction of data:** Should we become aware that a major breach in our plan to protect subject confidentiality and trial data has occurred, the IRB will be notified.

### **6.1.1 Source Documents**

Source documents are original documents, data and records which include hospital records, clinic and office charts, laboratory data/information, patient diaries or evaluation checklists, pharmacy dispensing and other records, recorded data from automated instruments, microfiches, photographic negatives, microfilm or magnetic media, x-rays. The Principal Investigator will permit trial-related monitoring, audits, IRB review, and regulatory inspections, providing direct access to source data documents.

## **6.2 ROUTINE DATA COLLECTION**

Following registration, all adverse events will be described in the source documents, reviewed by the designated research nurse, and captured in C3D.

- During the follow up period (more than 30 days following the last treatment), only those events that are serious, unexpected, and related to the treatment will be captured in C3D.
- All toxicities occurring within 30 days of treatment will be followed until resolution or return to baseline.

## **6.3 EXCLUSIONS TO ROUTINE DATA COLLECTION:**

### **6.3.1 Adverse Events**

The following Adverse Events will be captured only in the source documents and will not be reported in C3D

- Laboratory values that do not support the diagnosis of a reportable event
- All grade 1 events, except for bleeding events, which will all be captured regardless of grade.
- Grade 2 events that are not related to the treatment
- Grade 1, 2, and 3 lab values drawn outside of the protocol specified time points that are not assessed as clinically significant by the PI or his designee and are not associated with an adverse event
- Events related to vascular access devices (occlusion, thrombi, hospitalizations for insertion or removal)

**Note:** Events that result in a hospitalization for convenience will not be reported.

### **6.3.2 Concomitant medications/measures:**

- All concomitant medications and measures will be captured in the source documents. Only those medications that the patient is taking at baseline on a routine basis or medications

that cause an AE will be captured in C3D (e.g., onetime medications, PRN medications, supportive medications, electrolyte replacement and medications given to treat adverse events will not be captured in C3D).

## **6.4 DATA SHARING PLANS**

### **6.4.1 Human Data Sharing Plan**

I will share coded, linked human data generated in this research for future research

- in a NIH-funded or approved public repository [clinicaltrials.gov](http://clinicaltrials.gov)
- in BTRIS
- in publication and/or public presentations

at the time of publication or shortly thereafter

### **6.4.2 Genomic Data Sharing Plan**

The study is not subject to the NIH genomic data sharing policy. The gene and microRNA as well as DNA methylation studies performed under this protocol are not genome wide.

## **6.5 RESPONSE CRITERIA**

Objective response and progression will be evaluated in this study using the new international criteria proposed by the Response Evaluation Criteria in Solid Tumors (RECIST) Committee [version 1.1 <sup>83</sup>]. Note: Lesions are either measurable or non-measurable using the criteria provided below. The term “evaluable” in reference to measurability will not be used because it does not provide additional meaning or accuracy.

### **6.5.1 Response Criteria for Radiographic Studies**

#### **6.5.1.1 Measuring of Soft Tissue Disease**

Response and progression will be evaluated in this study using the new international criteria proposed by the revised Response Evaluation Criteria in Solid Tumors (RECIST) guideline [version 1.1 <sup>83</sup>]. Changes in the largest diameter (unidimensional measurement) of the tumor lesions and the shortest diameter in the case of malignant lymph nodes are used in the RECIST criteria.

#### **a. Evaluation of Target Lesions**

##### **Complete Response (CR)**

Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to <10 mm

##### **Partial Response (PR)**

At least a 30% decrease in the sum of the diameters of target lesions, taking as reference the baseline sum diameters

##### **Progressive Disease (PD)**

At least a 20% increase in the sum of the diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase

of at least 5 mm. (Note: the appearance of one or more new lesions is also considered progressions)

Note: determination of PD will not be made prior to the Day 60 evaluation.

**Stable Disease (SD)**

Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study

b. Evaluation of Non-Target Lesions

**Complete Response (CR)**

Disappearance of all non-target lesions and normalization of tumor marker level. All lymph nodes must be non-pathological in size (<10 mm short axis)

Note: If tumor markers are initially above the upper normal limit, they must normalize for a patient to be considered in complete clinical response.

**Non-CR/Non-PD (Stable Disease, SD)**

Persistence of one or more non-target lesion(s) and/or maintenance of tumor marker level above the normal limits.

**Progressive Disease (PD)**

Appearance of one or more new lesions and/or *unequivocal progression* of existing non-target lesions. *Unequivocal progression* should not normally trump target lesion status. It must be representative of overall disease status change, not a single lesion increase.

Although a clear progression of “non-target” lesions only is exceptional, the opinion of the treating physician should prevail in such circumstances, and the progression status should be confirmed at a later time by the review panel (or Principal Investigator).

c. Evaluation of Best Overall Response

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for progressive disease the smallest measurements recorded since the treatment started). The patient's best response assignment will depend on the achievement of both measurement and confirmation criteria.

Target Lesions	Non-Target Lesions	New Lesions	Overall Response	Best Overall Response when Confirmation is Required*
CR	CR	No	CR	≥4 wks. Confirmation**
CR	Non-CR Non-PD	No	PR	≥4 wks. Confirmation**
CR	Not evaluated	No	PR	
PR	Non-CR Non-PD Not evaluated	No	PR	
SD	Non-CR Non-PD Not evaluated	No	SD	Documented at least once ≥4 wks. from baseline**

Target Lesions	Non-Target Lesions	New Lesions	Overall Response	Best Overall Response when Confirmation is Required*
PD	Any	Yes or No	PD	No prior SD, PR or CR
Any	PD***	Yes or No	PD	
Any	Any	Yes	PD	
<p>*See RECIST 1.1 manuscript for further details on what is evidence of a new lesion.  **Only for non-randomized trials with response as primary endpoint.  ***In exceptional circumstances, unequivocal progression in non-target lesions may be accepted as disease progression</p>				

- d. Patients with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be reported as “*symptomatic deterioration.*” Every effort should be made to document the objective progression even after discontinuation of treatment.
- e. In some circumstances, it may be difficult to distinguish residual disease from normal tissue. When the evaluation of complete response depends on this determination, it is recommended that the residual lesions be investigated (fine needle aspirate/biopsy) before confirming the complete response status.

### **6.5.2 Confirmatory Measurement/Duration of Response**

- Confirmation

To be assigned a status of PR or CR, changes in tumor measurements must be confirmed by repeat assessments that should be performed at least 4 weeks after the criteria for response are first met.

- Duration of Overall Response

The duration of overall response is measured from the time measurement criteria are met for CR or PR (whichever is first recorded) until the first date that recurrent or progressive disease is objectively documented (taking as reference for progressive disease the smallest measurements recorded since the treatment started).

The duration of overall CR is measured from the time measurement criteria are first met for CR until the first date that progressive disease is objectively documented.

### **6.5.3 Measurable Disease**

Measurable lesions are defined as those that can be accurately measured in at least one dimension (longest diameter to be recorded) as:

- By chest x-ray:  $\geq 20$  mm;
- By CT scan:
  - Scan slice thickness 5 mm or under as  $\geq 10$  mm with CT scan
  - Scan slice thickness  $> 5$  mm: double the slice thickness
- With calipers on clinical exam:  $\geq 10$  mm.

All tumor measurements must be recorded in millimeters (or decimal fractions of centimeters).

Note: Tumor lesions that are situated in a previously irradiated area might or might not be considered measurable. *If the investigator thinks it appropriate to include them, the conditions under which such lesions should be considered must be defined in the protocol.*

#### **6.5.4 Malignant Lymph Nodes**

To be considered pathologically enlarged and measurable, a lymph node must be  $\geq 15$  mm in short axis when assessed by CT scan (CT scan slice thickness recommended to be no greater than 5 mm). At baseline and in follow-up, only the short axis will be measured and followed.

#### **6.5.5 Non-Measurable Disease**

All other lesions (or sites of disease), including small lesions (longest diameter  $< 10$  mm or pathological lymph nodes with  $\geq 10$  to  $< 15$  mm short axis), are considered non-measurable disease. Bone lesions, leptomeningeal disease, ascites, pleural/pericardial effusions, lymphangitis cutis/pulmonitis, inflammatory breast disease, and abdominal masses (not followed by CT or MRI), are considered as non-measurable.

Note: Cystic lesions that meet the criteria for radiographically defined simple cysts should not be considered as malignant lesions (neither measurable nor non-measurable) since they are, by definition, simple cysts.

‘Cystic lesions’ thought to represent cystic metastases can be considered as measurable lesions, if they meet the definition of measurability described above. However, if non-cystic lesions are present in the same patient, these are preferred for selection as target lesions.

#### **6.5.6 Target Lesions**

All measurable lesions up to a maximum of 2 lesions per organ and 5 lesions in total, representative of all involved organs, should be identified as **target lesions** and recorded and measured at baseline. Target lesions should be selected on the basis of their size (lesions with the longest diameter), be representative of all involved organs, but in addition should be those that lend themselves to reproducible repeated measurements. It may be the case that, on occasion, the largest lesion does not lend itself to reproducible measurement in which circumstance the next largest lesion which can be measured reproducibly should be selected. A sum of the diameters (longest for non-nodal lesions, short axis for nodal lesions) for all target lesions will be calculated and reported as the baseline sum diameters. If lymph nodes are to be included in the sum, then only the short axis is added into the sum. The baseline sum diameters will be used as reference to further characterize any objective tumor regression in the measurable dimension of the disease.

Progressive disease by RECIST criteria [1] noted after the first re-staging scan may represent disease that was not detected on the pre-study scan, and a confirmatory scan will be required at the next scheduled re-staging evaluation unless clinically not indicated. If confirmed, progression should be dated by the initial time when the lesions are first detected. If progressive disease by RECIST criteria is seen after cycle 2, but not confirmed on subsequent restaging scan, the scans from after cycle 2 would serve as the baseline scan to evaluate for disease progression <sup>84</sup>.

#### **6.5.7 Non-Target Lesions**

All other lesions (or sites of disease) including any measurable lesions over and above the 5 target lesions should be identified as **non-target lesions** and should also be recorded at baseline.



Measurements of these lesions are not required, but the presence, absence, or in rare cases unequivocal progression of each should be noted throughout follow-up.

#### **6.5.8 Metastatic Bone Lesions**

Disease progression is considered if a minimum of two new lesions is observed on bone scan. New lesions seen by the end of cycle 2 or before cycle 3 (with the first re-staging bone scan) may represent disease that was not detected on the pre-study scan, and a confirmatory scan will be required at the next scheduled re-staging bone scan unless clinically not indicated. If confirmed, progression should be dated by the initial time when the lesions are first detected. If new lesions are seen after cycle 2, but no additional lesions are seen on confirmatory scans, the scans from post-cycle 2 would serve as the baseline scan to evaluate for disease progression <sup>84</sup>.

#### **6.5.9 Clinical Lesions**

Clinical lesions will only be considered measurable when they are superficial (e.g., skin nodules and palpable lymph nodes) and  $\geq 10$  mm diameter as assessed using calipers (e.g., skin nodules). In the case of skin lesions, documentation by color photography, including a ruler to estimate the size of the lesion, is recommended.

#### **6.5.10 Guidelines for Evaluation of Measurable Disease**

All measurements should be taken and recorded in metric notation using a ruler or calipers. All baseline evaluations should be performed as closely as possible to the beginning of treatment and never more than 4 weeks before the beginning of the treatment.

The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up. Imaging-based evaluation is preferred to evaluation by clinical examination unless the lesion(s) being followed cannot be imaged but are assessable by clinical exam.

#### **6.5.11 Methods of Measurement**

Chest X-ray - Lesions on chest x-ray are acceptable as measurable lesions when they are clearly defined and surrounded by aerated lung. However, CT is preferable.

CT and MRI - CT and MRI are the best currently available and reproducible methods to measure target lesions selected for response assessment. For this study helical Multi-detector CT will be performed with cuts of 5 mm in slice thickness for chest, abdomen and pelvis lesions and 2-3 mm thickness for head and neck lesions.

#### **6.5.12 Additional response evaluation using volumetric analysis**

In addition, the utility of volumetric tumor measurement in patients with measurable disease will be prospectively evaluated and compared to 1D and 2D measurements.

### **6.6 TOXICITY CRITERIA:**

The following adverse event management guidelines are intended to ensure the safety of each patient while on the study. The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for AE reporting. All appropriate treatment areas have access to a copy of the CTCAE version 4.0. A copy of the CTCAE version 4.0 can be downloaded from the CTEP web site (<http://ctep.cancer.gov>).

## **7 SAFETY REPORTING REQUIREMENTS/DATA AND SAFETY MONITORING PLAN**

### **7.1 ADVERSE EVENT DEFINITIONS**

#### **7.1.1 Adverse Event**

An adverse event is any untoward medical occurrence in a human subject, including any abnormal sign (for example, abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the subject's participation in research, whether or not considered related to the subject's participation in the research

#### **7.1.2 Suspected adverse reaction**

Suspected adverse reaction means any adverse event for which there is a reasonable possibility that the drug caused the adverse event. For the purposes of IND safety reporting, 'reasonable possibility' means there is evidence to suggest a causal relationship between the drug and the adverse event. A suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug.

#### **7.1.3 Unexpected adverse reaction**

An adverse event or suspected adverse reaction is considered "unexpected" if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed; or, if an investigator brochure is not required or available, is not consistent with the risk information described in the general investigational plan or elsewhere in the current application. "Unexpected" also refers to adverse events or suspected adverse reactions that are mentioned in the investigator brochure as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but are not specifically mentioned as occurring with the particular drug under investigation.

#### **7.1.4 Serious**

An Unanticipated Problem or Protocol Deviation is serious if it meets the definition of a Serious Adverse Event or if it compromises the safety, welfare or rights of subjects or others.

#### **7.1.5 Serious Adverse Event**

An adverse event or suspected adverse reaction is considered serious if in the view of the investigator or the sponsor, it results in any of the following:

- Death
- A life-threatening adverse drug experience
- Inpatient hospitalization or prolongation of existing hospitalization
- Persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
- A congenital anomaly/birth defect.
- Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse drug experience when, based upon

appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

#### **7.1.6 Disability**

A substantial disruption of a person's ability to conduct normal life functions.

#### **7.1.7 Life-threatening adverse drug experience**

Any adverse event or suspected adverse reaction that places the patient or subject, in the view of the investigator or sponsor, at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that had it occurred in a more severe form, might have caused death.

#### **7.1.8 Protocol Deviation (NIH Definition)**

Any change, divergence, or departure from the IRB-approved research protocol.

#### **7.1.9 Non-compliance (NIH Definition)**

The failure to comply with applicable NIH Human Research Protections Program (HRPP) policies, IRB requirements, or regulatory requirements for the protection of human research subjects.

#### **7.1.10 Unanticipated Problem**

Any incident, experience, or outcome that:

- Is unexpected in terms of nature, severity, or frequency in relation to
  - (a) the research risks that are described in the IRB-approved research protocol and informed consent document; Investigator's Brochure or other study documents, and
  - (b) the characteristics of the subject population being studied; **AND**
- Is related or possibly related to participation in the research; **AND**
- Suggests that the research places subjects or others at a *greater risk of harm* (including physical, psychological, economic, or social harm) than was previously known or recognized.

### **7.2 NCI-IRB AND CLINICAL DIRECTOR REPORTING**

#### **7.2.1 NCI-IRB and NCI CD Expedited Reporting of Unanticipated Problems and Deaths**

The Protocol PI will report in the NIH Problem Form to the NCI-IRB and NCI Clinical Director:

- All deaths, except deaths due to progressive disease
- All Protocol Deviations
- All Unanticipated Problems
- All non-compliance

Reports must be received within 7 days of PI awareness via iRIS.

#### **7.2.2 NCI-IRB Requirements for PI Reporting Continuing Review**

The protocol PI will report to the NCI-IRB:

1. A summary of all protocol deviations in a tabular format to include the date the deviation occurred, a brief description of the deviation and any corrective action.
2. A summary of any instances of non-compliance
3. A tabular summary of the following adverse events:
  - All Grade 2 **unexpected** events that are possibly, probably or definitely related to the research;
  - All Grade 3 and 4 events that are possibly, probably or definitely related to the research;
  - All Grade 5 events regardless of attribution;
  - All Serious Events regardless of attribution.

**NOTE:** Grade 1 events are not required to be reported.

### **7.2.3 NCI-IRB Reporting of IND Safety Reports**

Only IND Safety Reports that meet the definition of an unanticipated problem will need to be reported to the NCI IRB.

## **7.3 IND SPONSOR REPORTING CRITERIA**

### **7.3.1 Expedited Adverse Event Reporting Criteria to the IND Sponsor**

During the first 30 days after the subject receives investigational agent/intervention, the investigator must immediately report to the sponsor, using the mandatory MedWatch form 3500a or equivalent, any serious adverse event, whether or not considered drug related, including those listed in the protocol or investigator brochure and must include an assessment of whether there is a reasonable possibility that the drug caused the event. For serious adverse events that occur more than 30 days after the last administration of investigational agent/intervention, only report those that have an attribution of at least possibly related to the agent/intervention.

Required timing for reporting per the above guideline:

- Deaths (except death due to progressive disease) must be reported via email within 24 hours. A complete report must be submitted within one business day.
- Other serious adverse events as well as deaths due to progressive disease must be reported within one business day

Events will be submitted to the Center for Cancer Research (CCR) at: [CCRsafety@mail.nih.gov](mailto:CCRsafety@mail.nih.gov) and to the CCR PI and study coordinator.

### **7.3.2 Reporting Pregnancy**

#### **7.3.2.1 Maternal exposure**

If a patient becomes pregnant during the course of the study, the study treatment should be discontinued immediately and the pregnancy reported to the Sponsor. The potential risk of exposure of the fetus to the investigational agent(s) or chemotherapy agents (s) should be documented in box B5 of the MedWatch form “Describe Event or Problem”.

Pregnancy itself is not regarded as an SAE. However, as patients who become pregnant on study risk intrauterine exposure of the fetus to agents which may be teratogenic, the CCR is requesting that pregnancy should be reported in an expedited manner as **Grade 3 “Pregnancy, puerperium and perinatal conditions - Other (pregnancy)”** under the *Pregnancy, puerperium and perinatal conditions* SOC.

Congenital abnormalities or birth defects and spontaneous miscarriages should be reported and handled as SAEs. Elective abortions without complications should not be handled as AEs. The outcome of all pregnancies (spontaneous miscarriage, elective termination, ectopic pregnancy, normal birth, or congenital abnormality) should be followed up and documented.

If any pregnancy occurs in the course of the study, then the investigator should inform the Sponsor within 1 day, i.e., immediately, but **no later than 24 hours** of when he or she becomes aware of it.

The designated Sponsor representative will work with the investigator to ensure that all relevant information is provided to the Sponsor within 1 to 5 calendar days for SAEs and within 30 days for all other pregnancies.

The same timelines apply when outcome information is available.

#### **7.3.2.2 Paternal exposure**

Male patients should refrain from fathering a child or donating sperm during the study and for 60 days after the last dose of Mithramycin.

Pregnancy of the patient’s partner is not considered to be an AE. However, the outcome of all pregnancies (spontaneous miscarriage, elective termination, ectopic pregnancy, normal birth, or congenital abnormality) occurring from the date of the first dose until 60 after the last dose should, if possible, be followed up and documented.

### **7.4 DATA AND SAFETY MONITORING PLAN**

#### **7.4.1 Principal Investigator/Research Team**

The clinical research team will meet regularly when patients are being actively treated on the trial to discuss each patient in detail.

All data will be collected in a timely manner and reviewed by the principal investigator or a clinical associate investigator in a timely manner. Adverse events will be reported as required above. Any safety concerns, new information that might affect either the ethical and or scientific conduct of the trial, or protocol deviations will be immediately reported to the IRB using iRIS.

The principal investigator will review adverse event and response data on each patient to ensure safety and data accuracy. The principal investigator will personally conduct or supervise the investigation and provide appropriate delegation of responsibilities to other members of the research staff.

#### **7.4.2 Sponsor Monitoring Plan**

As a sponsor for clinical trials, FDA regulations require the CCR to maintain a monitoring program. The CCR’s program allows for confirmation of: study data, specifically data that could affect the interpretation of primary study endpoints; adherence to the protocol, regulations, and

SOPs; and human subjects protection. This is done through independent verification of study data with source documentation focusing on:

- Informed consent process
- Eligibility confirmation
- Drug administration and accountability
- Adverse events monitoring
- Response assessment.

The monitoring program also extends to multi-site research when the CCR is the coordinating center.

This trial will be monitored by personnel employed by an CCR contractor. Monitors are qualified by training and experience to monitor the progress of clinical trials. Personnel monitoring this study will not be affiliated in any way with the trial conduct.

## **8 STATISTICAL CONSIDERATIONS**

Prior to amendment F, the protocol enrolled 12 evaluable patients, and there were no clinical responses, but dose-limiting hepatotoxicity associated with ABCB4 and ABCB11 genotypes was identified. Thus, the results from these initial 12 patients will be reported as a separate group, and accrual will begin again using the same phase II design as initially proposed, but restricted to patients with favorable SNPs, and receiving the modified dose regimen described in amendment F. Thus, beginning with amendment F, the primary endpoint of this study will be objective response by RECIST criteria v 1.1. Patients will be analyzed in two cohorts (primary thoracic malignancy; extra-thoracic malignancy metastatic to the chest). The best response to mithramycin will be examined for each cohort. In each cohort, the trial will seek to rule out an unacceptably low 5% objective response rate (ORR;  $p_0=0.05$ ), in favor of a higher response rate of 30% ( $p_1=0.30$ ). With  $\alpha=0.10$  (probability of accepting a poor treatment=0.10) and  $\beta = 0.10$  (probability of rejecting a good treatment=0.10), the study will initially enroll 7 evaluable patients in each stratum, and if 0 of the 7 have a response, then no further patients will be accrued in that cohort. If 1 or more the first 7 in a cohort have a response, then accrual would continue until a total of 21 evaluable patients have enrolled in that cohort. As it may take several weeks to determine if a patient has experienced a response, a temporary pause in the accrual to the trial may be necessary to ensure that enrollment to the second stage is warranted. If there are 1 to 2 responses in 21 patients, this would be an uninterestingly low response rate, while if there were 3 or more responses in 21 patients, then this would be sufficiently interesting to warrant further study of the patients from that cohort in later trials. Under the null hypothesis (5% response rate), the probability of early termination in each cohort is 70%.

With two cohorts, up to 42 evaluable patients may be required to be accrued, in addition to the 12 enrolled prior to amendment F. It is expected that 10-15 patients per year may enroll onto this study. Thus, 3-4 years additional may be needed to enroll 42 additional evaluable patients. To allow for a small number of inevaluable patients, and to account for the 12 enrolled prior to amendment F, the accrual ceiling will be set at 57.

The following table summarizes the two stage design that will be used for each cohort in the trial beginning with Amendment F.

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	Cumulative Number of Responses	Decision
Stage 1: Enter 7 patients	0	Terminate the trial for that cohort: agent ineffective
	1 or more	Inconclusive result, continue trial (proceed to Stage 2)
Stage 2: Enter 14 additional patients	2 or less	Terminate the trial for that cohort: agent ineffective
	3 or more	Terminate the trial: agent effective

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## 8.1 METHODS OF ANALYSIS

Patients who are considered evaluable for response will be included in an analysis of time to progression. Time to progression will be estimated using the product-limit method of Kaplan and Meier. The probability of progression free survival at 6 months will be summarized. Response rates will be calculated as the percent of patients whose best response is a CR or PR, and the fraction of responses obtained will have a 95% confidence interval, which takes into consideration the two-stage nature of the design<sup>85</sup>. Toxicity information recorded will include the type, severity, time of onset, time of resolution, and the probable association with the study regimen. Tables will be constructed to summarize the observed incidence by severity and type of toxicity.

**Time to progression** will be taken as the number of days from enrollment until: (1) disease progression; (2) death because of treatment complications; (3) resection of measurable tumor; or (4) last patient follow-up whichever is first. Patients will be considered to have experienced a progression event if (1) or (2) occurs. Otherwise, the patient will be considered censored for time to progression.

## 8.2 EVALUATION OF RESPONSE

Any patient who is enrolled and receives at least one full course (4 cycles, 28 doses) of mithramycin will be considered evaluable for response provided: (1) the patient demonstrates progressive disease or death while on protocol therapy; (2) the patient is observed on protocol therapy for at least one course and the tumor is not removed surgically prior to the time complete response or partial response is confirmed; or (3) the patient demonstrates a complete or partial response as confirmed according to protocol criteria. Patients who electively terminate therapy before receiving all 7 doses of mithramycin during the first treatment cycle and do not expire within 28 days from start of treatment will be replaced. Patients who experience disease progression after the first course but who did not receive a full course (all 28 doses) will be taken off-treatment and will only be evaluable for toxicity (not evaluable for response).

## 8.3 SECONDARY ENDPOINTS:

Pharmacokinetic analysis will be conducted using non-compartmental methods. For pharmacodynamic endpoints, descriptive statistics will be used for each endpoint. Briefly,

pharmacokinetics and molecular endpoints in target tissues will be assessed before and after mithramycin treatment, with the primary focus being comparison of microarray, qRT-PCR and IHC results relative to profiles identified in our preclinical studies. It is anticipated that non-parametric statistical methods will be used to compare paired results before and after treatment (but not between groups), as well as to perform comparisons or correlations among parameters. As the exact set of comparisons and analyses to be performed will be determined following completion of the trials, and will be based on limited numbers of patients, the analyses will be considered exploratory and hypothesis generating rather than definitive. The results of these descriptive analyses will be used to design further studies.

## **9 COLLABORATIVE AGREEMENTS**

### **9.1 AGREEMENT TYPE**

*N/A*

## **10 HUMAN SUBJECTS PROTECTIONS**

### **10.1 RATIONALE FOR SUBJECT SELECTION**

The patients to be entered on this protocol have advanced malignancies involving lungs, esophagus, or pleura and have limited life expectancies. This population was selected because of the unknown outcome of this treatment in terms of its effectiveness. The experimental treatment has a chance to provide clinical benefit although this is unknown. Subjects of both genders and all racial/ethnic groups are eligible. Efforts will be made to extend accrual to a representative population, but in this preliminary study, a balance must be struck between patient safety considerations and limitations on the number of individuals exposed to potentially toxic and/or ineffective treatments on the one hand and the need to explore gender and ethnic aspects of clinical research on the other hand. If differences in outcome that correlate with gender or ethnic identity are noted, accrual may be expanded, or a follow-up study may be written to investigate these differences more fully. One strategy for recruitment may be to distribute an IRB approved protocol recruitment letter to General and Oncology physicians and nurses.

### **10.2 PARTICIPATION OF CHILDREN**

It is anticipated that most children with cancer will not have disease that is appropriate for study. Because the effects of mithramycin have not been evaluated in children, individuals <18 will be excluded from this study until more data are available.

### **10.3 PARTICIPATION OF SUBJECTS UNABLE TO GIVE CONSENT**

Adults unable to give consent are excluded from enrolling in the protocol. However, re-consent may be necessary and there is a possibility, though unlikely, that subjects could become decisionally impaired. For this reason and because there is a prospect of direct benefit from research participation (section 10.4), all subjects will be offered the opportunity to fill in their wishes for research and care, and assign a substitute decision maker on the “NIH Advance Directive for Health Care and Medical Research Participation” form so that another person can make decisions about their medical care in the event that they become incapacitated or cognitively impaired during the course of the study. Note: The PI or AI will contact the NIH Ability to Consent Assessment Team (ACAT) for evaluation as needed for the following: an independent assessment of whether an individual has the capacity to provide consent; assistance in identifying and assessing an appropriate surrogate when indicated; and/or an assessment of the capacity to appoint



a surrogate. For those subjects that become incapacitated and do not have pre-determined substitute decision maker, the procedures described in MAS Policy 87-4 and NIH HRPP SOP 14E for appointing a surrogate decision maker for adult subjects who are (a) decisionally impaired, and (b) who do not have a legal guardian or durable power of attorney, will be followed.

#### **10.4 EVALUATION OF BENEFITS AND RISKS/DISCOMFORTS FOR ALL PARTICIPANTS**

There may be some direct benefit to patients who participate in this trial since it is anticipated that mithramycin infusion may cause tumor stabilization or some tumor regression. The greatest benefit will be the information regarding the feasibility, toxicity and dosing of mithramycin administration, as well as the information on changes in cancer stem cell gene expression. The risks to the patient participating in this trial are anticipated to be small and are primarily the risks associated with administering the two agents and the radiation risk of up to 2 CT guided research biopsies. The amount of radiation from the research CT scans is 1.6 rem which is below the guidance if 5 rem per year allowed for research subjects by the NIH Radiation Safety Committee. These potential risks and benefits will be carefully discussed with the patient at the time consent is obtained.

#### **10.5 CONSENT PROCESS AND DOCUMENTATION**

All patients will be thoroughly screened both via telephone and during clinic evaluation by the physician and the research nurse prior to completing the consent. During the initial consultation, the patient and family or friends, if present, will be presented with a forthright and detailed overview of the treatment option available to them at the NIH. The experimental nature of the treatment, its objectives, its theoretical advantages and disadvantages will be presented. The Informed Consent document is given to the patient and they are asked to review it, make notes and ask questions prior to agreeing to participate in this protocol. The patient is reassured that participation on this trial is entirely voluntary and that they can withdraw or decide against treatment at any time without adverse consequences. The physician assures the patient that if alternative therapy or no therapy at all is preferred, that we will do all that we can to facilitate obtaining consultation with the appropriate referral organizations. The Informed Consent document may be obtained from the patient by the principal investigator, associate investigators, or the medical staff fellow under the supervision of the principal investigator.

Reconsent on this study may be obtained via telephone according to the following procedure: the informed consent document will be sent to the subject. An explanation of the study will be provided over the telephone after the subject has had the opportunity to read the consent form. The subject will sign and date the informed consent. A witness to the subject's signature will sign and date the consent. The original informed consent document will be sent back to the consenting investigator who will sign and date the consent form with the date the consent was obtained via telephone. A fully executed copy will be returned via mail for the subject's records. The informed consent process will be documented on a progress note by the consenting investigator.

##### ***10.5.1 Informed consent of non-English speaking subjects***

If there is an unexpected enrollment of a research participant for whom there is no translated extant IRB approved consent document, the principal investigator and/or those authorized to obtain informed consent will use the Short Form Oral Consent Process as described in MAS Policy M77-2, OHSRP SOP 12, 45 CFR 46.117 (b) (2), and 21 CFR 50.27 (b) (2). The summary that will be used is the English version of the extant IRB approved consent document. Signed copies of both

the English version of the consent and the translated short form will be given to the subject or their legally authorized representative and the signed original will be filed in the medical record.

Unless the PI is fluent in the prospective subject's language, an interpreter will be present to facilitate the conversation. Preferably someone who is independent of the subject (i.e., not a family member) will assist in presenting information and obtaining consent. Whenever possible, interpreters will be provided copies of the relevant consent documents well before the consent conversation with the subject (24 to 48 hours if possible).

We request prospective IRB approval of the use of the short form process for non-English speaking subjects and will notify the IRB at the time of continuing review of the frequency of the use of the Short Form.

## **11 PHARMACEUTICAL INFORMATION**

### **11.1 MITHRAMYCIN (PLICAMYCIN)**

Mithramycin is an antineoplastic antibiotic (oligosaccharide) produced by the growth of *Streptomyces argillaceus*, *S. plicatus* and *S. tanashiensis*. Previously used in the treatment of inoperable metastatic neoplasms of the testes, and for the symptomatic treatment of hypercalcemia and hypercalciuria.

#### **11.1.1 Source**

Mithramycin was supplied by IriSys LLC to the NIH Clinical Center Pharmacy, which will be providing the mithramycin under an IND held by the CCR.

#### **11.1.2 Toxicity**

The following effects have been observed in previous human administration (possible signs and symptoms in parentheses where appropriate)

##### More frequent:

- a) Hypocalcemia (muscle and abdominal cramps)
- b) Anorexia (loss of appetite)
- c) Diarrhea
- d) Stomatitis
- e) Nausea or vomiting —may occur 1 to 2 hours after initiation of therapy and continue for 12 to 24 hours

**Note:** Incidence and severity of *gastrointestinal side effects* may increase with too rapid a rate of administration.

##### Less frequent

- Drowsiness
- Fever
- Headache
- Mental depression
- Pain, redness, soreness, or swelling at injection site
- Unusual tiredness or weakness

- f) Gastrointestinal bleeding (bloody or black, tarry stools; vomiting of blood)
- g) hepatotoxicity (yellow eyes or skin)
- h) Epistaxis, hematemesis
- i) Leukopenia (sore throat and fever)—incidence about 6%
- j) Petechial bleeding
- k) Thrombocytopenia
- l) Toxic epidermal necrolysis (flushing or redness or swelling of face; skin rash)—possible early symptoms of overdose

Note: Hemorrhagic diathesis—Incidence more frequent with doses of more than 30 mcg (0.03 mg) per kg of body weight a day and/or for more than 10 doses.

### **11.1.3 Formulation and preparation**

Mithramycin is supplied as 2 mg/vial, freeze dried powder for injection. To prepare the initial dilution of 500 mcg (0.5 mg) of mithramycin per mL, add 3.9 mL of sterile water for injection to the 2 mg vial and shake to dissolve. After the appropriate dose has been withdrawn from the vial, discard the unused portion.

For intravenous infusion, doses should be diluted as follows:

Mithramycin dose (mcg)	Infusion Solution: 0.9% Sodium Chloride or 5% Dextrose Injection (mL)
1000 or above	1000
500-999	500
250-499	250

All dilutions will be infused over 6 hours.

### **11.1.4 Stability and Storage**

Prior to reconstitution, store between -20 and -10 °C (-4 and 14 °F). Store in a light-resistant container.

Reconstituted solution (500 mcg per mL) should be freshly prepared for each dose and used immediately. Studies in Clinical Center Pharmacy Department indicate that the drug is stable for 24 hours at room temperature when further diluted in 0.9% Sodium Chloride or 5% Dextrose Injection to concentrations between 1 and 25 mcg/ml when protected from light. Because this is a pharmacokinetic study and the drug concentrate does not contain a preservative, infusion solutions should be prepared daily within four hours of the scheduled infusion. Discard any unused portion of either solution.

### **11.1.5 Administration procedures**

Infuse intravenously over 6 hours using chemotherapy precautions. Infusion bags have to be protected from light from the time of preparation through completion of the infusion. Observe frequently for signs of extravasation. Should extravasation occur, the infusion should be terminated at that site and reinstated at another site. Moderate heat should be applied to the site of extravasation to disperse the drug and minimize local tissue irritation and discomfort.

### **11.1.6 Contraindications**

The following medications are contraindicated:

- Anticoagulants, coumadin- or indandione-derivatives

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- Heparin other than heparin flushes
- Thrombolytic agents
- Aspirin or salicylate-containing products, which may increase risk of hemorrhage
- Dextran
- Dipyridamole
- Sulfinpyrazone
- Valproic acid
- Clopidogrel

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## 13 TABLES, FIGURES AND APPENDICES

### 13.1 TABLES

#### 13.1.1 Table 1: Genes Differentially Expressed *in vitro* and *in vivo* by Mithramycin

Ingenuity Canonical Pathways - <i>in vitro</i>	Differentially expressed genes
Molecular Mechanisms of Cancer	MAPK1, SMAD3, ARHGEF7, LRP6, BMPR2, MAP3K5, RBL1, EP300, PTK2, BMPR1A, SOS1, TGFB2, PRKCE, AKT3, GSK3B, HIPK2, PRKD1, PRKCA, PMAIP1, GNA12, CREBBP, SMAD6, GNAQ, CDK6, TCF3, FZD8, BMPR1B, PLCB4, NF1, IRS1, PIK3CB, ARHGEF18, CFLAR
TGF- $\beta$ Signaling *	MAPK1, SMAD3, SKI, CREBBP, SMAD6, BMPR2, SMURF1, EP300, BMPR1B, BMPR1A, SOS1, TGFB2, SMURF2
TR/RXR Activation	SLC2A1, NCOA3, EP300, LDLR, SREBF1, AKT3, STRBP, NCOR1, ACACA, PIK3CB, NCOR2, TBL1XR1, RXRA
HER-2 Signaling in Breast Cancer	SOS1, CDK6, PRKCE, AKT3, PIK3CB, MAP3K5, GSK3B, PARD3, PRKD1, PRKCA, EGFR
Non-Small Cell Lung Cancer Signaling	STK4, MAPK1, SOS1, CDK6, AKT3, PDPK1, PIK3CB, RXRA, PRKCA, EGFR
mTOR Signaling *	MAPKAP1, MAPK1, PPP2R5C, PPP2CA, PDPK1, RICTOR, EIF4E, IRS1, PRKAA1, EIF3A, PRKCE, AKT3, PIK3CB, PPP2R5E, PRKD1, PRKCA
BMP signaling pathway *	BMPR1B, MAPK1, BMPR1A, CREB1, SOS1, CREBBP, SMAD6, BMPR2, SMURF1, ATF2
Cyclins and Cell Cycle Regulation	HDAC4, PPP2R5C, PPP2CA, CDK6, TGFB2, GSK3B, PPP2R5E
Wnt/ $\beta$ -catenin Signaling *	PPP2R5C, PPP2CA, CSNK1G2, TGFB3, CREBBP, LRP6, GNAQ, CSNK1A1, TCF3, EP300, FZD8, TGFB2, AKT3, GSK3B, PPP2R5E, TCF7L2
Human Embryonic Stem Cell Pluripotency *	SMAD3, SMAD6, BMPR2, PDPK1, TCF3, FZD8, BMPR1B, BMPR1A, TGFB2, AKT3, PIK3CB, GSK3B, TCF7L2
DNA Methylation and Transcriptional Repression Signaling	MECP2, ARID4B, SAP18
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency *	C3orf63, CCNF, JARID2, IGF2BP1, WWP2
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency *	FZD8, BMPR1B, MAPK1, BMPR1A, SOS1, BMPR2, AKT3, PIK3CB, GSK3B
Notch Signaling *	MAML2, NUMB, MAML3, JAG1
NF- $\kappa$ B Signaling	TRAF3, TNFRSF1A, TGFB3, CREBBP, BMPR2, MAP4K4, MALT1, EP300, BMPR1B, BMPR1A, IGF1R, AKT3, PIK3CB, GSK3B, EGFR
p53 Signaling	PMAIP1, STAG1, AKT3, PIK3CB, GSK3B, HIPK2, EP300

<b>Ingenuity Canonical Pathways - in vitro</b>	<b>Differentially expressed genes</b>
Molecular Mechanisms of Cancer	MAP2K6, PMAIP1, TCF4, TGFBR1, BMPR2, CRK, RBL1, TGFBR2, FZD8, BMPR1B, HIPK2, BRCA1, PRKCA
TGF- $\beta$ Signaling *	MAP2K6, TGFBR2, BMPR1B, TGFBR1, BMPR2
TR/RXR Activation	LDLR, TBL1XR1, RXRA, NCOA3
HER-2 Signaling in Breast Cancer	PARD3, PRKCA
Non-Small Cell Lung Cancer Signaling	STK4, RXRA, PRKCA
mTOR Signaling *	PRKAB2, PPP2R5C, PPP2R5E, PDGFC, PRKCA
BMP signaling pathway *	BMPR1B, BMPR2
Cyclins and Cell Cycle Regulation	HDAC4, PPP2R5C, CCNB2, PPP2R5E
Wnt/ $\beta$ -catenin Signaling *	TGFBR2, FZD8, TCF4, TGFBR1, PPP2R5C, PPP2R5E
Human Embryonic Stem Cell Pluripotency *	TGFBR2, FZD8, BMPR1B, TCF4, TGFBR1, BMPR2, PDGFC
DNA Methylation and Transcriptional Repression Signaling	SAP30
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency *	JARID2, BRCA1
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency *	FZD8, BMPR1B, BMPR2
Notch Signaling *	MAML2
NF- $\kappa$ B Signaling	MAP2K6, TGFBR2, BMPR1B, TGFBR1, BMPR2
p53 Signaling	PMAIP1, TP53INP1, HIPK2, BRCA1

\* Stem cell related signaling pathway

**13.1.2 Table 2: Tissue Mithramycin Concentrations in Mice following IV or IP Injection**

Assumptions:

- 1) Specific activity of mithramycin = 147.5 microCi/mg (from reference # 49)
- 2) Counting efficiency of scintillation counter = 15% (from reference # 49)
- 3) Specific gravity of C<sub>3</sub>H mouse liver tissue = 1.086 (from reference # 50 )
- 4) Specific gravity of C<sub>3</sub>H mouse kidney tissue = 1.070 (from reference #50)

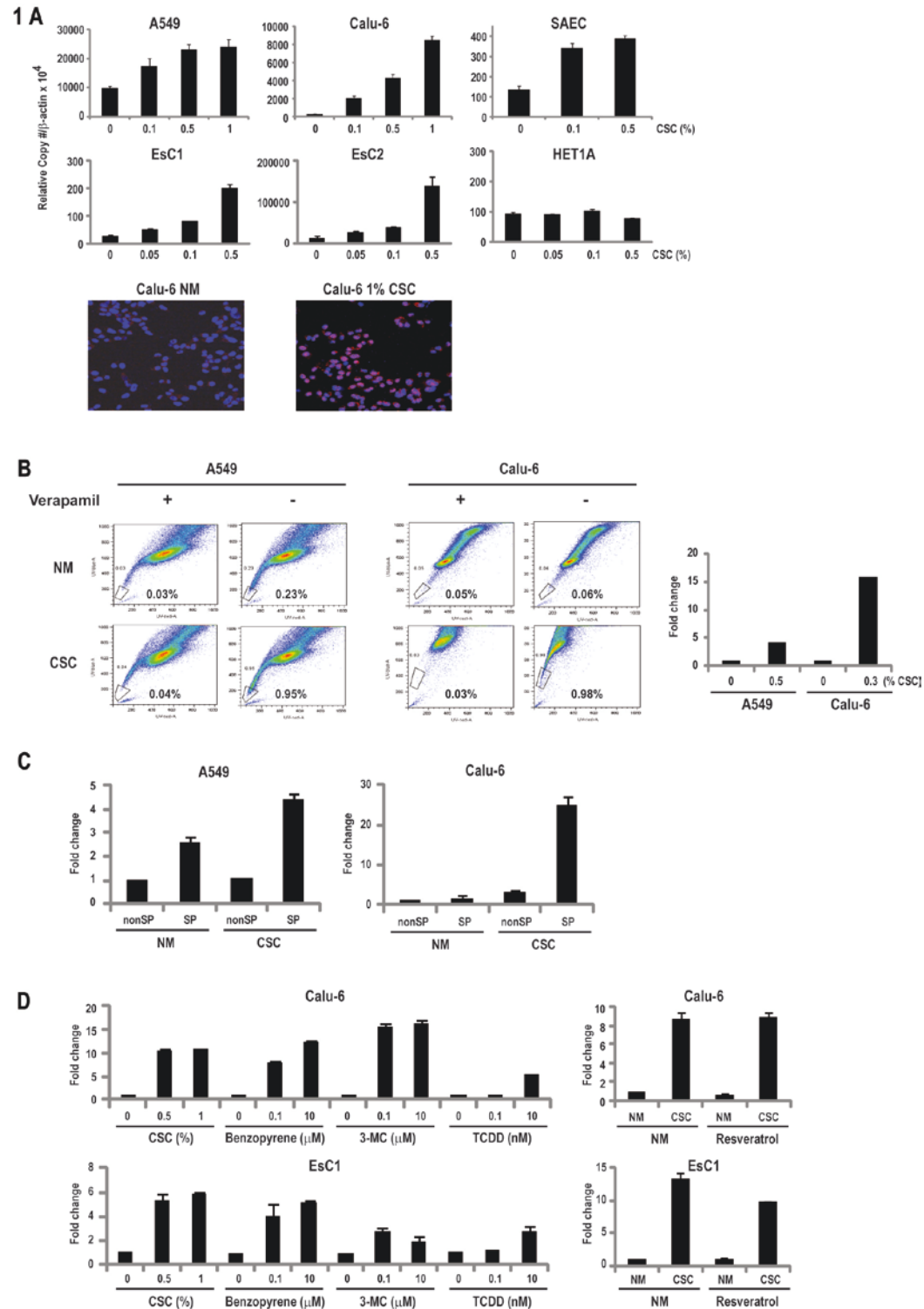
$$\left(\frac{147\text{microCi}}{\text{mg}}\right)\left(\frac{2.22\times 10^6\text{cpm}}{\text{microCi}}\right)\left(\frac{1\text{mg}}{10^6\text{ng}}\right)$$

$$= \frac{327.5\text{cpm}}{\text{ng}} (.15 \text{ factor for counting efficiency})$$

$$1\text{nM} = \frac{1.085\text{ng}}{\text{mL}}$$

	30 min post injection		Route	2 hrs post injection	
	Radioactivity	Average		Radioactivity	Average
Liver (nM)	672	692	IV	489	336
	713		IV	183	
	916	865	IP	366	
	814		IP	407	
Kidney (nM)	703	1055	IV	479	361
	1410		IV	261	
	1144	1075	IP	361	
	996		IP	482	
Blood (nM)	187	187	IV	76.6	90.4
	188		IV	104	
	325	315	IP	93.2	
	305		IP	78.5	

## 13.2 FIGURES



### 13.2.1 Figure 1: Effects of CSC on ABCG2 expression in lung and esophageal cancers

A. Upper panel: qRT-PCR analysis demonstrating dose-dependent induction of *ABCG2* expression in A549, Calu-6, EsC1 and EsC2 cells as well as cultured normal SAEC and

immortalized squamous esophageal epithelia. Basal levels of *ABCG2* in cancer cells were higher than corresponding normal cells.

Lower panel: Representative immunofluorescence demonstrating increase in *ABCG2* expression in Calu-6 cells exposed to CSC.

**B.** Left panel: Representative flow cytometry analysis demonstrating that CSC increases SP fraction in Calu-6 and A549 lung cancer cells. The effects of CSC were more pronounced in Calu-6 relative to A549 cells, exhibiting low vs. high endogenous *ABCG2* levels, respectively.

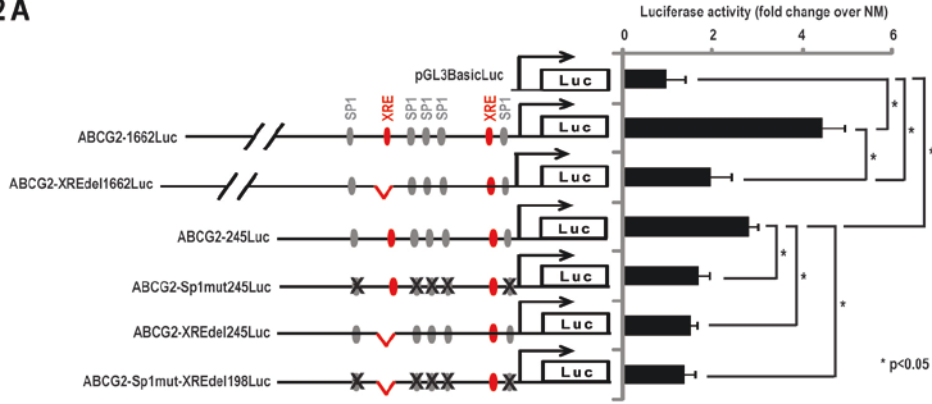
Right panel: Summary of *ABCG2* expression for left panel.

**C.** qRT-PCR analysis of *ABCG2* expression in SP and non-SP fractions in A549 and Calu-6 cells.

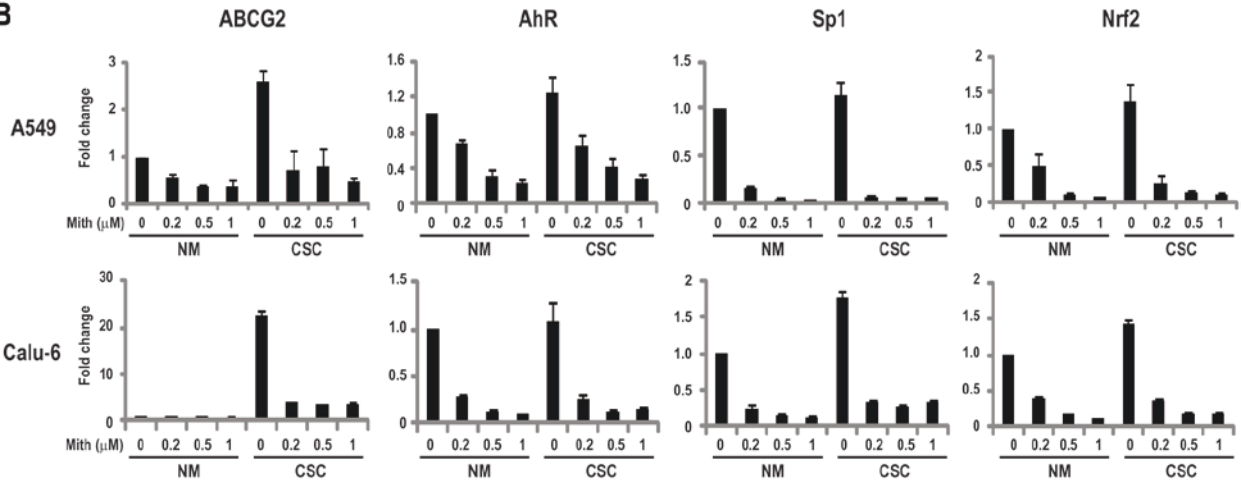
**D.** Left panel: qRT-PCR analysis of *ABCG2* expression in Calu-6 and EsC1 cells following exposure to CSC, or purified carcinogens, which activate AhR signaling.

Right panel: qRT-PCR analysis demonstrating relatively modest inhibition of 1% CSC-mediated induction of *ABCG2* by the AhR antagonist, resveratrol.

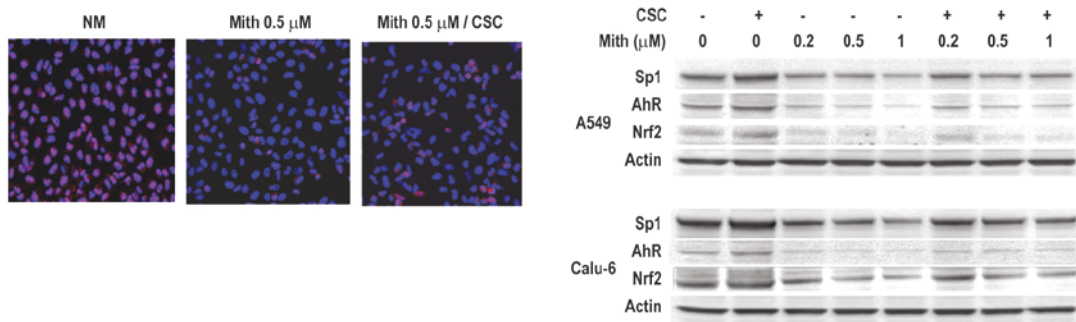
2A



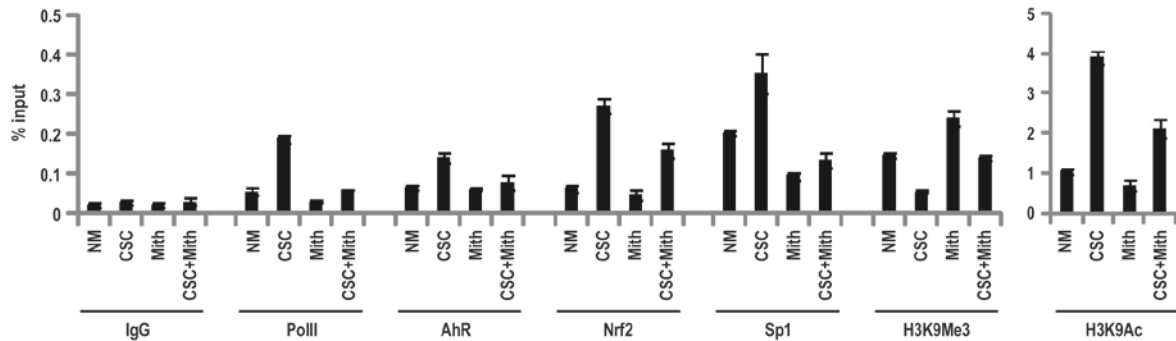
B



C



D





**13.2.2 Figure 2: Role of AhR, Sp1 and Nrf2 in ABCG2 activation by CSC.**

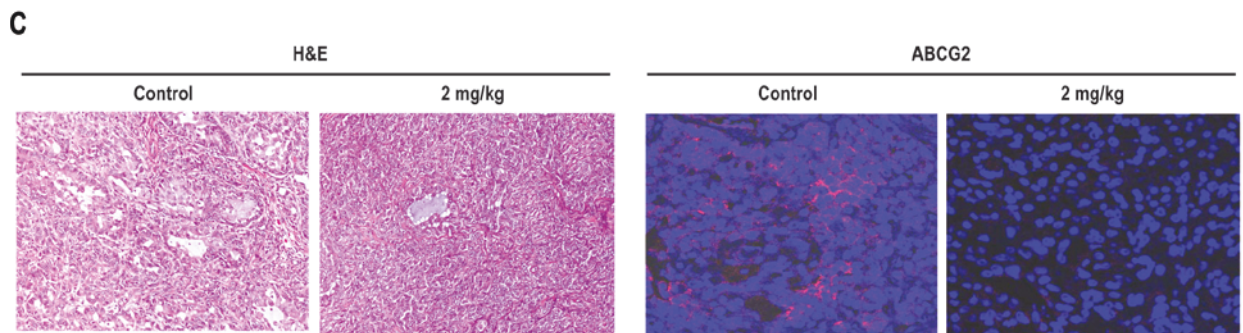
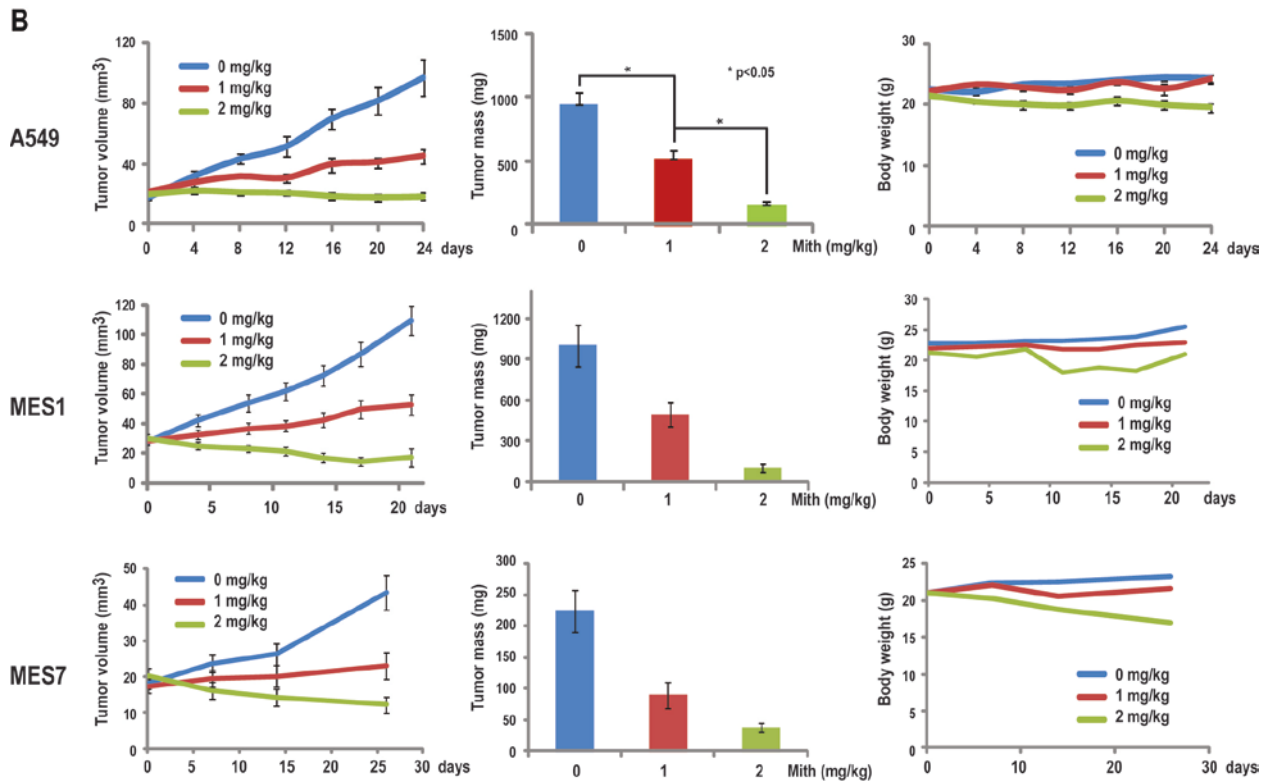
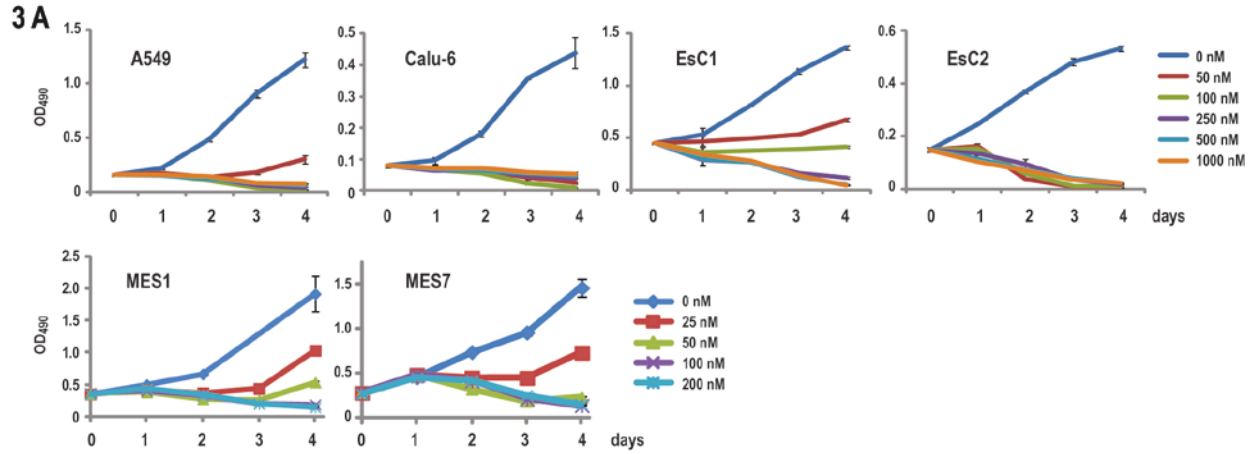
**A.** Luciferase activity of *ABCG2* promoter reporter constructs following transient transfection into Calu-6 cells. Relative to full length promoter (*ABCG2*-1662-LUC), luciferase activities of *ABCG2* promoter constructs decreased following serial deletions or mutations of XRE and Sp1 elements.

**B.** qRT-PCR analysis of *ABCG2*, *AhR*, *Sp1*, and *Nrf2* expression in A549 and Calu-6 cells cultured in NM with or without mithramycin in the presence or absence of CSC. See text for details.

**C.** Left panel: Immunofluorescence analysis of *ABCG2* expression in A549 cells cultured in the presence or absence of mithramycin with or without CSC.

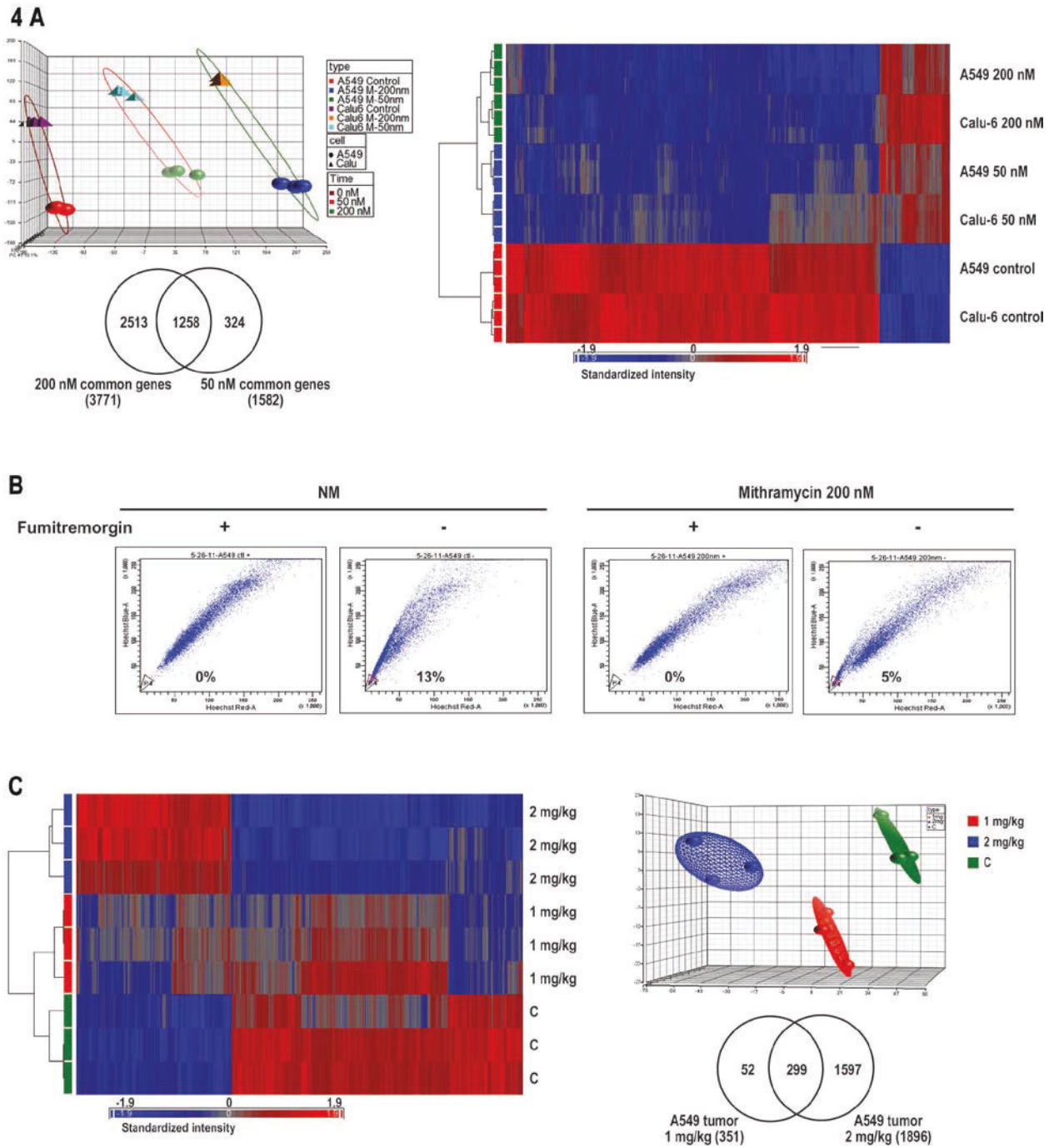
Right panel: Immunoblot analysis of Sp1, AhR, and Nrf2 expression in A549 and Calu-6 cells cultured in the presence or absence of mithramycin and/or CSC.

**D.** Quantitative ChIP analysis of the *ABCG2* promoter region in Calu-6 cells cultured in NM with or without CSC in the presence or absence of mithramycin. The dose of mithramycin for ChIP was optimized in preliminary experiments. See text for details.



**13.2.3 Figure 3: Effects of mithramycin in lung and esophageal cancer and malignant pleural mesothelioma (MPM) cells.**

- A.** MTS assays depicting effects of 24h mithramycin exposure on proliferation of lung and esophageal cancer and MPM cells.
- B.** Effects of IP mithramycin (1 mg/kg or 2 mg/kg M-W-F x3) on growth of established subcutaneous A549, MES1 and MES7 xenografts. Left panel: tumor volumes; middle panel: tumor masses; right panel: effects of mithramycin on body mass.
- C.** Representative tissue sections from A549 xenografts from control and mithramycin-treated mice. Left panel: H&E stains. Right panel: representative immunofluorescence results depicting ABCG2 expression in control tumors, and xenografts from mice treated with mithramycin.



13.2.4 Figure 4: Microarray analysis of mithramycin effects on gene expression cultured A549 and Calu-6 cells, and A549 xenografts related to respective controls.

Left panel (bottom): Venn diagram demonstrating overlap of genes simultaneously modulated in A549 and Calu-6 cells under two in-vitro exposure conditions.

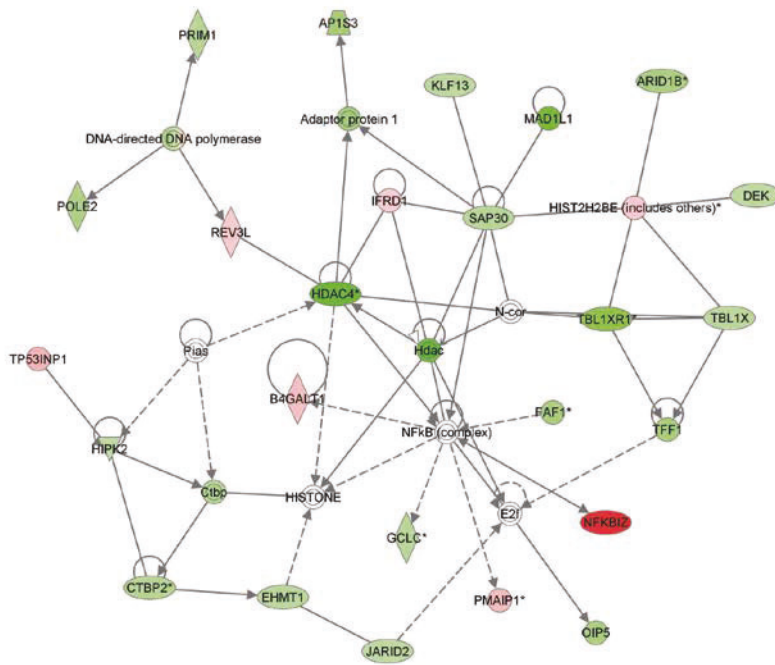
Right panel: Heat map depicting 1258 differentially expressed genes modulated by mithramycin. A marked dose-dependent alteration of gene expression profiles was observed in these cells (triplicate samples).

**B.** Flow cytometry analysis demonstrating that mithramycin decreases SP in A549 cells.

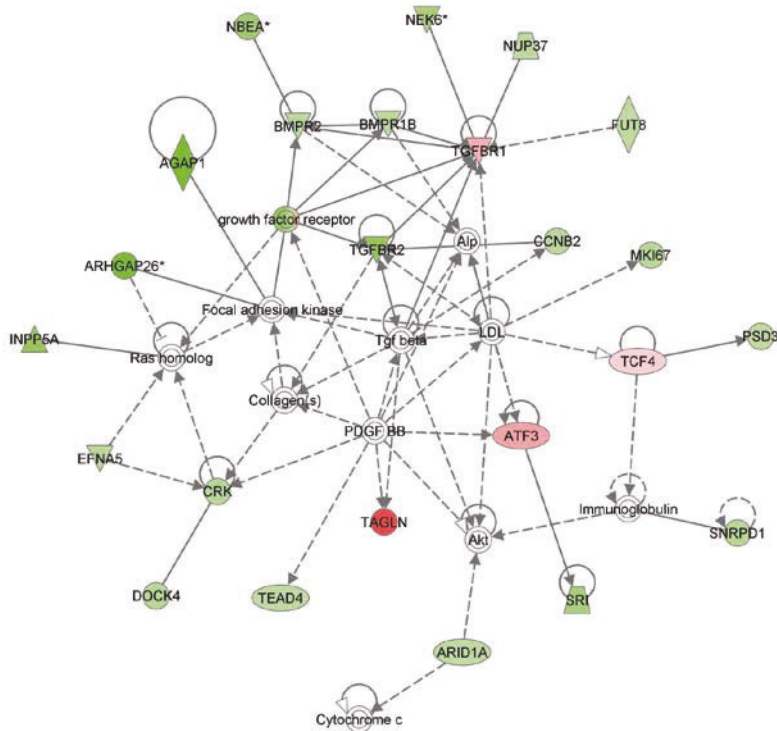
**C.** Left panel: Heat map revealing dose-dependent modulation of gene expression by mithramycin in tumor xenografts.

Right panel: top, PCA demonstrating highly reproducible results of triplicate samples (derived from 9 tumors for such conditions); bottom, Venn diagram depicting overlap of genes modulated in-vivo under both mithramycin doses.

5 A



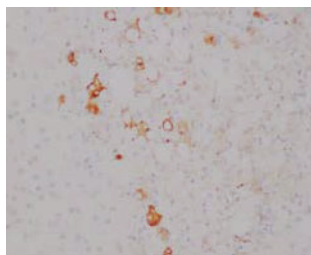
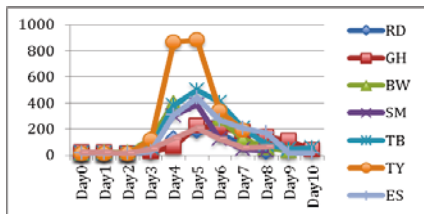
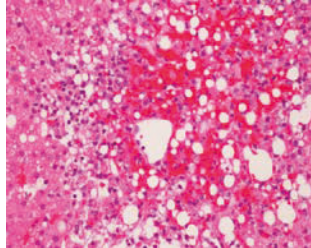
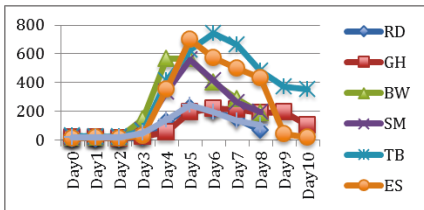
B



13.2.5 Figure 5: Ingenuity Pathway Analysis depicting representative cancer network targeted by mithramycin in tumor xenografts following systemic administration of mithramycin in athymic nude mice.

6A

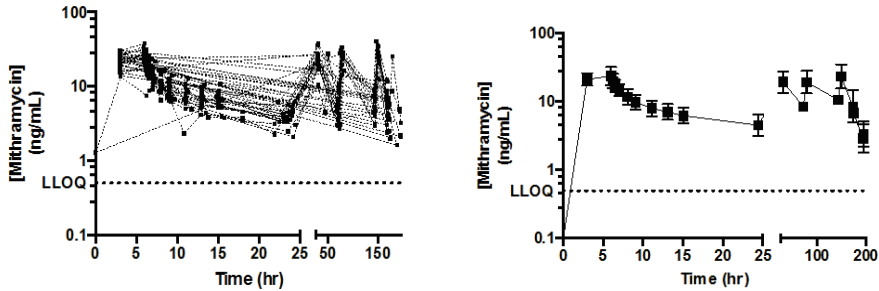
### Mithramycin Induces Hepatotoxicity in Some Cancer Patients



Cleaved Caspase 3

6B

## Mithramycin Plasma Concentration vs Time (20 cycles, 12 pts)



- Peak and steady state concentrations were lower than predicted
- No significant differences in PK were apparent in patients with or without hepatotoxicity

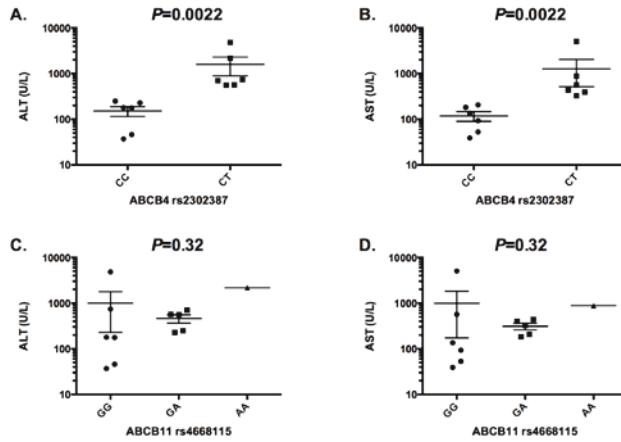
### 13.2.6 Figure 6: Serum AST and ALT levels and representative liver biopsy from patients with mithramycin mediated hepatotoxicity.

Transaminitis was associated with apoptotic hepatocyte death (A). Mithramycin levels during and after 6 hour infusions. No associations were observed between drug levels and hepatotoxicity. Drug levels were lower than previously predicted (B).



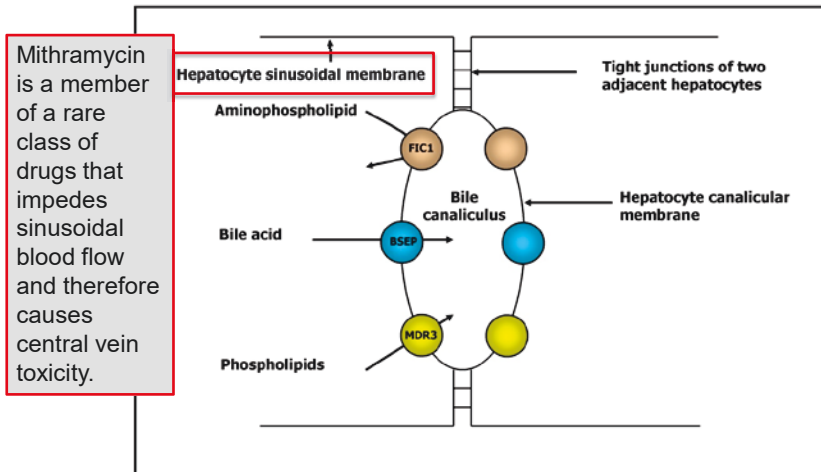
7A

### Mithramycin-Induced Hepatotoxicity Correlates with ABCB4 SNP

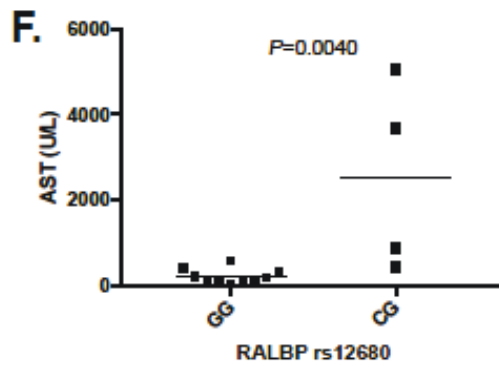
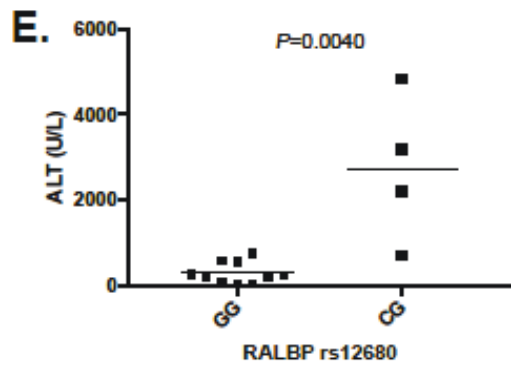
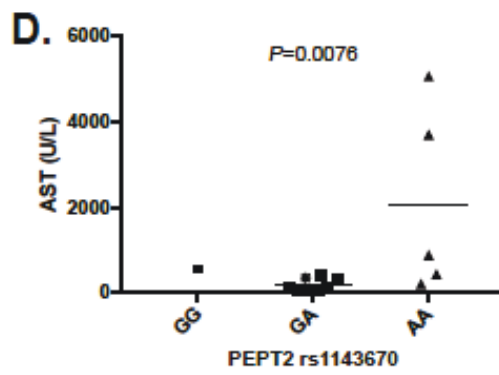
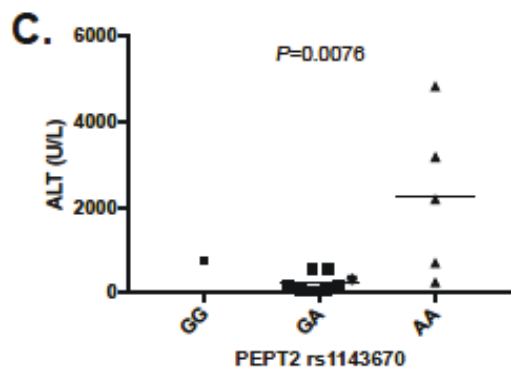
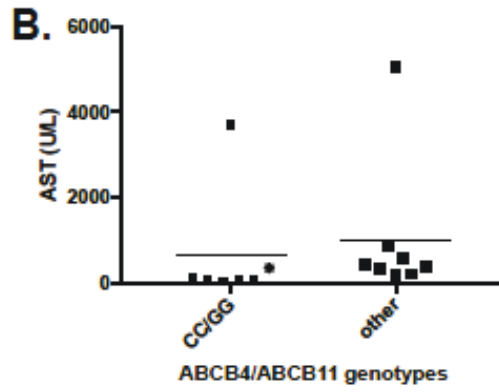
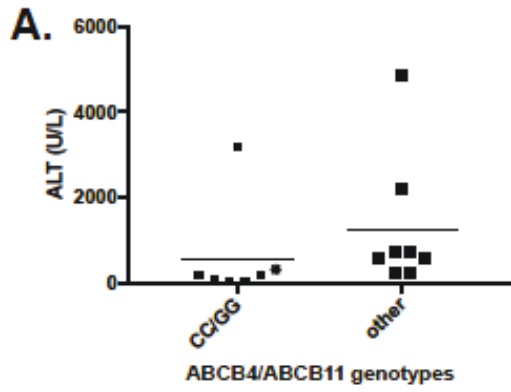


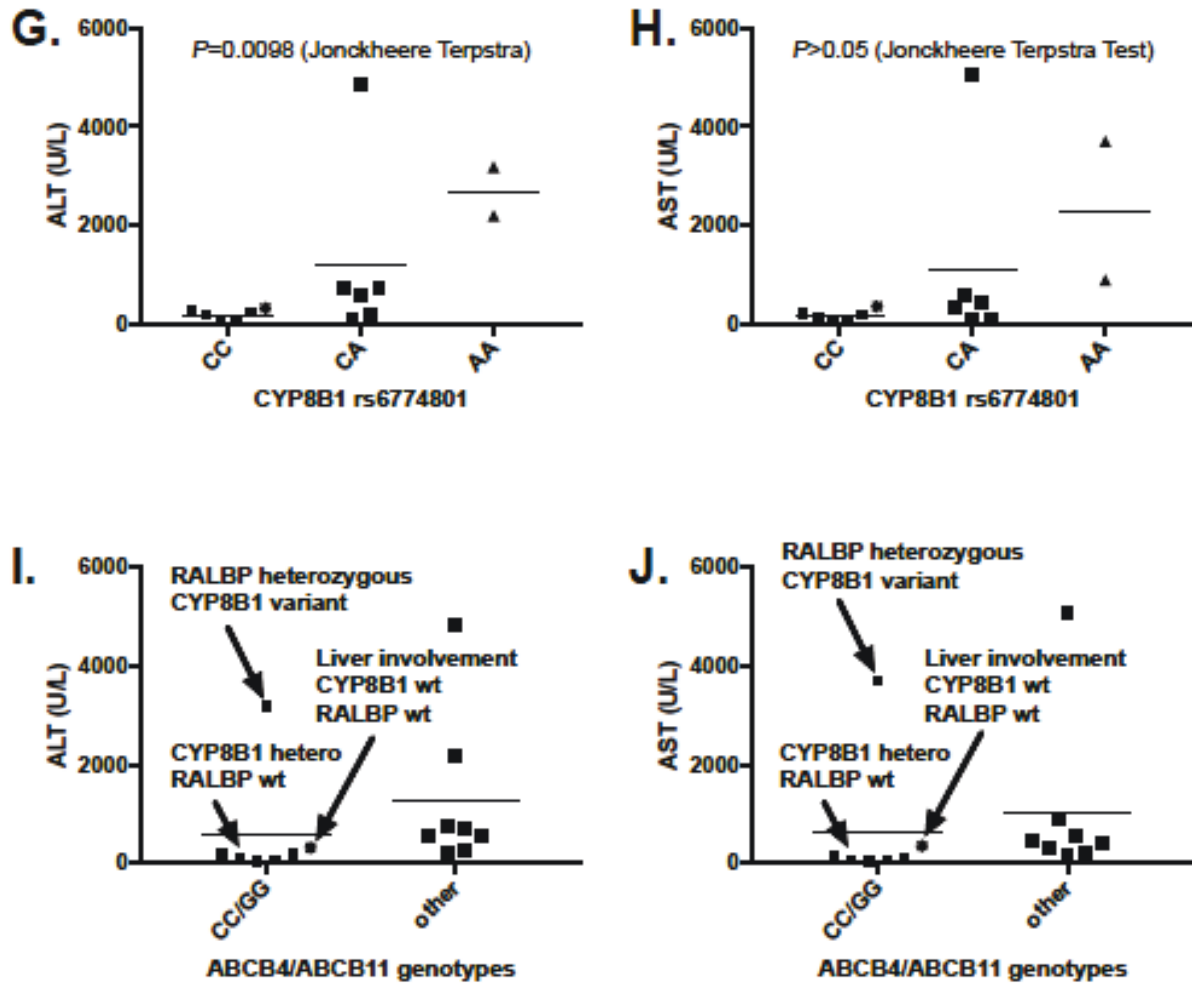
7B

### ABCB4 and ABCB11 Regulate Bile Acid Flow



13.2.7 Figure 7: Associations between ABCB4 and ABCB11 SNPs in mithramycin mediated hepatotoxicity in cancer patients





13.2.8 Figure 8: Results of DMET analysis depicting associations between AST and ALT elevations with SNPs in ABCB4, ABCB11, PEPT2, and RALB from patients receiving mithramycin

### 13.3 APPENDICES

#### 13.3.1 Appendix 1: Mithramycin Clinical Experience Reported in the Literature

Author	Year	Patient Age	Disease	Response rate	Dose	Schedule	Toxicity In cycle 1	Toxicity subsequent cycles	MTD?	PK
Donaldson <sup>86</sup> Kofman <sup>42</sup> "Tx of Disseminated."	1985 1963	NONE 10-74 (10 yo ESFT) 4 under 22	Various 1 ESFT	84 pts (68 evaluated) 6 regressions 2 subjective improvements	25 ug/kg/day as 24 hour infusion	Daily as tolerated (up to 16) -a few got 24	Hemorrhage- 3 deaths "usually related to thrombocytopenia" -one patient with bleeding time of 90 minutes -no deaths at 25 ug/kg with continuous infusions -Nausea, Vomiting, anorexia, restlessness irritability	"a surprising factor was severe cumulative toxicity -again hemorrhage on second course with PLT >50	50 ug/kg tried	
Dutcher <sup>72</sup>	1997	35-74 (median 51)	CML (mithra + interferon)	13 pts 2SD 3PR 1CR	25 ug/kg over 2-4 hrs	M, W, F for 2 weeks then one dose Qmonth	1 patient removed for "hepatotoxicity" not defined	Not defined	Not defined	
Thurlimann, B <sup>79</sup>	1992	27-74 (median 62)	Hypercalcemia	N/A	25 ug/kg	Day 1	-Vomiting Moderate 18% Severe 5% -Phlebitis 11% -Fever 9% -Mean 2x AST/ALT -DEATH arterial bleed from duodenal ulcer 1 Patient *thrombocytopenia 5%			
Lebbin, D <sup>45</sup>	1974	47-74 yrs	10 patients with Paget's	10/10 with improved pain and increased activity (all regimens effective)	25 ug/kg	Daily x 10 for cycle 1 Then A = Daily x5 every week for a month Vs B = Q week for 14 months Vs	-N/V – responded to anti-emetics NO dose reductions -uremia- 7/10 -thrombocytopenia 1/10 (<150k) NO hemorrhage-withheld drug if PLT<150k -Isocitrate Dehydrogenase elevation 1/10	-no evidence of cumulative hepatotox.		

Author	Year	Patient Age	Disease	Response rate	Dose	Schedule	Toxicity In cycle 1	Toxicity subsequent cycles	MTD?	PK
Johnson, P.R. <sup>E</sup>	1991	28-57	CML in blast crisis	9 Patients -1 returned to chronic phase	25 ug/kg + hydroxyurea	B1 = twice weekly for 5 months QOD for 3 weeks	-LFT elevations after 7 doses (3 Pt) resolved -hypocalcemia (4 pt) -Sepsis (1 pt) -severe bone pain (1 pt) -n/v 2 patients			
Baum, M. <sup>88</sup>	1968	42-83 (mean 61)	Various -1 fibrosarcoma	32 patients 12- no change 7 minor regression 1 major regression (rectal CA)	25 ug/kg over 12 hrs	Daily x 8	11 patients tolerated 8 day course -15 4-7 days -6 < 4days -none-10 -Anorexia/nausea 9 pts -Severe vomiting 5 pts -thrombocytopenia 4 pts -diarrhea 3 pts -CNS symptoms 3 patients -thrombophlebitis 3 pts	Only one patient got a second course separated by a month -stopped with vomiting		
Sewell, I. A. <sup>69</sup>	1966	30-89 (mean 56)	Various -1 alveolar celled -1 leiomyosarcoma	26 patients -4 pts with "quantitative remission" -6 stable disease -another rectal cancer	25 ug/kg over 12 hours	8 or 10 doses over 8 to 14 days based on symptoms	-FEW -17 patients with nausea/vomiting controlled with phenothiazine (5 pts) OR droperidol -dizziness and headaches (poorly responsive to antihistamines or analgesia) -seizures in patient with cerebral mets - ONLY 2 required cessation of doses -NO LFT problems -thrombocytopenia in 1	Not reported NB slow infusion attributed to the low side effects		
Ransohoff <sup>71</sup>	1965	26-64	Glioblastoma (8 patients with improvement) 3 (3+), 2 (2+), 2 (2+)	14 -tx with surgery + radiation +mithra	25 ug/kg over 8 hours	Daily times 8	2 required held drug for thrombocytopenia, N/V -otherwise limited toxicity			<sup>3</sup> H drug crosses BBB *****
Walker <sup>67</sup>	1976	Median age 53 years	Anaplastic gliomas	96 patients at NIH 116 total 58 got mithra	25 ug/kg/day over 6-8 hours	Daily times 21	-N/V in 58% of patients -mucositis -anemia (60%) mild to moderate -leukopenia (20%)			Discussion of PK data at the end

Author	Year	Patient Age	Disease	Response rate	Dose	Schedule	Toxicity In cycle 1	Toxicity subsequent cycles	MTD?	PK
Kofman "metastatic ewings, s 73	1973	Two reported 21 and 23 yo	-no difference in survival	-tx with surgery + randomized to mithra +/- radiation	25 ug/kg/day as 8-24 hour infusion NOTE ORAL INACTIV E	-then 6 wks later daily times 12 -then 6 wks later daily times 12 -83% got 11 doses -median is 21 doses	-thrombocytopenia -LFTs-mild 30%, moderate 50% in ALT/AST -hemorrhage into tumor bed in 3 patients- 2 DEATHS -chart summarizing tox			of the paper -crosses BBB but should not (inhibits MDR1)
Kofman, s 70	1964	22 10 15 Much younger 3 16 yo 2 23 yo	? of redundant patients 3 ESFT reported (2 repeats)	5 patients 1 durable CR		-q3wks total of 6- 10 courses	Thrombocytopenia Fever with infusion			
Curreri, AR 68	1960	36 pts no age	Variety A synovial	5/26 Embryonal CA 2 Chorio 1 Wilms 1 Breast 1 (all at 40 ug/day or more)	25, 30, 35, 40, 50, 60, 70, 80, 90	Daily times 5 q 5-7 wks	n/v. thrombocytopenia, hemorrhage, hepatic damage (Transaminases, PT, Alk Phos) Hemorrhage described in some detail		50 µg/dose	
Spear 74	1963	58 patients (only 29 with performance status >50	Variety of adult melanoma, bronchogeni c CA, hepatipma	NONE	50 ug/kg/dau	50 x 1 then 60 ug/kg/day q week x 8	N/V, severe diarrhea in 3, LFTs -thrombocytopenia in 1 patient -GI hemorrhage causing death in 1	N/A	N/A	N/A

Author	Year	Patient Age	Disease	Response rate	Dose	Schedule	Toxicity In cycle 1	Toxicity subsequent cycles	MTD?	PK
Package insert Pfizer <sup>66</sup>	1971	1600 patients	Testicular CA		25 ug/kg/day over 12 hours daily x 8 q month  dilute in 1L of d5 and give over 4-6hrs	Q month	Contraindicated: thrombocytopenia, coagulopathy, poor bone marrow, follow PLTs, PT and bleeding time -hemorrhagic syndrome is dose related if 30 ug/kg/day or less 10 or fewer bleeds –drug mortality rate is 1.6%  IF >30 12% bleeding with mortality rate of 5.7% COMMON: Anorexia, N/V, stomatitis LESS COMMON: Fever, drowsiness, lethargy, malaise, H/A, depression, phlebitis, flushing, skin arash LABS: Thrombocytopenia, leucopenia (6%), increased clotting, bleeding time, abnormal clot retraction, LFTs, AP, LDH, bili  Renal, Increased BUN, CR and proteinuria Hypocalcemia, hypophosphatemia, hypokalemia			

13.3.2 Appendix 2: Study Calendar

		Cycles 1 and 2							
Research Eligibility Evaluation/Baseline		Week 1							Week 2
		day 1	day 2	day 3	day 4	day 5	day 6	day 7	
Mithramycin		x	x	x	x	x	x	x	
Histologic path confirmation	x								
ABCB4 and ABCB11 status	X								
Informed consent	x								
Medical history	x								
Concurrent meds	x								
Physical examination	x	x <sup>i</sup>							
Performance status	x	x <sup>i</sup>							
General labs	x	x	x	x	x	x	x	x	xx <sup>b</sup>
Serum bile acids (total and fractionated)	x	x						x	
Coagulation labs	x				x				x <sup>c</sup>
Pregnancy test	x								
Cardiac function tests	x								
Imaging studies incl liver Elastograpy by ultrasound	x								
Adverse events		←—————→							
Tumor biopsy	x								
Research labs		x <sup>d</sup>							x <sup>c</sup>
PK	x	x <sup>df</sup>	x <sup>df</sup>		x <sup>df</sup>			x <sup>df</sup>	x <sup>g</sup>
EKG	X	x <sup>d</sup>			x <sup>d</sup>			x <sup>d</sup>	

		Cycles 3 and beyond*						
		Week 1					Week 2	
		day 1	day 2	day 3	day 4	day 5	day 6	day 7
Mithramycin		x	x	x	x	x	x	x
Histologic path confirmation								
Informed consent								
Medical history								
Concurrent meds								
Physical examination		x <sup>i</sup>						



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	Cycles 3 and beyond*							
	Week 1							Week 2
	day 1	day 2	day 3	day 4	day 5	day 6	day 7	
Performance status	x <sup>i</sup>							
General labs				x <sup>h</sup>			x	x <sup>a</sup>
Coagulation labs				x				x <sup>c</sup>
Serum bile acids (total and fractionated)	x						x	x <sup>e</sup> (course 1 only)
Pregnancy test								
Cardiac function tests								
Imaging studies including liver elastography by ultrasound								x <sup>e</sup>
Adverse events								
Tumor biopsy								
Research labs	x <sup>d</sup>							x <sup>c</sup>
EKG	x <sup>d</sup>						x <sup>d</sup>	
a - once weekly								
b - twice weekly								
c - day 8 of cycle (same as day 1 of week 2)								
d -prior to dosing								
e – two weeks (plus 12 or minus 3 days ) after the end of each course (1 course = 3 cycles)								
f - end of infusion								
g - 24 and 48 hours after day 7 infusion								
h- CBC with differential only								
i – within 4 days prior to the start of each cycle								
*Patients will continue to receive treatment until criteria for removal from protocol or off-study criteria are met (See section 3.6)								

13.3.3 Appendix 3: SAMPLE Pharmacokinetic and EKG Worksheet for Mithramycin CYCLE 1

TEL research sample # (2 10 mL red top tubes & 4 10 mL purple top tubes)	Coag reference sample # (obtain kit from TEL)	PK Sample # (two 3cc lavender top tube)	Hour (pre dose = within 1 hour prior to infusion)	Target Time	Actual Time	EKG Time	initials
<b>C1D1</b>	(date)						
<b>R1</b>	<b>C1</b>	<b>PK 1</b>	pre dose				
n/a	n/a	<b>PK 2</b>	3 h post-start of infusion			n/a	
n/a	n/a	<b>PK 3</b>	Just prior to the end of the 6 hour infusion			n/a	
n/a	n/a	<b>PK 4</b>	0.25 h post-infusion			n/a	
n/a	n/a	<b>PK 5</b>	0.5 h post-infusion			n/a	
n/a	n/a	<b>PK 6</b>	1 h post-infusion			n/a	
n/a	n/a	<b>PK 7</b>	2 h post-infusion			n/a	
n/a	n/a	<b>PK 8</b>	3 h post-infusion			n/a	
n/a	n/a	<b>PK 9</b>	5 h post-infusion			n/a	
n/a	n/a	<b>PK 10</b>	7 h post-infusion			n/a	
n/a	n/a	<b>PK 11</b>	9-12 h post-infusion			n/a	
<b>C1D2</b>	(date)						
n/a	n/a	<b>PK 12</b>	pre dose			n/a	
n/a	n/a	<b>PK 13</b>	end of infusion			n/a	
<b>C1D4</b>	(date)						
n/a	n/a	<b>PK 14</b>	pre dose			n/a	
n/a	n/a	<b>PK 15</b>	end of infusion			n/a	
<b>C1D7</b>	(date)						
n/a	n/a	<b>PK 16</b>	pre dose			n/a	
n/a	n/a	<b>PK 17</b>	end of infusion			n/a	
<b>C1D8</b>	(date)						
<b>R2</b>	n/a	<b>PK 18</b>	24 hrs p/dose #7			n/a	
n/a	n/a	<b>PK 19</b>	24.5 hrs/p dose #7			n/a	
<b>C1D9</b>	(date)						
n/a	n/a	<b>PK 20</b>	48 hrs/p dose #7			n/a	
n/a	n/a	<b>PK 21</b>	48.5 hrs/p dose #7			n/a	

13.3.4 Appendix 4: SAMPLE Pharmacokinetic and EKG Worksheet for Mithramycin CYCLE 2

TEL research samples (2 10 mL red top tubes & 4 10 mL purple top tubes)	Coag reference # (obtain kit from TEL)	PK Sample # (two 3cc lavender top tube)	Hour (pre dose = within 1 hour prior to infusion)	Target Time	Actual Time	EKG Time	initials
<b>C2D1</b>	(date)						
n/a	<b>C 2</b>	<b>PK 1</b>	pre dose				
n/a	n/a	<b>PK 2</b>	3 h post-start of infusion			n/a	
n/a	n/a	<b>PK 3</b>	Just prior to the end of the 6 hour infusion			n/a	
n/a	n/a	<b>PK 4</b>	0.25 h post-infusion			n/a	
n/a	n/a	<b>PK 5</b>	0.5 h post-infusion			n/a	
n/a	n/a	<b>PK 6</b>	1 h post-infusion			n/a	
n/a	n/a	<b>PK 7</b>	2 h post-infusion			n/a	
n/a	n/a	<b>PK 8</b>	3 h post-infusion			n/a	
n/a	n/a	<b>PK 9</b>	5 h post-infusion			n/a	
n/a	n/a	<b>PK 10</b>	7 h post-infusion			n/a	
n/a	n/a	<b>PK 11</b>	9-12 h post-infusion			n/a	
<b>C2D2</b>	(date)						
n/a	n/a	<b>PK 12</b>	pre dose			n/a	
n/a	n/a	<b>PK 13</b>	end of infusion			n/a	
<b>C2D4</b>	(date)						
n/a	n/a	<b>PK 14</b>	pre dose				
n/a	n/a	<b>PK 15</b>	end of infusion			n/a	
<b>C2D7</b>	(date)						
n/a	n/a	<b>PK 16</b>	pre dose				
n/a	n/a	<b>PK 17</b>	end of infusion			n/a	
<b>C2D8</b>	(date)						
<b>R2</b>	n/a	<b>PK 18</b>	24 hrs p/dose #7			n/a	
n/a	n/a	<b>PK 19</b>	24.5 hrs/p dose #7			n/a	
<b>C2D9</b>	(date)						

TEL research samples (2 10 mL red top tubes & 4 10 mL purple top tubes)	Coag reference # (obtain kit from TEL)	PK Sample # (two 3cc lavender top tube)	Hour (pre dose = within 1 hour prior to infusion)	Target Time	Actual Time	EKG Time	initials
n/a	n/a	PK 20	48 hrs/p dose #7			n/a	
n/a	n/a	PK 21	48.5 hrs/p dose #7			n/a	

### Instructions

### PK Studies

Collect 6 mL blood in lavender top tubes. Blood has to be drawn from a site away from the site of infusion (cannot be drawn through the line used to infuse drug, or through another lumen of that line). Following blood collection, the tubes should be inverted several times to ensure mixing with the anticoagulant. Red top tubes should be kept at room temperature whereas lavender top tubes should be placed immediately on crushed ice. Samples should be transferred immediately to the TEL. Lavender top tubes should be centrifuged for 15 minutes at approximately 1000 x g at 0-5 °C within 15 minutes of collection. The plasma should be transferred to separate pre-labeled screw-capped polypropylene tubes and stored at -80 °C until shipped to the analytical site. Total time from blood draw to freeze down of plasma should be less than 1 hour.

### Research Labs

Samples will be collected in 2 red top and 4 lavender top tubes and transported on wet ice immediately to the TEL. Samples in lavender top tubes should be centrifuged at 1000 x g at 0-5°C for 15 minutes. Supernatant (plasma) should be transferred to separate pre-labeled screw-capped polypropylene tubes and stored at -80 °C for subsequent analysis. Pellet (PBMC) should be transferred to separate pre-labeled screw-capped polypropylene tubes and stored at -80 °C for subsequent analysis. Total time from blood draw to freeze down of plasma should be less than 1 hour. Red top tubes should be kept at room temperature for 1 hour. Thereafter, clot should be removed, and then samples centrifuged at 1000 x g at 0-5°C for 15 minutes. Supernatant (serum) should be transferred to separate pre-labeled screw-capped polypropylene tubes and stored at -80 °C for subsequent analysis. Total time from blood draw to freeze down of serum should be less than 2 hr.

### Coagulation Studies

Samples will transported to the TEL lab on ice within 30 minutes. After centrifugation at 1000 x g at 0-5°C for 15 minutes, supernatant should be filtered through a 0.22 micron filter, and transferred to separate pre-labeled screw-capped polypropylene tubes and stored at -80 °C for subsequent analysis.

**Labeling**

Prior to the infusion, the PK /research lab sheet and labels will be placed in the patient's room and a separate set of labels will be given to the TEL staff. The labels will list the patient identifiers and either "Cycle 1" or "Cycle 2" as appropriate. As the RN (or designee) draws the bloods, she will place a label on the tube and write the sample reference # on the label on the tube. When the TEL personnel or fellow processes the sample, she will label the storage tube with the label and will transcribe the corresponding sample reference # on the label on the tube.

**Tracking**

Upon completion of the PK/Research sample draws, the research nurse will make 3 copies of the PK sheet. The original and one copy should be placed in the research binder. 2 copies should be given to the TEL personnel. The TEL will retain one copy for their records and will send the other copy with the PK samples to Dr. Figg's lab. Note: Prior to sending the PK sheets to Dr. Figg's lab, the patient name should be crossed out.



**13.3.6 Appendix 6: Sample Patient Log for Concomitant Medication**

NAME: \_\_\_\_\_ Cycle: \_\_\_\_\_

Dates of last Mithramycin infusion: \_\_\_\_\_

<u>Medication Taken</u>	<u>Start Date</u>	<u>End Date</u>

\*Contact your study center team or local physician right away if you experience signs of bleeding. This may include, but is not limited to, petechiae (red or purple spots on the skin), nosebleeds; bruising; or blood in urine, stool or vomit.

Patient signature: \_\_\_\_\_ Date: \_\_\_\_\_

Reviewed by: \_\_\_\_\_ RN/MD Date: \_\_\_\_\_

**13.3.7 Appendix 7: SAMPLE Monitoring Toxicities Between Mithramycin Cycles with Local Physician**

Patient Name: \_\_\_\_\_

Dates and Doses of last Mithramycin: \_\_\_\_\_ Cycle: \_\_\_\_\_

Dear Dr. \_\_\_\_\_,

The patient named above is currently participating on a Phase I-II Mithramycin study at the National Cancer Institute at the National Institutes of Health in Bethesda, Maryland, and requires the following lab tests and evaluations between \_\_\_\_\_ and \_\_\_\_\_:

(date)

(date)

- **History, physical exam:** Once Weekly
- **Weight:** Once Weekly
  
- **Labs:** (Circle one) Twice Weekly during Cycles 1-3 or Once Weekly after Cycle 3.  
**CBC/Diff**

**Chemistry Panel** (include sodium, potassium, chloride, CO<sub>2</sub>, creatinine, glucose, BUN, albumin, ionized calcium\*\*, magnesium, LDH, phosphorus, uric acid)

**LFT** (include alk phos, ALT/AST, Total bilirubin, Direct bilirubin, Total protein)

**Coagulation profiles - ONLY If clinically indicated.\***

\*\* only if covered by insurance

Please FAX all lab results and medical summaries to Dr. David Schrump, M.D. or Tricia Kunst, R.N. at 301-451-6934

For any questions, please call 240-760-6239, 240-760-6234, or 240-760-6233 or email [tricia.kunst@mail.nih.gov](mailto:tricia.kunst@mail.nih.gov), or [cara.kenney@nih.gov](mailto:cara.kenney@nih.gov)

*For urgent issues, contact the Attending Physician, Dr. David Schrump, through the NIH Operator at (301) 496-1211*

**\*If the patient experiences any signs of bleeding (≤ Grade 2) including, epistaxis, hematuria, hematemesis or blood in the stool, the patient will require immediate comprehensive clinical evaluation and coagulation profile (including PT/PTT/fibrinogen, thrombin time, PFA-100, D-dimer, Ca<sup>2+</sup> and ionized calcium)**

**Please contact the NIH page operator at 301-496-1211 and ask for the Thoracic Surgery attending on call or Dr. David Schrump with any clinically significant bleed and support with appropriate blood product support. Bleeding that does not stop within 6 hours with appropriate treatment will be considered a dose limiting toxicity and further dosing of mithramycin will be discontinued.**

\_\_\_\_\_  
Physician Signature

\_\_\_\_\_  
Print Name

NIH/NCI/TGIB