Abbreviated Title: 24h Mithramycin Infusion
CC Protocol Number: 16C0152 F
Version Date: 12/22/2020
NCT Number: NCT02859415

Title: Phase I/II Evaluation of Continuous 24h Intravenous Infusion of Mithramycin, an Inhibitor of Cancer Stem Cell Signaling, in Patients with Primary Thoracic Malignancies or Carcinomas, Sarcomas or Germ Cell Neoplasms with Pleuropulmonary Metastases

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IND Information:

Drug Name:	Mithramycin
IND Number:	115272
Sponsor:	NCI, Center for Cancer Research
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, 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 malignant pleural mesothelioma (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 Objectives:

- Phase I component: To determine pharmacokinetics, toxicities, and maximum tolerated dose (MTD) of mithramycin administered as a continuous 24h infusion in patients with primary thoracic malignancies or carcinomas, sarcomas or germ cell tumors metastatic to the chest.
- Phase II component: To determine objective response rates (CR+PR) of mithramycin administered as 24h intravenous infusions in patients with primary thoracic malignancies or carcinomas, sarcomas or germ cell tumors metastatic to the chest.

Eligibility:

- Patients with measurable inoperable, histologically confirmed 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 favorable 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 or equal to 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 X upper limits of normal (ULN) and AST/ALT $\leq$ 3 X ULN. Serum creatinine within normal institutional limits or creatinine clearance $\geq$ 60 mL/min/1.73 m^2 for patients with creatinine levels above institutional normal.

Design:

- Single arm Phase I dose escalation to define pharmacokinetics, toxicities and MTD.

- Patient cohorts will receive 24h infusions of mithramycin targeting total doses previously administered during 7 daily six hour infusions at 30-50 mcg/kg.
- The 24h infusions will be administered every 14 days (1 cycle). Four cycles will constitute one course of therapy.
- Pharmacokinetics and toxicity assessment to define MTD will be assessed during Cycle 1 of the first course of therapy.
- Due to uncertainties regarding potential cumulative toxicities, no intra-patient dose escalation will be allowed.
- Once MTD has been defined, patients will be enrolled into two cohorts (primary thoracic malignancy vs neoplasm of non-thoracic origin metastatic to the chest) to determine clinical response rates at the MTD, using a Simon Optimal Two Stage Design for Phase II Clinical Trials targeting an objective response rate (RECIST) of 30%.
- 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 4 of the first cycle of therapy for analysis of pharmacodynamic endpoints. Optional tumor biopsies may be requested at the completion of Course 1 (4 cycles) and in patients exhibiting objective responses.

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1 INTRODUCTION

1.1 STUDY OBJECTIVES

1.1.1 Primary Objectives

- To determine pharmacokinetics, toxicities, and maximum tolerated dose (MTD) of mithramycin administered as a continuous 24h infusion in patients with primary thoracic malignancies or carcinomas, sarcomas or germ cell tumors metastatic to the chest.
- To determine objective response rates (CR and PR) of mithramycin administered as 24h intravenous infusions in patients with primary malignancies involving lungs, esophagus, thymus, pleura, chest wall or mediastinum; or carcinomas, sarcomas or germ cell tumors metastatic to the chest following mithramycin infusions administered at the MTD.

1.1.2 Secondary Objective

- 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 (1). 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 (2). Presently, 80% of lung cancers, and 50% of esophageal carcinomas are directly attributable to cigarette smoke (3, 4). 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 (5). 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 (6).

In addition to being a significant risk factor for major morbidity and mortality in individuals undergoing potentially curative resections (7, 8), 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 (9-12); 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 (13). 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* (14). In more recent studies (15), we observed that cigarette smoke mediates epigenetic repression of miR-487b in lung cancer cells, resulting in over-expression of polycomb group proteins SUZ12 and BMI1 as well as Wnt5A, k-ras and C-myc, all of which have been implicated in modulating stem cell pluripotency (16-19); consistent with these observations, knock-down of miR-487b increases proliferation and tumorigenicity of lung cancer cells (15). 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*.

Cigarette smoke Condensate (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, NCI to further examine mechanisms by which cigarette smoke enhances "stemness" of thoracic neoplasms (20). 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 and esophageal 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 (21). 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 (22). Furthermore, ABCG2 binds to heme, thereby diminishing intracellular porphyrin levels, rendering stem cells resistant to hypoxia (23). Constitutive expression of *ABCG2* protects cardiac stem cells from oxidative stress (24), and enhances expansion, while impairing maturation of hematopoietic progenitor cells (25). Of particular relevance regarding thoracic malignancies are recent reports demonstrating that increased expression of *ABCG2* correlates with chemo-resistance and stem-like

phenotype of lung and esophageal carcinomas (26-30) as well as pleural mesotheliomas (31-33), and decreased survival of patients with these neoplasms (34-36).

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 (27, 29). 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 romidepsin induce *ABCG2* expression in cancer cells by aryl hydrocarbon receptor (AhR) signaling (37, 38). 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 (39), 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 (36) 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* promotor 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 (40) 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 (41). 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 2 mg/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 2 mg/kg, but not 1 mg/kg mithramycin. All 8 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 polyauroleic acid isolated from streptomyces, was initially evaluated as a chemotherapeutic agent in cancer patients during the 1960's and 70's (42), but was discontinued due to excessive systemic toxicities (43). 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 (40), thereby down-regulating numerous genes mediating stemness, chemoresistance, invasion and metastasis of cancer cells (20, 44-46). 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 (47). 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 pleiotrophic 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 (48-51) .

Recent studies have been performed to evaluate the potential efficacy of mithramycin for therapy of MPM. qRT-PCR, immunoblot and immunofluorescence experiments demonstrated markedly higher Sp1 mRNA and protein levels in MPM cells and primary MPMs relative to control cells/tissues. Knockdown of Sp1 significantly inhibited proliferation, migration and clonogenicity of MPM cells. Mithramycin dramatically inhibited growth and invasion of MPM cells *in vitro* and *in vivo* by inducing dose-dependent cell cycle arrest and senescence with subsequent apoptosis (**Figure 6**). Consistent with these findings, micro-array, qRT-PCR and ChIP experiments demonstrated that mithramycin depleted Sp1 and also activated p53 signaling, thereby modulating

genes mediating oncogene signaling, cell cycle regulation, senescence and apoptosis (Rao et al, submitted for publication).

Our preclinical studies have clearly shown that 24 hour mithramycin treatment is sufficient to mediate progressive decreases in cancer cell viability *in vitro*; furthermore, repeated IP administration of mithramycin (qM-W-F x 3 weeks) significantly inhibits growth of subcutaneous lung and pleural mesothelioma xenografts. Our micro-array experiments analyses (performed immediately following 24h mithramycin *in vitro*, or approximately 3 days following the last IP treatment of mice bearing tumor xenografts) demonstrated considerable overlap between *in vitro* and *in vivo* mithramycin exposures. These findings strongly suggest that the anti-proliferative effects of mithramycin are not primarily due to acute cytotoxicity, but are instead related to transcriptional reprogramming, which persists long after mithramycin exposure. These observations have direct translational implications regarding evaluation of mithramycin in patients with thoracic malignancies. Extrapolation of data from previous animal studies (52, 53) 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 (42). As such, gene expression signatures corresponding with treatment response in our preclinical studies may be relevant surrogate endpoints in patients receiving mithramycin infusions.

Although our 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_{50} was determined to be about 2000 mcg/kg (6000 mcg/m^2) 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^2) and monkeys (1200 mcg/m^2) (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^2 dog, 288-600 µcg/m^2 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 (20) as well as those reported by Grohar et al (65), indicate that IP doses of 1-2 mg/kg/day 3 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 ^{125}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^2 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^2.

Kennedy et al (52) administered tritiated 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 50-100 nM 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 (73). This impressive response rate may be due to the ability of mithramycin to inhibit expression of EWS-FLI fusion transcript (65). 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 (68). However, Spear et al (74) 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 (66).

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 (66)). 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 (43, 69, 75).

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 (42). Subsequent studies revealed that bleeding was dose-related, occurring in 5.4% of 1150 patients treated at 30 mcg/kg/day or more (66). 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 (76, 77), and possible potentiation of anthracycline-mediated cardiotoxicity (78). 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 (79). 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 (75).

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

1.3.4.1 12C0151 Phase II Trial

In the recent Phase II trial (12C0151) in TSB/CCR/NCI, 12 patients (10 males; 2 females; median age 51y; range 40-72y) with refractory thoracic malignancies (6 MPMs, 2 esophageal cancers, 2 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 were observed. No patients experienced bleeding. The lack of these toxicities, which have been associated with mithramycin treatment (66) 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 25 mcg/kg/infusions. This toxicity always occurred on Day 4 of the infusion. Percutaneous biopsies from three individuals revealed apoptotic hepatocellular death (**Figure 7A**). Four patients (2 with MPM) had no hepatotoxicity and tolerated dose escalation to 30 mcg/kg/infusion. Peak and steady state mithramycin levels were 20 and 8 nM, respectively, which were not different in patients with or without hepatotoxicity (**Figure 7B**).

Given the striking differences in transaminase levels in patients who did and did not experience liver toxicity, additional studies were performed to ascertain if transaminitis correlated with patient genotype. Briefly, DNA isolated from PBMC from the 12 patients was evaluated using the Affymetrix Drug Metabolizing Enzymes and Transporters (DMET) microarray, a CLIA-certified micro-array containing 1936 polymorphisms in 225 genes regulating disposition, distribution, metabolism and elimination of drugs. Results were compared to absolute elevations in ALT/AS (Grades 0-2 vs Grades 3-4) using chi-square test. Genotypes were also compared to the magnitude of ALT/AST elevations using Kruskall Wallis and Wilcoxin Rank Sum Test. This analysis revealed that the grade of MM-mediated hepatotoxicity correlated with SNPs in ABCB4 (P=0.017) and ABCB11 (P=0.0098), which encode proteins regulating bile flow (**Figure 8A** and **Figure 8B**). ABCB4 SNPs were associated with magnitude of ALT/AST elevation. Patients who were wild type for both genes had the lowest ALT/AST elevations. Additional studies using hepatocytes from warm autopsies demonstrated that both polymorphisms had the greatest predictive value of expression of both transporters. 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 8A** and **Figure 8B**).

Following this observation, 12C0151 was amended 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 9A-B**). Variants in three other genes were found to be associated with LFT elevations: rs1143670 in *PEPT2* (**Figure 9C-D**), rs12680 in *RALBP* (**Figure 9E-F**), and rs6774801 in *CYP8B1* (**Figure 9G-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 and regulates cellular accumulation of doxorubicin by a poorly-characterized non-ABC-transport mechanism. 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. 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, the enrollment criteria of the clinical trial is now amended to include the following genotypes: RALBP rs12680 (GG), CYP8B1 rs6774801 (CC or CA), ABCB4 rs2302387 (CC), and ABCB11 rs4668115 (GG) (**Figure 9I-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. Plasma mithramycin levels measured in the 12 patients enabled simulation of pharmacokinetics of mithramycin administered via 24h infusions (**Figure 10**). Based on these data, doses in the range of 25-200 mcg/kg are estimated to produce plasma levels mediating pronounced antitumor effects in preclinical murine xenograft experiments.

1.3.4.2 Current Trial

In the current trial (16C0152), 3 patients with stage IV malignancies received 24-hour mithramycin infusions at 60 mcg/kg. Patient #1 with HLRCC exhibited Grade 4 AST/ALT elevations immediately following completion of his cycle infusion. AST/ALT levels peaked at over 6000, and were associated with Grade 2 nausea and vomiting. This transaminitis resolved without treatment over the next 7-10 days. Patients #2 and 3 both of whom had stage IV esophageal adenocarcinoma tolerated their first infusions well with no increase in AST/ALT until approximately 48 hours post-infusion when they developed Grade 4 transaminitis (peak ~2500) which was also associated with Grade 1-2 nausea and vomiting. The transaminitis in both patients resolved in approximately 7 days whereas the nausea and vomiting resolved within approximately 24 hours.

Due to the severity of the dose limiting transaminitis, Patient #1 requested to be removed from study to pursue other investigational therapy. He was removed from protocol treatment and study when appropriate criteria were met. Patients #2 and 3 were dose reduced for subsequent cycles, receiving mithramycin at 30 mcg/kg per infusion with no significant AST/ALT elevations. Interestingly, both of these patients had stable disease by imaging criteria at initial treatment and evaluation following the completion of the first course of therapy. Both of these patients appeared to exhibit clinical benefit from their treatment, as evidenced by significant resolution of dysphagia/odynophagia, improved oral intake and overall sense of well-being, and have continued on study. PK results pertaining to Cycles 1 and 2 infusions in these patients are summarized in **Figure 11**, and closely approximate predicted levels based on modeling from drug levels obtained in the Phase II mithramycin study (12C0151). The lack of significant liver toxicities observed in Patients #2 and 3 who received mithramycin infusions at 30 mcg/kg per infusion and possible clinical benefit warrant evaluation of additional patients at this dose level, and escalation if appropriate to a new intermediate level (45 mcg/kg per infusion), to optimize mithramycin delivery to solid tumors.

2 ELIGIBILITY ASSESSMENT AND ENROLLMENT FOR PHASE I AND PHASE II

2.1 ELIGIBILITY CRITERIA

2.1.1 Inclusion Criteria

2.1.1.1 Patients with measurable (per RECIST see Section **6.3.3.1**), inoperable, histologically confirmed non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), esophageal carcinomas, thymic neoplasms, germ cell tumors, malignant pleural mesotheliomas or chest wall sarcomas, as well as patients with gastric, colorectal, pancreas or renal cancers and sarcomas metastatic to 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 ≥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 6 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 ≥ 1500/mm^3
   ii. Platelets ≥ 100,000/ mm^3 (transfusion independent)
   iii. Hemoglobin ≥ 8 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) ≤ 3.0 X ULN
   iii. Albumin > 2 g/dL

c) Renal Function
   i. Creatinine within normal institutional limits or creatinine clearance ≥ 60 mL/min/1.73 m^2 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 2 months after treatment, if female of childbearing potential or male having 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 unfavorable 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, < 30% predicted; DLCO, < 30% predicted (post-bronchodilator); Oxygen saturation ≤ 92% on room air (per vital sign measurement). 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. intralumenal 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.

2.1.3 Recruitment Strategies

This protocol may be abstracted into a plain language announcement posted on NIH websites and on NIH social media platforms. Participants may also be recruited through self-referrals, physician referrals, and referrals from the NIH Clinical Center (CC) Office of Patient Recruitment.

2.2 RESEARCH ELIGIBILITY EVALUATION

Note: Screening evaluation testing/procedures are conducted under the separate screening protocol 06C0014 (Prospective Evaluation of Genetic and Epigenetic Alterations in Patients with Thoracic Malignancies).

Pre-treatment blood tests should be performed within one week 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
    - Chemistries: LDH, SGPT (ALT), SGOT (AST), alkaline phosphatase, bilirubin (total and direct), BUN, creatinine, amylase, lipase, electrolytes, ionized calcium, magnesium, phosphorus, uric acid, albumin
    - Total and fractionated serum bile acids
    - Thyroid panel
    - HIV, HBV and HCV serologies
  - Affymetrix Drug Metabolizing Enzymes and Transporters (DMET) microarray genotyping of PBMC (to be performed in Dr. William D. Figg's lab under protocol 06C0014). (**May be performed at any time prior to enrollment.**)
  - Urinalysis
  - Women of child-bearing potential will have a urine or serum β-hCG pregnancy test.
    - Note: This test is to be done within 48 hours 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.
- Radiographic Evaluation

  Assessment of disease sites by appropriate radiological evaluation. This should include a CT scan of chest, abdomen and pelvis, brain MR and PET-CT. Ultrasonographic elastography of liver (all patients).
- Arterial blood gas (ABG) if clinically indicated
- Pulmonary function tests (PFTs)

- 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 PARTICIPANT REGISTRATION AND STATUS UPDATE PROCEDURES

Registration and status updates (e.g. when a participant is taken off protocol therapy and when a participant is taken off-study) will take place per CCR SOP ADCR-2: CCR Participant Registration & Status Updates found here.

2.4 TREATMENT ASSIGNMENT PROCEDURES

Cohorts

Number	Name	Description
1	Phase I	Patients with primary malignancies involving lungs, esophagus, thymus, pleura, chest wall or mediastinum, or extra-thoracic malignancies metastatic to the chest
2	Phase II (Thoracic Malignancies)	Patients with primary thoracic malignancies
3	Phase II (Non-Thoracic Malignancies)	Patients with neoplasms of non-thoracic origin metastatic to the chest

Arms

Number	Name	Description
1	Phase I	Escalating doses of Mithramycin
2	Phase II	Mithramycin administered at MTD

Arm Assignment

- Up to 54 patients in Cohort 1 may be initially assigned to Arm 1 until MTD is determined.
- After MTD is determined:
  - Up to 7 evaluable patients in Cohort 2 will be directly assigned to Arm 2. An additional 14 may be directly assigned if interim efficacy analysis is positive.
  - Up to 7 evaluable patients in Cohort 3 will be directly assigned to Arm 2. An additional 14 may be directly assigned if interim efficacy analysis is positive.

3 STUDY IMPLEMENTATION

3.1 STUDY DESIGN

This Phase I/II study is designed primarily to evaluate the toxicities, pharmacokinetics and clinical efficacy of mithramycin administered as a continuous 24-hour infusion in patients with thoracic malignancies. All patients will be evaluated initially in the Thoracic Surgery Clinic, Clinical Center, NIH. Once appropriate consents have been signed, patients will undergo biopsy of their tumors for analysis of pharmacodynamics endpoints via endoscopic procedures performed in the operating room by the Thoracic Surgery attending physicians or by percutaneous FNA methods performed by interventional radiology staff at the Clinical Center, NIH. Specimens will be processed for DNA/RNA isolation, and immunohistochemistry studies. If sufficient materials are available from a previous biopsy either at the NIH or elsewhere, this baseline biopsy may be omitted as outlined in **Appendix 2**. Patients will be hospitalized on 3NW, NIH Clinical Center, and receive 24h infusions of mithramycin as outlined below using a conventional Phase I study design, with no intra-patient dose escalation, and no routine cytokine support. Treatment cycles will be separated by 2 weeks to allow recovery from systemic toxicities. Due to potential cardiac toxicity related to mithramycin, all patients will have EKGs (12 lead) prior to, and daily EKGs for 4 days following mithramycin infusions (first cycle only unless otherwise clinically indicated), and will be on telemetry during the Cycle 1 infusion (and subsequent cycles if clinically indicated per Cardiology recommendations). Patients will also have routine bloodwork as outlined in Section **3.5**. Blood will be obtained at designated time points to assess mithramycin levels are outlined in Section **5.1**. Unless medical difficulties arose during the collection of the first biopsy, a post-treatment tumor biopsy will be obtained on Cycle 1 Day 4 (+/- 3 days) to assess molecular endpoints. At the time of each biopsy session, representative samples will be evaluated by frozen section techniques to verify the histology and quality of the tumor tissue for subsequent analysis. An attempt will be made to biopsy the same tumor site for consistency of sampling during the treatment cycles. Approximately 14 days following initiation of the first treatment cycle, a second treatment cycle will commence. If possible, an optional tumor biopsy may be taken at treatment evaluation following the end of Course 1, and additional optional tumor biopsies requested if a patient experiences a dramatic clinical response to treatment, provided that the biopsies are easy to obtain and pose little risk to the patients (refer to Section **5.2** and **Appendix 2**). CT guided research biopsies will not exceed 5 per year on this study. At the end of the fourth cycle (approximately 56-60 days following the initiation of the first treatment cycle), patients will return to the Thoracic Surgery Clinic for repeat staging studies and blood work to analyze treatment response using criteria outlined in Section **6.3.3**. In patients who exhibit response to therapy and commence a second course of mithramycin treatment an additional tissue biopsy may be requested during this course of therapy for confirmatory analysis of pharmacodynamics endpoints identified in preclinical experiments to be associated with regressions of human cancer xenografts in mice.

Summary of Mithramycin Treatment Cycle 1

	Day 1	**Day 2**	**Day 4**	**Day 15**
Initial Evaluation including imaging, blood work and biopsy of tumor tissue for molecular analysis*	Commence Mithramycin Infusion	Complete Mithramycin Infusion	Tumor Biopsy in OR ***or*** Interventional Radiology Suite	Commence Second Cycle.
	Obtain blood for Pharmacokinetic Studies → **and Toxicity Analysis as outlined in Sections 3.5 and 5.1**			

*May be obtained at any point prior to treatment either on this protocol or from clinically indicated procedures (surgeries and/or biopsies) obtained on 06C0014. If sufficient tissue is available from a biopsy performed at a previous time at NIH or elsewhere, this baseline biopsy may be omitted.
Note: Blood work will be collected as described in Section 3.5.

3.1.1 Dose Limiting Toxicity

In the Phase I portion of the study, dose limiting toxicity is defined as any of the following occurring during Cycle 1 of therapy:

- Grade 4 or greater anemia or neutropenia exceeding 5 days duration
- Thrombocytopenia requiring transfusion
- Grade 3 or greater nonhematologic toxicity possibly, probably or definitely related to the investigational therapy – excluding alopecia;

Full toxicity criteria outlined in Section 6.4.

3.1.2 Dose Escalation

3.1.2.1 Dose Escalation for MTD Determination

The doses of mithramycin which will be used at each level are indicated below, and are based on modeling performed using pharmacokinetic results obtained in our recent Phase II mithramycin trial (protocol 12C0151) and of initial patient results in this trial (16C0152). The schema of drug exposure has been selected to optimize growth inhibition and transcriptional reprogramming in clinical settings. The overall goal is to administer a dose of mithramycin during a 24-hour infusion that is equivalent to the total dose administered during 7 daily 6 hour infusions at a dose of 30-50 mcg/kg per infusion (210-350 mcg/kg) used in our recent Phase II trial (12C0151). This dose range would be expected to produce plasma levels approximating 50-100 nM by the end of infusion (**Figure 10**). The selection of only those patients with favorable ABCB4, ABCB11, RALBP and

CYP8B1 genotypes should allow more efficient escalation of mithramycin doses with acceptable toxicities.

Dose Escalation Schema for 24h Mithramycin Infusions
(For MTD Determination in Initial Cohort 1 Arm 1 Patients)

Dose Level	Dose (mcg/kg)
-1	**30**
-1A	**45**
1	**60**

The first 3 patients in Cohort 1 received mithramycin and experienced Grade 4 ALT/AST elevations. Two of these three patients continued mithramycin at a reduced dose (30 mcg/kg; DL-1) without exhibiting dose-limiting toxicity (refer to Section **1.3.4.2**).

As such in Amendment E of this protocol (Version Date 11/01/2019) the dose levels have been amended and MTD determination will begin anew with 3 patients at dose level DL-1 (30 mcg/kg), to be assessed during the first treatment cycle for DLT. The MTD is the dose level at which no more than 1 of up to 6 patients experience DLT during Cycle 1 of treatment, and the dose below that at which at least 2 (of ≤ 6) patients have DLT.

If the 3 patients at DL-1 do not exhibit DLT, the next 3 patients will receive mithramycin at an increased dose of 45 mcg/kg (DL-1A) provided that at least 2 weeks have elapsed since the third patient of dose level DL-1 was treated to rule out unacceptable delayed toxicities (primarily hepatic and hematologic). Patients who receive mithramycin infusions without dose limiting toxicity (DLT) during Cycle 1 will be eligible for treatment during Cycle 2 at the same dose level in order to assess clinical as well as molecular effects of cumulative mithramycin exposures. Nausea, vomiting and diarrhea will not be considered dose limiting toxicities unless refractory to maximal standard prophylactic measures.

Dose escalation will continue at DL-1A (45 mcg/kg) as indicated above using 3 patients per dose level until DLT (assessed during the first treatment cycle) is observed. The MTD is the dose level at which no more than 1 of up to 6 patients experience DLT during Cycle 1 of treatment, and the dose below that at which at least 2 (of ≤ 6) patients have DLT. We anticipate that the MTD will be 45 mcg/kg.

If 1 of 3 patients in DL-1 experiences DLT, up to 3 additional patients will be treated at this dose level.

- If only 1 of 6 patients exhibits DLT, subsequent patients will be enrolled into DL-1A. As soon as 2 patients in a cohort at any given level develop DLT, no additional patients will be entered at that level and subsequent patients will be accrued into the preceding dose level; if DLT is observed in less than 2 of 6 patients treated at this lower level, this dose will represent maximum tolerated dose (MTD).
- If more than 1 of 6 patients experience DLT, if necessary*, 3 additional patients will be accrued to DL-1, which will be designated the MTD if fewer than 2 of 6 subjects

experience DLT. (*It may be necessary to accrue 3 additional patients to this dose level if 6 subjects have not been treated.) Accrual will continue until an MTD is determined.

Following completion of the fourth cycle of therapy, all patients will undergo evaluation consisting of physical exam, blood work, and appropriate staging studies to define response to treatment. Those individuals who exhibit disease progression will be taken off study. Patients who exhibit stabilization or regression of their disease will be eligible for 4 additional mithramycin infusions at the same dose level at which they were originally treated. Patients will continue therapy in this manner until off-study criteria have been met.

3.1.2.2 After MTD Has Been Defined

Once the MTD has been defined, an additional 14 patients (7 with primary thoracic malignancies and 7 with neoplasms of non-thoracic origin metastatic to the chest) will initially be enrolled in a Phase II setting, to more fully evaluate clinical and molecular responses at the MTD (Phase II – Simon Stage 1). If one or more patients exhibit response to therapy, 14 additional patients will be accrued to the respective cohort(s) to more fully assess clinical efficacy of the four cycle treatment regimen (Phase II-Simon Stage 2).

Patients 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 hepatotoxicity or bleeding. Patients will be hospitalized in the NIH Clinical Center during the infusion. Following each course of therapy (four 14-day cycles) patients will undergo appropriate staging studies to determine response to therapy using RECIST criteria.

3.2 DRUG ADMINISTRATION

Given our recent findings within our recent Phase II mithramycin trial (12C0151) pertaining to ABCB4, ABCB11, RALBP and CYP8B1 SNPs that distinguish patients who did and did not experience mithramycin-mediated hepatotoxicity, this protocol will accrue only those patients with favorable ABCB4/ABCB11/RALBP/CYP8B1 genotypes.

Mithramycin will be administered intravenously via central line over 24 hours once every 14 days (+7 days/-3 days). 12 mg IV of dexamethasone will be administered one hour prior to each infusion. Four cycles will constitute one course. Intravenous hydration will be provided as clinically indicated.

3.3 DOSING DELAYS AND MODIFICATIONS

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

Summary of dose holding/interruptions and dose de-escalation recommendations for mithramycin in case of mithramycin -related adverse events (graded according to NCI-CTCAEv5.0)

Reduction to the next lower dose as indicated in the table in Section **3.1.2**.

General Adverse Events	Action
Non-hematological, Grade 1 or 2 (excluding hemorrhage)	• Continue mithramycin therapy at full dose prescribed dose. • Apply maximum supportive care recommendations. • If prolonged duration of Grade 2 adverse event (≥ 7 days) is affecting quality of life, start the next treatment cycle at 1 dose level lower. • If event persists and continues to affect quality of life for ≥ 7 more days following initial dose reduction, start the next treatment cycle at an additional dose level lower. • If events continue to persist, discontinue drug.
Grade 3 ALT and AST	• Hold mithramycin therapy until recovery to Grade ≤ 1 (up to 21 days of start of cycle). • If ALT and AST resolve to Grade ≤ 1within 21 days, maintain current dose level. • If ALT and AST do NOT resolve to Grade ≤ 1within 21 days, dose reduce 1 level. • If Grade 3 ALT or AST occur in a subsequent cycle, dose reduce 1 level independent of the date of resolution.
Grade 4 ALT and AST	• Hold mithramycin therapy until recovery to Grade ≤ 1 (up to 21 days of start of cycle). • If ALT and AST resolve to Grade ≤ 1within 21 days, reduce 1 level. • If Grade 4 ALT or AST occur in a subsequent cycle, dose reduce 1 level independent of the date of resolution.
Non-hematological, Grade 3 or 4 (excluding ALT, AST, hemorrhage and QT prolongation)	• Apply maximum supportive care recommendations. Hold mithramycin therapy until recovery to Grade ≤ 1 (up to 21 days from start of cycle) and start the next treatment cycle at the next lower dose level. • 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 ≤ 1 (up to 21 days) and start the next treatment cycle *1 dose level lower*. ***Note:*** *If patient's toxicity recovers to Grade ≤ 1 prior to Day 7 of treatment cycle, dosing for that cycle may resume 1 dose level lower.* • If symptoms continue to persist at Grade 3 or 4, following a maximum of 2 dose reductions, discontinue drug.
QT prolongation, Grade 3 or 4	• 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. • If therapy is continued, dose reduce 1 level for Grade 4 events.
Bleeding Events (hemorrhage)	

General Adverse Events	Action
Grade 1 events	• Continue mithramycin therapy at full dose prescribed. • Apply maximum supportive care recommendations.
Grade 2 events	• Apply maximum supportive care recommendations. Hold mithramycin therapy until recovery to Grade ≤ 1 (up to 21 days from start of treatment cycle) and start the next treatment cycle at 1 dose level lower. • 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 ≤ 1 (up to 21 days from start of treatment cycle) and start the next treatment cycle at 1 additional dose level lower. • If symptoms continue to persist at any grade, discontinue drug.
Grade 3 events	• Discontinue mithramycin
Grade 4 events	• Discontinue mithramycin
*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.*	
Hematological Adverse Events	
Grades 1 and 2 events	• Continue mithramycin therapy at full dose prescribed. Apply maximum supportive care recommendations.
Grade 3 events	• Apply supportive care • If toxicities resolved to allow retreatment by Day 14, resume at current dose. • If toxicities resolve between Days 14-21 to allow retreatment (See Section **3.4**), resume at 1 dose level lower. • Following dose reduction, if toxicities persist and do not allow treatment at Day 15, but are sufficiently resolved to allow retreatment by Day 21, resume at 1 additional dose level lower.
Grade 4 events	• Apply maximum supportive care recommendations. • 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 1 dose level lower. • If recurrence of hematological adverse event after drug hold/ interruptions is observed with treatment at 1 dose level lower, 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 1 additional dose level lower. • If symptoms continue to persist at any grade, discontinue drug.

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 **6.2**) 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 ≥ 75,000/µL (transfusion independent), and an ANC ≥ 1000/µL, and
- meets other laboratory parameters defined in the eligibility criteria (Section **2.1**); 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 Evaluation

- Complete history and physical examination including assessment of vital signs and ECOG status (within 4 days prior to treatment).
- Laboratory
  - ROUTINE LABS:
    - Complete blood count, differential
    - Chemistries: 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 (within one week prior to treatment).
    - Total and fractionated serum bile acid levels (within 2 weeks prior to treatment). *Note: Results need not be back prior to commencing treatment.*
  - Coagulation Studies: PT/PTT; lupus anticoagulant (LA) test if indicated (within one week prior to treatment).
  - Urinalysis.
  - Women of child-bearing potential will have a urine or serum βhCG pregnancy test. *Note: this test is to be done within 2 days prior to start of treatment.*
- Radiographic Evaluations (refer to **Appendix 2** for details). *Note: Obtain within 4 weeks prior to treatment*.
- Liver elastography assessment by ultrasound (within 2 weeks prior to treatment).
- Research Labs (refer to **Appendix 3** for details). *Note: May be obtained at any point within 2 weeks of commencing 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.

- Laboratory Evaluations (Days 1-5 while hospitalized, unless otherwise indicated below*):
  - CBC with differential count.
  - Chemistry: sodium, potassium, chloride, CO2, creatinine, glucose, BUN, albumin, ionized calcium**, magnesium, alkaline phosphatase, ALT/GPT, AST/GOT, total bilirubin, total protein.
  - Total and fractionated serum bile acids on Day 1 (prior to infusion) and Day 5 of Cycle 1(+/- 3 days) and at treatment evaluation.
  - Coagulation Studies: PT/PTT/Thrombin Time, Fibrinogen.

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

    *** Note:** Day 1 labs can be drawn on the day prior to infusion. In between treatments, the above labs may be drawn twice weekly (+48 hours between labs). For patients not experiencing Grade 3 or 4 toxicities these labs may be drawn once weekly in between treatments per PI discretion instead.

    ****** Ionized calcium is not part of a standard lab panel and may be omitted if not available or not covered by insurance when performed outside NIH.12-Lead EKG:
  - Cycle 1: Days 1 (within 2 hours prior to infusion), 2 (within 2 hours post-infusion), 3, 4 and 5, to evaluate for QT prolongation potential.

    Note: EKGs may be done outside of the specified times if unexpected events in clinic prevent staff from conducting the study within the specified timeframe. EKGs performed outside of this window will NOT be considered protocol deviations.
  - Cycle 2 and subsequent cycles: within 2 hours prior to start and within 2 hours following completion of infusion.
- Ultrasonic liver elastography Day 4 of Cycle 1 (+/- 3 days) and Day 1 (+/- 3 days) of subsequent cycles and at treatment evaluation.

3.5.3 Research Evaluations (Refer to Appendix 3, Unless Otherwise Indicated Below)

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

- Coagulation Studies.
- Pharmacokinetic (PK) Studies.
- Radiological Evaluations (refer to **Appendix 2**).
- Research Labs for PD studies on plasma and PMBC.
- Tumor Biopsies, to be collected as described in Section **5.2** (refer to **Appendix 2** for details).

  Note: *Within 2 days prior to biopsies:* Normal PT/PTT with exception of lupus anticoagulant, platelets ≥75,000/µL, peripheral ANC ≥750/µL.

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 or more frequently if indicated until all clinically significant toxicities resolve to less than or equal to Grade 1 or baseline as follows:

- Toxicity Evaluation
  - Laboratory evaluation: CBC, Chemistries (Sodium, potassium, chloride, bicarbonate, creatinine, glucose, BUN, albumin, total calcium, ionized calcium*, magnesium, inorganic phosphorus, alkaline phosphatase, ALT/SGPT, AST/SGOT, total bilirubin, direct bilirubin, lactate dehydrogenase, total protein, creatine kinase, uric acid), 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, (Sodium, potassium, chloride, bicarbonate, creatinine, glucose, BUN, albumin, total calcium, ionized calcium*, magnesium, inorganic phosphorus, alkaline phosphatase, ALT/SGPT, AST/SGOT, total bilirubin, direct bilirubin, lactate dehydrogenase, total protein, creatine kinase, uric acid), PT/PTT
  - Radiographic Evaluations (see **Appendix 2**).

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

Prior to removal from study, effort must be made to have all subjects complete a safety visit a minimum of 2-4 weeks following the last dose of study therapy in person or via telephone/videocall or other NIH approved remote platform as detailed in Section **3.5.4**.

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.
- Progressive disease.
- A patient who becomes pregnant will be immediately taken off therapy.
- Excessive toxicity as defined in Section **3.3**.
- Failure to meet criteria for additional treatment as defined in Section **3.4**.

Note: Patients will be followed until toxicity resolves to less than or equal to Grade 1 or baseline (as detailed in Section **3.5.4**).

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 with no ongoing treatment related toxicities above Grade 1 or baseline.
- If the investigator determines that it is in the best interest of the patient to discontinue follow up.
- Investigator decision to end study.
- Lost to follow up.
- Death.

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

Additional antiemetic therapy will be prescribed using clinical standard of care practices, per the discretion of the Principal Investigator. The provided antiemetic therapy may be either intravenous or oral route as needed. (See Section **3.2** for antiemetic therapy prior to mithramycin infusion.)

4.3 ANTIDIARRHEALS

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 collected as outlined in **Appendix 3**. PK analysis will be performed in the NCI, Genitourinary Malignancies Branch (GMB). Samples will be stored in the Thoracic Epigenetics Lab (TEL) and batched and later transferred to the Figg Lab on dry ice for analysis.

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* if sufficient tissue is not available from a previous biopsy (all efforts will be made to avoid repeat biopsy) and on Cycle 1 Day 4 (± 3 days). If possible, an optional biopsy may be taken at treatment evaluation

following Course 1. If insufficient tissue was obtained or if there was harm associated with 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 (refer to **Appendix 2**). 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 focused microarray and/or qRT-PCR analysis of ~50 genes modulated by mithramycin in murine xenograft experiments. 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 under direction of the PI.

***Note*:** If there is an optional biopsy for research in the protocol, then the patient will consent 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.

***** May be omitted as outlined in **Appendix 2: Study Calendar**.

5.3 SAMPLE STORAGE, TRACKING AND DISPOSITION

Samples will be ordered in CRIS and tracked through Clinical Trial Data Management system. Should a CRIS screen not be available, the CRIS downtime procedures will be followed. Samples will not be sent outside NIH without appropriate approvals and/or agreements, if required.

5.3.1 Thoracic Epigenetics Laboratory

This study will be conducted within the Thoracic Surgery Branch, 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 in the Figg Lab, GMB. 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 the Thoracic Surgery Branch, NCI with access limited to the PI or study coordinator. Focused gene expression microRNA, and DNA methylation signatures will be analyzed in Dr. David Schrump's laboratory using a panel of genes and miRs that were noted to be modulated in subQ cancer xenografts following IP mithramycin treatment.

5.3.2 Blood Processing Core (BPC)

5.3.2.1 BPC Contact Information

Please e-mail NCIBloodcore@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 NCIBloodcore@mail.nih.gov.

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

5.3.2.2 Sample Data Collection

All samples sent to the Blood Processing Core (BPC) will be barcoded, with data entered and stored in the Labmatrix utilized by the BPC. This is a secure program, with access to Labmatrix limited to defined Figg lab personnel, who are issued individual user accounts. Installation of Labmatrix is limited to computers specified by Dr. Figg. These computers all have a password restricted login screen.

Labmatrix creates a unique barcode ID for every sample and sample box, which cannot be traced back to patients without Labmatrix 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.).

5.3.2.3 Sample Storage and Destruction

Sample bar-codes are linked to patient demographics and limited clinical information. 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 Labmatrix. 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.

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). The PI will record any loss or unanticipated destruction of samples as a deviation. Reporting will be per the requirements of Section **7.2**.

5.3.3 Protocol Completion/Sample Destruction

All specimens obtained in the protocol are used as defined in the protocol. Any specimens that are remaining at the completion of the protocol will be stored in the conditions described below. The study will remain open so long as sample or data analysis continues.

If the patient withdraws consent the participants data will be excluded from future distributions, but data that have already been distributed for approved research use will not be able to be retrieved.

Following completion of this study, if the subject has co-enrolled on 06C0014, samples will be transferred to the Tissue Procurement protocol and remain in storage in the Blood Processing Core (BPC) as detailed within 06C0014. Access to these samples will only be granted following IRB approval of an additional protocol, granting the rights to use the material. If the subject has not co-enrolled on the Tissue Procurement protocol, the samples will be destroyed.

The PI will record any loss or unanticipated destruction of samples as a deviation. Reporting will be per the requirements of Section **7.2**.

6 DATA COLLECTION AND EVALUATION

6.1 DATA COLLECTION

For the purposes of the research sample analyses and correlation with clinical outcomes, demographic information, histology, operative and peri-operative interventions, pathologic findings, laboratory and imaging parameters (performed as part of routine or protocol specified patient care) may be collected on this study. The PI will be responsible for overseeing entry of data into an in-house password protected electronic system (C3D, Labmatrix) and ensuring data accuracy, consistency and timeliness. 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.

All AEs, including clinically significant abnormal findings on laboratory evaluations, regardless of severity, will be followed until return to baseline or stabilization of event.

Document AEs from the first study intervention, Study Day 1, through 2-4 weeks after the last dose of study therapy. Beyond 2-4 weeks after the last intervention, only adverse events which are serious and related to the study intervention need to be recorded.

An abnormal laboratory value will be considered an AE **only** if the laboratory abnormality is characterized by any of the following:

- Results in discontinuation from the study
- Is associated with clinical signs or symptoms
- Requires treatment or any other therapeutic intervention
- Is associated with death or another serious adverse event, including hospitalization
- Is judged by the Investigator to be of significant clinical impact
- If any abnormal laboratory result is considered clinically significant, the investigator will provide details about the action taken with respect to the test drug and about the patient's outcome.

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, this will be reported expeditiously per requirements in Section 7.2.1.

6.1.1 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 hospitalization, only the admission labs, first morning labs drawn after 4 am, and labs that support the diagnosis of a reportable event will be uploaded into C3D.
- During chemotherapy regimen, for laboratory values obtained at sites other than the NIH Clinical Center: only the following values will be captured in C3D:
  - Hemoglobin, total white blood cell count, absolute neutrophil count, platelet count
  - PTT, PT or INR
  - Creatinine, ALT, AST, Bilirubin (total and direct), lipase and amylase
  - Any unexpected laboratory abnormality ≥ Grade 2 possibly, probably or definitely related to the research
- 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 to Grade 1 or return to baseline.

6.1.2 Exclusions to Routine Data Collection

6.1.2.1 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.

6.1.2.2 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).

6.2 DATA SHARING PLANS

6.2.1 Human Data Sharing Plan

What data will be shared?

I will share human data generated in this research for future research as follows:

- Coded, linked data in an NIH-funded or approved public repository.
- Coded, linked data in BTRIS (automatic for activities in the Clinical Center).

How and where will the data be shared?
Data will be shared through:

- An NIH-funded or approved public repository, clinicaltrial.gov.
- BTRIS (automatic for activities in the Clinical Center).
- Publication and/or public presentations.

When will the data be shared?

- Before publication.
- At the time of publication or shortly thereafter.

6.2.2 Genomic Data Sharing Plan

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

6.3 RESPONSE CRITERIA

For the purposes of this study, patients should be re-evaluated for response every 8 weeks (1 course) (± 2 weeks). In addition to a baseline scan, confirmatory scans should also be obtained not less than 4 weeks following initial documentation of objective response.

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) [(Version 1.1)(80)]. 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.

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.

6.3.1 Disease Parameters

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: ≥ 20 mm;
- By CT scan:
    - Scan slice thickness 5 mm or under: as ≥ 10 mm
    - Scan slice thickness > 5 mm: double the slice thickness
- With calipers on clinical exam: ≥ 10 mm.

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

6.3.1.1 Malignant Lymph Nodes

To be considered pathologically enlarged and measurable, a lymph node must be ≥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.

6.3.1.2 Non-Measurable Disease

All other lesions (or sites of disease), including small lesions (longest diameter < 10 mm or pathological lymph nodes with ≥ 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.

6.3.1.3 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 (80) 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 (81).

6.3.1.4 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.

6.3.2 Methods of Evaluation of Measurable Disease

6.3.2.1 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.

6.3.2.2 Metastatic Bone Lesions

Disease progression is considered if any new lesion 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 (80).

6.3.2.3 Clinical Lesions

Clinical lesions will only be considered measurable when they are superficial (e.g., skin nodules and palpable lymph nodes) and ≥ 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.

6.3.2.4 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.

6.3.2.5 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.

6.3.3 Response Criteria for Radiographic Studies

6.3.3.1 Measuring of Soft Tissue Disease

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: For this study, 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: Tumor markers alone cannot be used to assess response. 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.

For Patients with Measurable Disease (i.e., Target Disease)

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	

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

*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.

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.

6.3.4 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.

6.4 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 5.0 will be utilized for AE reporting.

All appropriate treatment areas have access to a copy of the CTCAE version 5.0. A copy of the CTCAE version 5.0 can be downloaded from the CTEP web site (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm).

7 NIH REPORTING REQUIREMENTS/DATA AND SAFETY MONITORING PLAN

7.1 DEFINITIONS

Please refer to definitions provided in Policy 801: Reporting Research Events found here.

7.2 OHSRP OFFICE OF COMPLIANCE AND TRAINING/IRB REPORTING

7.2.1 Expedited Reporting

Please refer to the reporting requirements in Policy 801: Reporting Research Events and Policy 802: Non-Compliance Human Subjects Research found here. Note: Only IND Safety Reports that meet the definition of an unanticipated problem will need to be reported per these policies.

7.2.2 IRB Requirements for PI Reporting Continuing Review

Please refer to the reporting requirements in Policy 801: Reporting Research Events found here.

7.2.3 NCI Clinical Director Reporting

Problems expeditiously reported to the OHSRP in iRIS will also be reported to the NCI Clinical Director. A separate submission is not necessary as reports in iRIS will be available to the Clinical Director.

In addition to those reports, all deaths that occur within 30 days after receiving a research intervention should be reported via email to the Clinical Director unless they are due to progressive disease.

To report these deaths, please send an email describing the circumstances of the death to Dr. Dahut at NCICCRQA@mail.nih.gov within one business day of learning of the death.

7.3 NIH REQUIRED DATA AND SAFETY MONITORING PLAN

7.3.1 Principal Investigator/Research Team

The clinical research team will meet on a weekly basis when patients are being actively treated on the trial to discuss each patient in detail. Decisions about dose level enrollment and dose escalation if applicable will be made based on the toxicity data from prior patients.

All data will be collected in a timely manner and reviewed by the principal investigator or a clinical associate investigator. Events meeting requirements for expedited reporting as described in Section **7.2.1** will be submitted within the appropriate timelines.

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.

8 SPONSOR SAFETY REPORTING

8.1 DEFINITIONS

8.1.1 Adverse Event

Any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment. An adverse event (AE) can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product (ICH E6 (R2)).

8.1.2 Serious Adverse Event (SAE)

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 event (see Section **8.1.3**).
- Inpatient hospitalization or prolongation of existing hospitalization.
    - A hospitalization/admission that is pre-planned (i.e., elective or scheduled surgery arranged prior to the start of the study), a planned hospitalization for pre-existing condition, or a procedure required by the protocol, without a serious deterioration in health, is not considered a serious adverse event.
    - A hospitalization/admission that is solely driven by non-medical reasons (e.g., hospitalization for patient convenience) is not considered a serious adverse event.
    - Emergency room visits or stays in observation units that do not result in admission to the hospital would not be considered a serious adverse event. The reason for seeking medical care should be evaluated for meeting one of the other serious criteria.
- 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.

8.1.3 Life-threatening

An adverse event or suspected adverse reaction is considered "life-threatening" if, in the view of either the investigator or sponsor, its occurrence places the patient or subject at immediate risk of death. It does not include an adverse event or suspected adverse reaction that, had it occurred in a more severe form, might have caused death. (21CFR312.32)

8.1.4 Severity

The severity of each Adverse Event will be assessed utilizing the CTCAE version 5.0.

8.1.5 Relationship to Study Product

All AEs will have their relationship to study product assessed using the terms: related or not related.

- Related – There is a reasonable possibility that the study product caused the adverse event. Reasonable possibility means that there is evidence to suggest a causal relationship between the study product and the adverse event.

- Not Related – There is not a reasonable possibility that the administration of the study product caused the event.

8.2 Assessment of Safety Events

AE information collected will include event description, date of onset, assessment of severity and relationship to study product and alternate etiology (if not related to study product), date of resolution of the event, seriousness and outcome. The assessment of severity and relationship to the study product will be done only by those with the training and authority to make a diagnosis and listed on the Form FDA 1572 as the site principal investigator or sub-investigator. AEs occurring during the collection and reporting period will be documented appropriately regardless of relationship. AEs will be followed through resolution.

SAEs will be:

- Assessed for severity and relationship to study product and alternate etiology (if not related to study product) by a licensed study physician listed on the Form FDA 1572 as the site principal investigator or sub-investigator.
- Recorded on the appropriate SAE report form, the medical record and captured in the clinical database.
- Followed through resolution by a licensed study physician listed on the Form FDA 1572 as the site principal investigator or sub-investigator.

For timeframe of recording adverse events, please refer to Section **6.1**. All serious adverse events recorded from the time of first investigational product administration must be reported to the Sponsor. Any exceptions to the expedited reporting requirements are found in Section **Error! Reference source not found.**.

8.3 Reporting of Serious Adverse Events

Any AE that meets protocol-defined serious criteria or meets the definition of Adverse Event of Special Interest that require expedited reporting must be submitted immediately (within 24 hours of awareness) to OSRO Safety using the CCR SAE report form. Any exceptions to the expedited reporting requirements are found in Section **Error! Reference source not found.**.

All SAE reporting must include the elements described in Section **8.2**.

SAE reports will be submitted to the Center for Cancer Research (CCR) at: OSROSafety@mail.nih.gov and to the CCR PI and study coordinator. CCR SAE report form and instructions can be found at: https://ccrod.cancer.gov/confluence/pages/viewpage.action?pageId=157942842

Following the assessment of the SAE by OSRO, other supporting documentation of the event may be requested by the OSRO Safety and should be provided as soon as possible.

8.4 REPORTING PREGNANCY

8.4.1 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 no later than 24 hours of when the Investigator becomes aware of it. The Investigator should notify the Sponsor no later than 24 hours of when the outcome of the Pregnancy becomes known.

Pregnancy itself is not regarded as an SAE. However, congenital abnormalities or birth defects and spontaneous miscarriages that meet serious criteria (Section **8.1.2**) should be reported as SAEs.

The outcome of all pregnancies (spontaneous miscarriage, elective termination, ectopic pregnancy, normal birth, or congenital abnormality) should be followed up and documented.

8.4.2 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 days after the last dose should, if possible, be followed up and documented.

8.5 REGULATORY REPORTING FOR STUDIES CONDUCTED UNDER CCR-SPONSORED IND

Following notification from the investigator, CCR, the IND Sponsor, will report any suspected adverse reaction that is both serious and unexpected in expedited manner to the FDA in accordance to 21 CFR 31.2.32. CCR will report an AE as a suspected adverse reaction only if there is evidence to suggest a causal relationship between the study product and the adverse event. CCR will notify FDA and all participating investigators (i.e., all investigators to whom the Sponsor is providing drug under its INDs or under any investigator's IND) in an IND safety report of potential serious risks from clinical trials or any other source, as soon as possible, in accordance to 21 CFR Part 312.32.

All serious events will be reported to the FDA at least annually in a summary format.

9 CLINICAL MONITORING

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 and secondary study endpoints; adherence to the protocol, regulations, ICH E6 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.

10 STATISTICAL CONSIDERATIONS

The Phase 1 dose escalation study of mithramycin infusion will be conducted as a single arm study to minimize the number of patients needed to define MTD. Pharmacokinetic analyses, as well as analyses of molecular endpoints such as HDAC4, KIAA1199, PMAIP and p21 expression in tumor tissues and PBMC before and after treatment will be performed, with the primary focus being with respect to results at the MTD. 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 trial and will be based on limited numbers of patients, the analyses will be considered exploratory and hypothesis generating rather than definitive. As many as 15 patients may theoretically need to be accrued to define the MTD if the amended dose levels (DL-1 and DL-1A) each require 6 patients, in addition to the 3 subjects that were already enrolled at dose level DL1.

The primary endpoint of the Phase II component study will be evaluated for objective response by RECIST criteria v 1.1. Patients will be enrolled into 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; p0=0.05), in favor of a higher response rate of 30% (p1=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 cohort and if 0 of the 7 have a response, then no further patients will be accrued in that cohort. If 1 or more of the first 7 in a cohort have a response, then accrual will 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%.

The following table summarizes the two stage design that will be used for each cohort in the Phase II component of this trial.

	Cumulative Number of Responses	Decision
Stage 1: Enter 7 patients	0	Terminate the trial: 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: agent ineffective
	3 or more	Terminate the trial: agent effective

With two cohorts, up to 42 evaluable patients may be required to be accrued to the Phase II portion. It is expected that 10-15 patients per year may enroll onto this study. Thus, 2 to 3 years may be needed to enroll up to 15 patients in Phase I plus 36 evaluable patients in Phase II (up to 6 patients may continue from the Phase I component of the trial). This may be completed much sooner depending on the number actually required. To allow for a small number of inevaluable patients, the accrual ceiling will be set at 60.

10.1 METHODS OF ANALYSIS

Response rates will be calculated as the percent of patients whose best response is a CR or PR. 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.

10.2 EVALUATION OF RESPONSE

Any patient who is enrolled and receives at least one full course (4 cycles) 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 completing a full course of mithramycin and do not expire within 14 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 will be taken off-treatment and will only be evaluable for toxicity (not evaluable for response).

10.3 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.

11 HUMAN SUBJECTS PROTECTIONS

11.1 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.

11.2 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.

11.3 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 **11.4**), all subjects ≥ age 18 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 Policy 403 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.

11.4 EVALUATION OF POTENTIAL BENEFITS AND RISKS

The risks and benefits of participation for adults who become unable to consent on study are no different than those described for the rest of the study population.

11.4.1 Risks

11.4.1.1 Blood Sampling

Side effects of blood draws include pain and bruising, lightheadedness, and rarely, fainting.

11.4.1.2 Urine Collection

There is no physical risk involved with urine collection.

11.4.1.3 Ultrasound and EKG

No risks are associated with these procedures. The patient may experience minimal discomfort during the procedure.

11.4.1.4 Pulmonary Function Tests

These tests are safe and side effects are unlikely, but may include brief light headedness or slight soreness of the chest.

11.4.1.5 Study Drug Risks

The risks to the patient participating in this trial are anticipated to be small and are primarily the risks associated with administering the study drug.

These potential risks and benefits will be carefully discussed with the patient at the time consent is obtained. Patients will be monitored throughout the study in order to limit the consequences of any adverse events.

11.4.1.6 Central Line

IV drugs on this study will be given through a central line, a “tunneled” catheter surgically inserted into one of the main blood vessels leading to the heart. The risks associated with the procedure include pain, bleeding, infection, puncture of the underlying lung and pulmonary embolism. Lung puncture can result in lung collapse, which might require that a chest tube be placed into the chest cavity (usually for a day or two) to help the lung re-expand. The long-term risks of the catheter include infection and clotting of the vein in which the catheter sits which can break off and travel to the veins near the neck, face, chest, arms or lungs (pulmonary embolism).

11.4.1.7 Biopsy Risks

The risks associated with biopsies are pain and small amounts of bleeding at the biopsy site, and rarely, a small infection at the site. Biopsy sites usually heal very well and with very little scarring. In order to minimize pain, local anesthesia will be used; side effects of the local anesthesia may include a mild burning sensation when the numbing medicine is injected into the skin CT guidance will be used in obtaining biopsies.

11.4.1.8 Scans and Contrast

The most common discomfort is the length of time a patient must lay still during a scan. Patients may also become uncomfortable with the closed space of the machines.

There is a small risk of reaction in scans involving contrast. Common reactions include pain in the vein where the contrast was given, a metallic or bitter taste in the mouth, headache, nausea and a warm or flushing feeling that lasts from 1-3 minutes. In very rare cases, severe reactions that affect breathing, heart rhythm or blood pressure have occurred.

An IV line may need to be inserted for administration of the contrast agent or anesthetic, which may cause pain at the site where the IV is placed and there is a small risk of bruising or infection.

11.4.1.9 Risks of Exposure to Ionizing Radiation

This research study involves the potential for 6 CT CAP scans, 6 PET/CT scans as well as a potential maximum of 5 CT-guided biopsies (2 required, see **Appendix 2**) over the course of the first year on study. This radiation exposure is not required for medical care and is for research purposes only. Subjects will be exposed to approximately 17.8 rem. This amount of radiation is above the guideline of 5 rem per year and will expose the subject to the roughly the same amount of radiation as 59.3 years of background radiation.

11.4.2 Benefits

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.

11.5 Consent Process and Documentation

The informed consent document will be provided to the participant or consent designee(s) (e.g., the legally authorized representative [LAR] if participant is an adult unable to consent) for review prior to consenting. A designated study investigator will carefully explain the procedures and tests involved in this study, and the associated risks, discomforts and benefits. In order to minimize potential coercion, as much time as is needed to review the document will be given, including an opportunity to discuss it with friends, family members and/or other advisors, and to ask questions of any designated study investigator. A signed informed consent document will be obtained prior to entry onto the study.

The initial consent process as well as re-consent, when required, may take place in person or remotely (e.g., via telephone or other NIH approved remote platforms) per discretion of the designated study investigator and with the agreement of the participant/consent designee(s). Whether in person or remote, the privacy of the subject will be maintained. Consenting investigators (and participant/consent designee, when in person) will be located in a private area (e.g., clinic consult room). When consent is conducted remotely, the participant/consent designee will be informed of the private nature of the discussion and will be encouraged to relocate to a more private setting if needed.

12 PHARMACEUTICAL AND INVESTIGATIONAL DEVICE INFORMATION

12.1 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.

12.1.1 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.

12.1.2 Toxicity

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

More frequent:

- Hypocalcemia (muscle and abdominal cramps)
- Anorexia (loss of appetite)
- Diarrhea
- Stomatitis
- 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
- Gastrointestinal bleeding (bloody or black, tarry stools; vomiting of blood)
- hepatotoxicity (yellow eyes or skin)
- Epistaxis, hematemesis
- Leukopenia (sore throat and fever)—incidence about 6%
- Petechial bleeding
- Thrombocytopenia
- 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.

12.1.3 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 24 hours.

12.1.4 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 48 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.

12.1.5 Administration Procedures

Infuse intravenously over 24 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 reinstituted at another site. Moderate heat should be applied to the site of extravasation to disperse the drug and minimize local tissue irritation and discomfort.

12.1.6 Contraindications

The following medications are contraindicated:

- Anticoagulants, coumadin- or indandione-derivatives
- 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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14 TABLES, FIGURES AND APPENDICES

14.1 TABLES

14.1.1 Table 1: Genes Differentially Expressed In Vitro and In Vivo by Mithramycin

Ingenuity Canonical Pathways - in vitro	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-β 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/β-catenin Signaling *	PPP2R5C, PPP2CA, CSNK1G2, TGFBR3, 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-κB Signaling	TRAF3, TNFRSF1A, TGFBR3, CREBBP, BMPR2, MAP4K4, MALT1, EP300, BMPR1B, BMPR1A, IGF1R, AKT3, PIK3CB, GSK3B, EGFR
p53 Signaling	PMAIP1, STAG1, AKT3, PIK3CB, GSK3B, HIPK2, EP300
Molecular Mechanisms of Cancer	MAP2K6, PMAIP1, TCF4, TGFBR1, BMPR2, CRK, RBL1, TGFBR2, FZD8, BMPR1B, HIPK2, BRCA1, PRKCA
TGF-β 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/β-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-κB Signaling	MAP2K6, TGFBR2, BMPR1B, TGFBR1, BMPR2
p53 Signaling	PMAIP1, TP53INP1, HIPK2, BRCA1

*** Stem cell related signaling pathway**

14.1.2 Table 2: Tissue Mithramycin Concentrations in Mice Following IV or IP Injection

Assumptions:

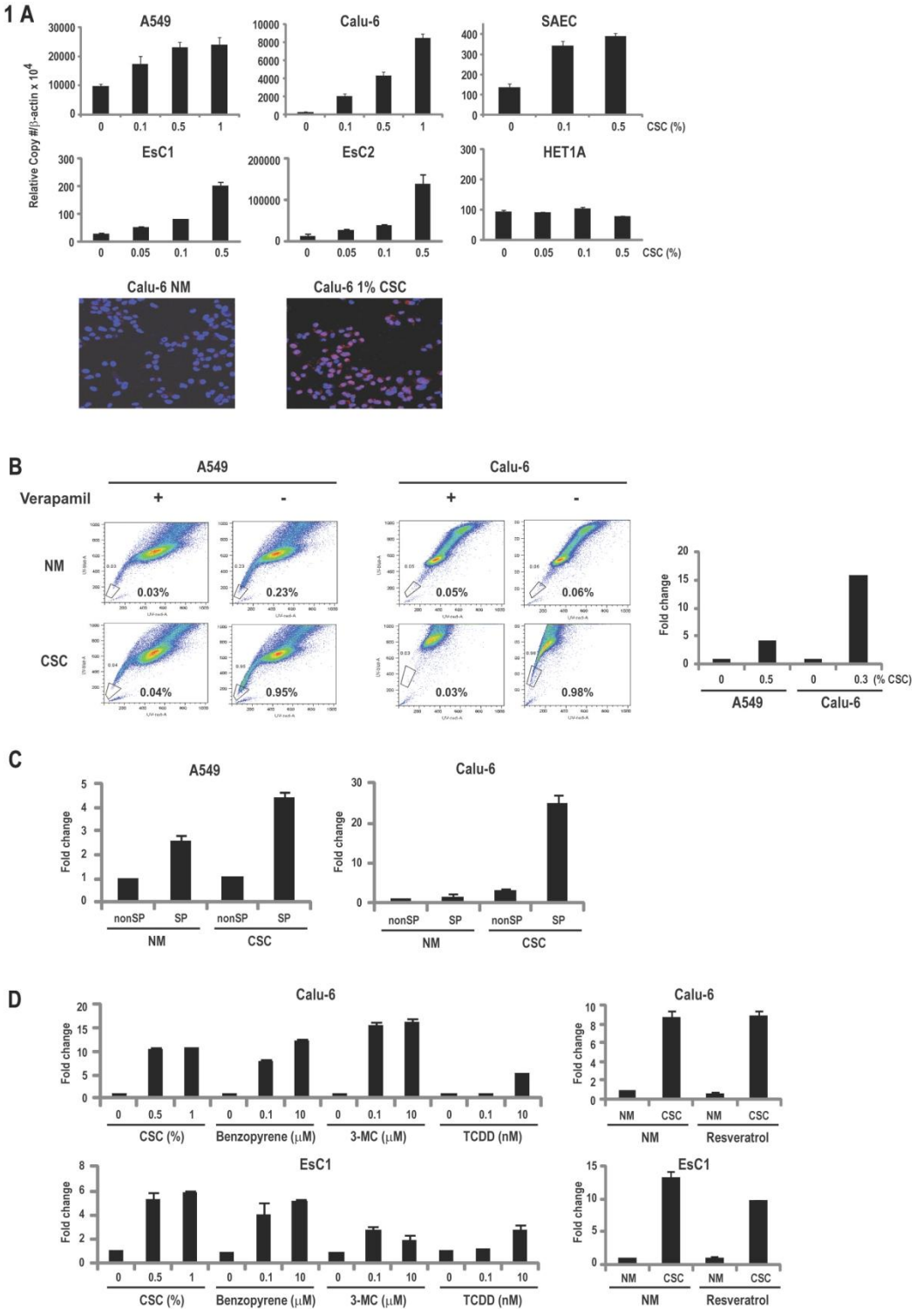
1) Specific activity of mithramycin = 147.5 microCi/mg (from reference # 52)
2) Counting efficiency of scintillation counter = 15% (from reference # 52)
3) Specific gravity of C3 H mouse liver tissue = 1.086 (from reference # 53)
4) Specific gravity of C 3 H mouse kidney tissue = 1.070 (from reference #53)

$$\left(\frac{147 microCi}{mg}\right)\left(\frac{2.22x10^6 cpm}{microCi}\right)\left(\frac{1mg}{10^6 ng}\right)=\frac{327.5cpm}{ng}(.15\ factor\ for\ counting\ efficiency)$$

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

	30 min post injection		Route	2 hrs post injection	
	Radioactivity	Average		Radioactivity	Average
Liver (nM)	672		IV	489	
	713	692	IV	183	336
	916		IP	366	
	814	865	IP	407	387
Kidney (nM)	703		IV	479	
	1410	1055	IV	261	361
	1144		IP	361	
	996	1075	IP	482	422
Blood (nM)	187		IV	76.6	
	188	187	IV	104	90.4
	325		IP	93.2	
	305	315	IP	78.5	91.3

14.2 FIGURES


1 A
A549
Calu-6
SAEC
EsC1
EsC2
HET1A
Relative Copy #/β-actin x 10^4
CSC (%)
Calu-6 NM
Calu-6 1% CSC
B
A549
Calu-6
Verapamil
+
-
NM
CSC
0.03%
0.23%
0.05%
0.06%
0.04%
0.95%
0.03%
0.98%
Fold change
0
0.5
0.3 (% CSC)
A549
Calu-6
C
A549
Calu-6
Fold change
nonSP
SP
NM
CSC
D
Calu-6
EsC1
CSC (%)
Benzopyrene (μM)
3-MC (μM)
TCDD (nM)
NM
CSC
Resveratrol

14.2.1 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, EsCl 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.

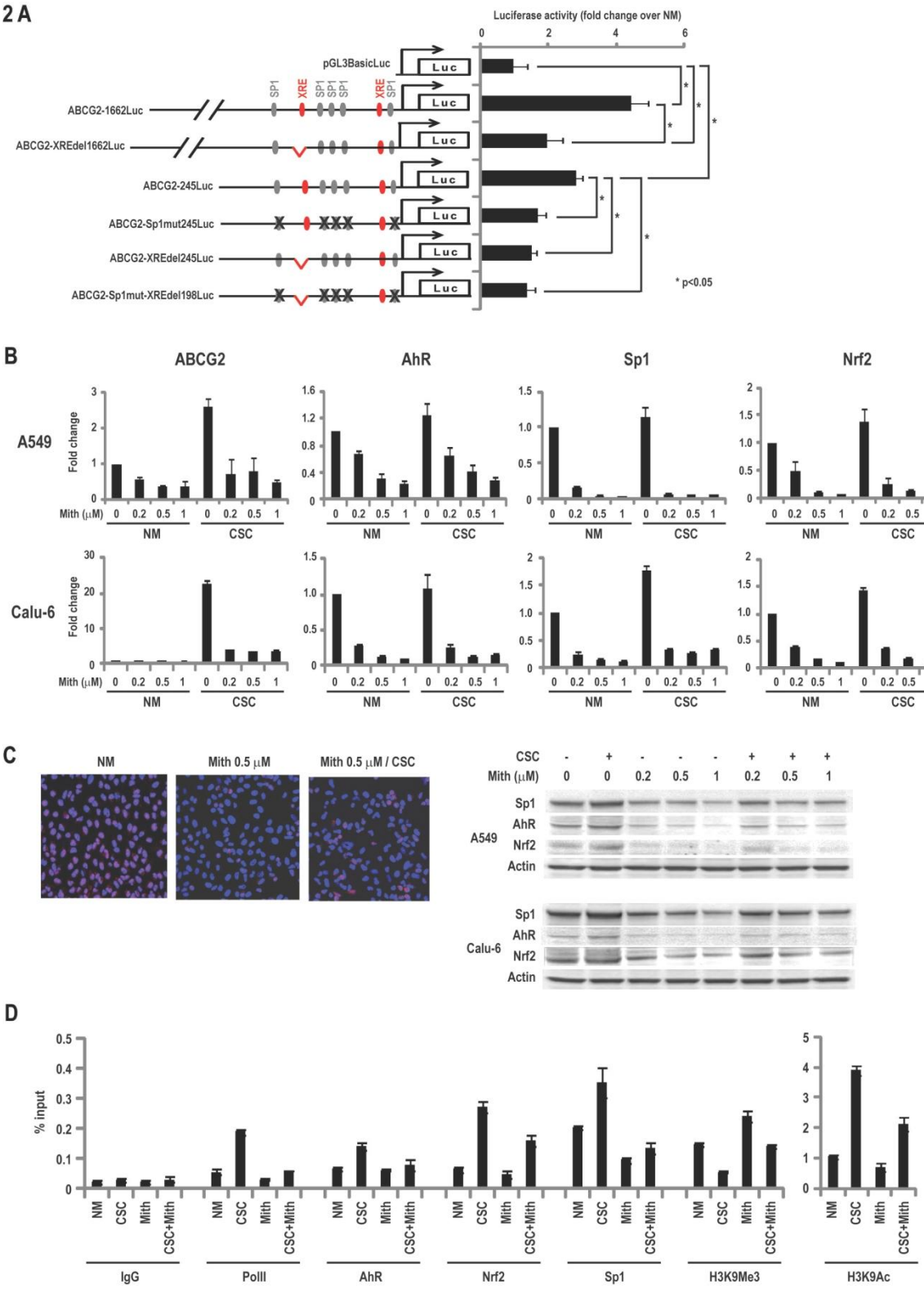
B. Left panel: Representative flow cytometry analysis demonstrating that CSC increases SP fraction in Calu-6 and A549 lung cancer cells. The effects of CSC were more pronounced in Calu-6 relative to A549 cells, exhibiting low vs. high endogenous *ABCG2* levels, respectively.

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.



14.2.2 Figure 2: Role of AhR, Sp1 and Nrf2 in ABCG2 Activation by CSC.

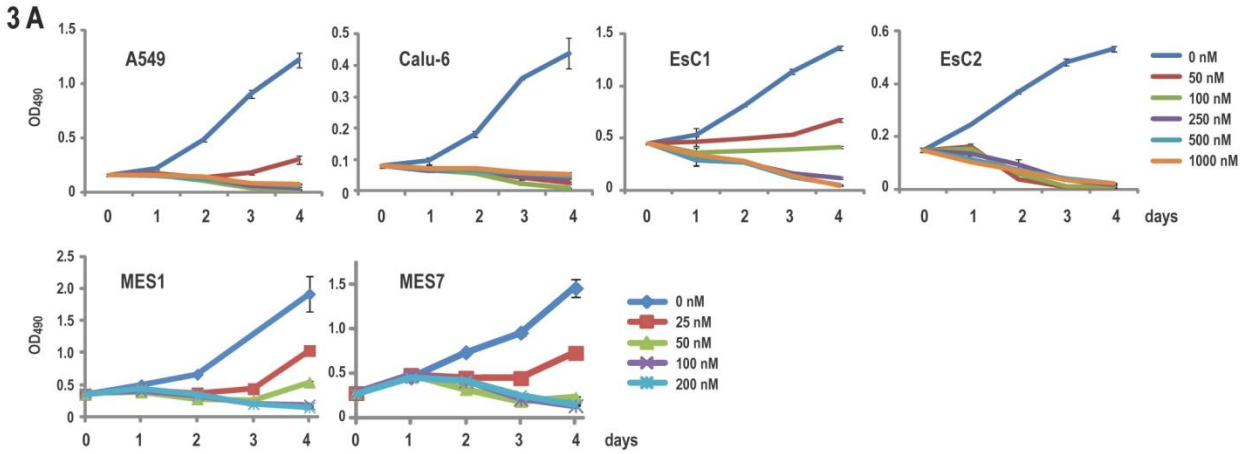
A. Luciferase activity of *ABCG2* promoter reporter constructs following transient transfection into Calu-6 cells. Relative to full length promoter (*ABCG2*-1662-LUC), luciferase activities of *ABCG2* promoter constructs decreased following serial deletions or mutations of XRE and Sp1 elements.

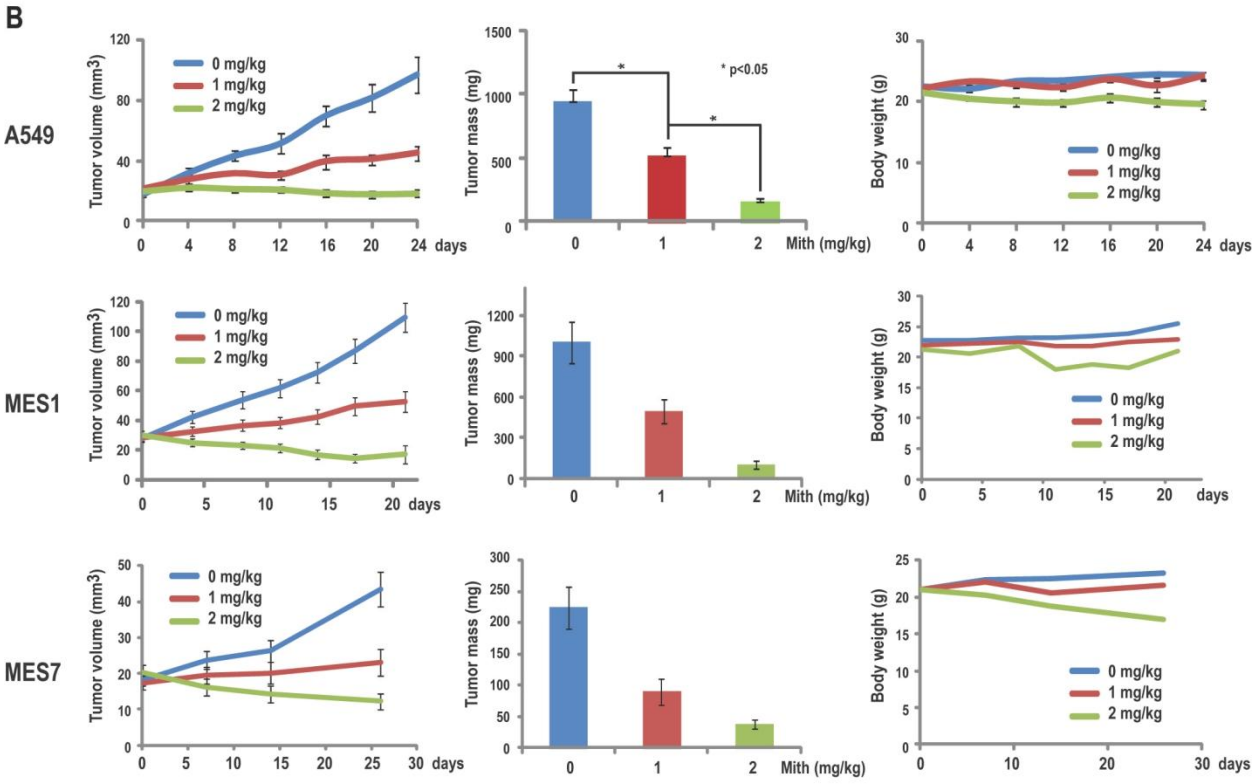
B. qRT-PCR analysis of *ABCG2*, *AhR, Sp1*, and *Nrf2* expression in A549 and Calu-6 cells cultured in NM with or without mithramycin in the presence or absence of CSC. See text for details.

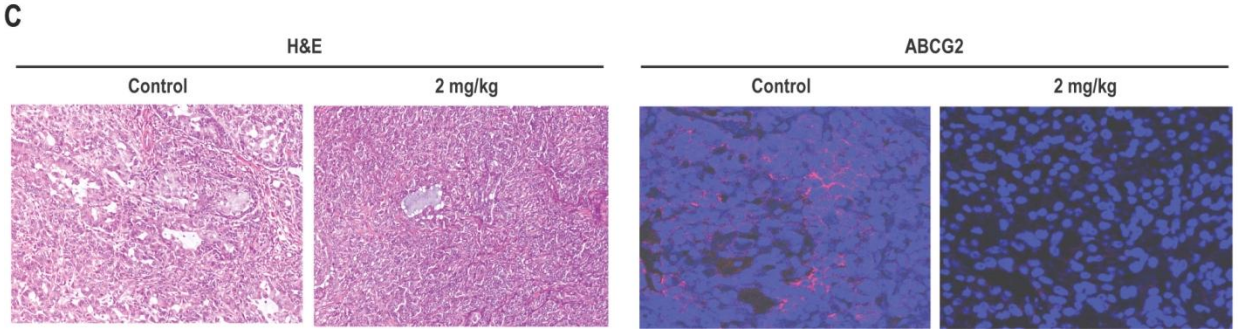
C. Left panel: Immunofluorescence analysis of *ABCG2* expression in A549 cells cultured in the presence or absence of mithramycin with or without CSC.

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.


3 A
A549
Calu-6
EsC1
EsC2
MES1
MES7
OD490
days
0 nM
50 nM
100 nM
250 nM
500 nM
1000 nM
0 nM
25 nM
50 nM
100 nM
200 nM


B
A549
MES1
MES7
Tumor volume (mm3)
Tumor mass (mg)
Body weight (g)
0 mg/kg
1 mg/kg
2 mg/kg
* p<0.05
Mith (mg/kg)
days

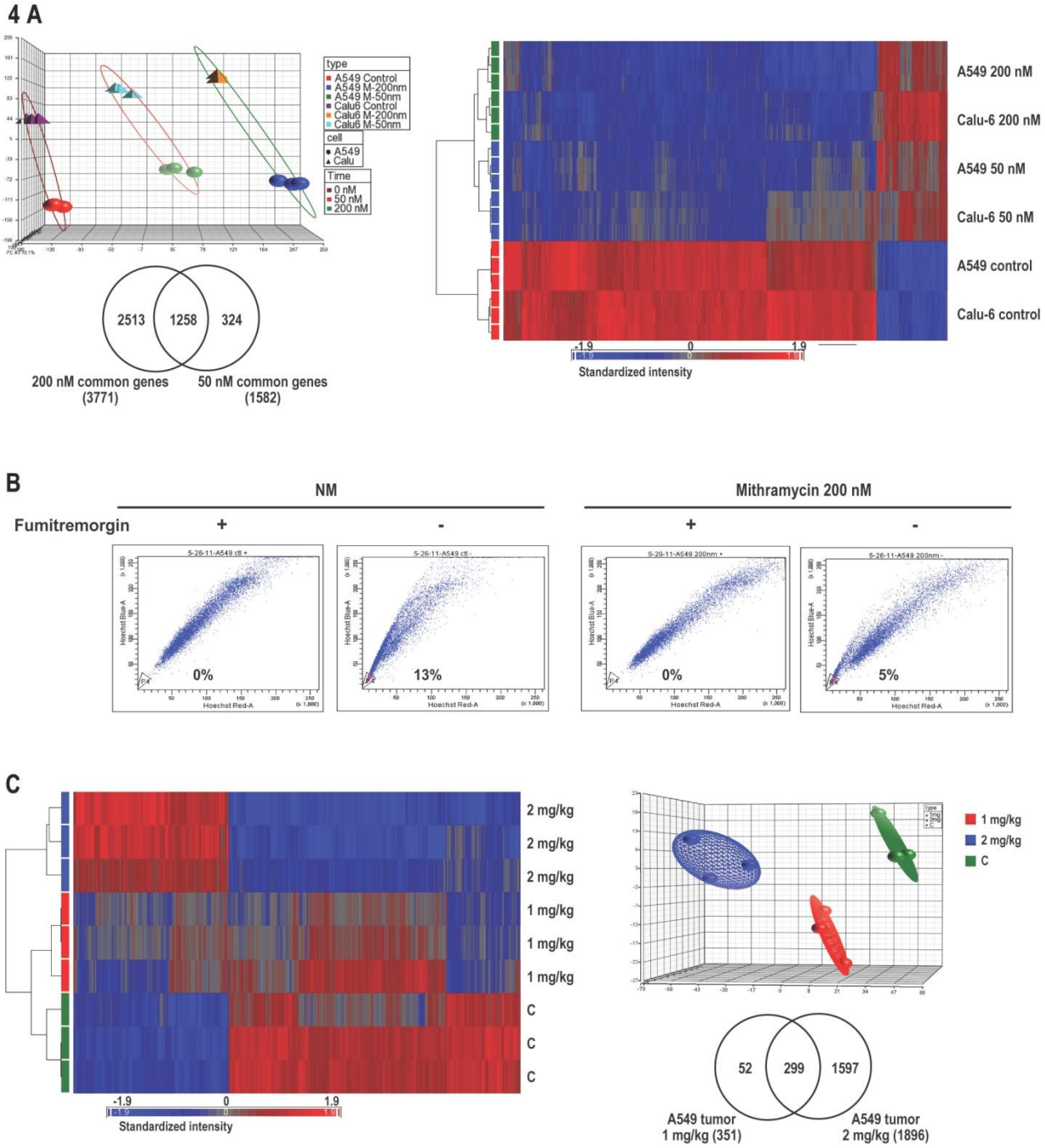

C
H&E
ABCG2
Control
2 mg/kg
Control
2 mg/kg

14.2.3 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.



14.2.4 Figure 4: Microarray Analysis of Mithramycin Effects on Gene Expression Cultured A549 and Calu-6 Cells, and A549 Xenografts Related to Respective Controls.

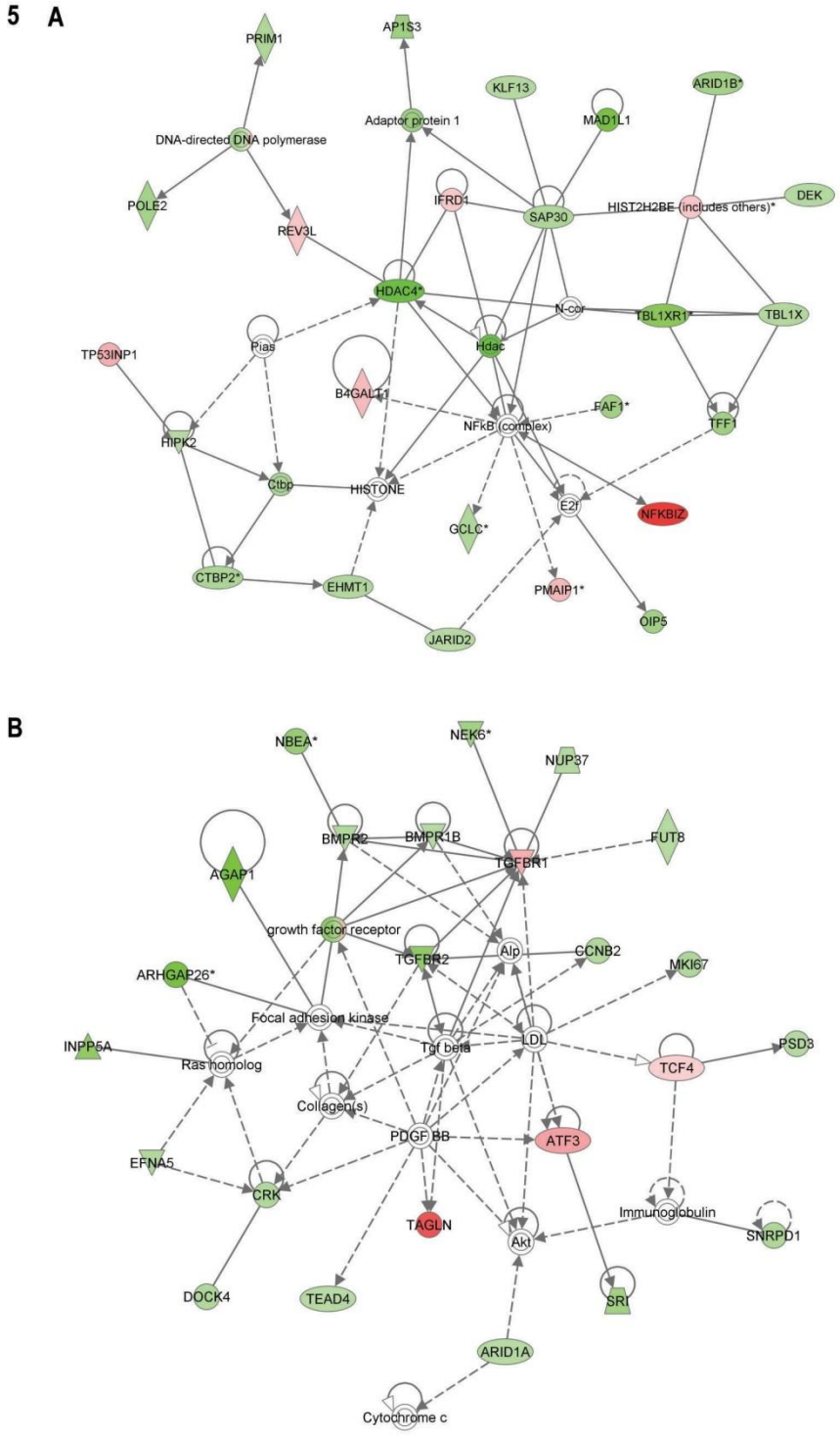
A. Left panel (bottom): Venn diagram demonstrating overlap of genes simultaneously modulated in A549 and Calu-6 cells under two in-vitro exposure conditions.

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.

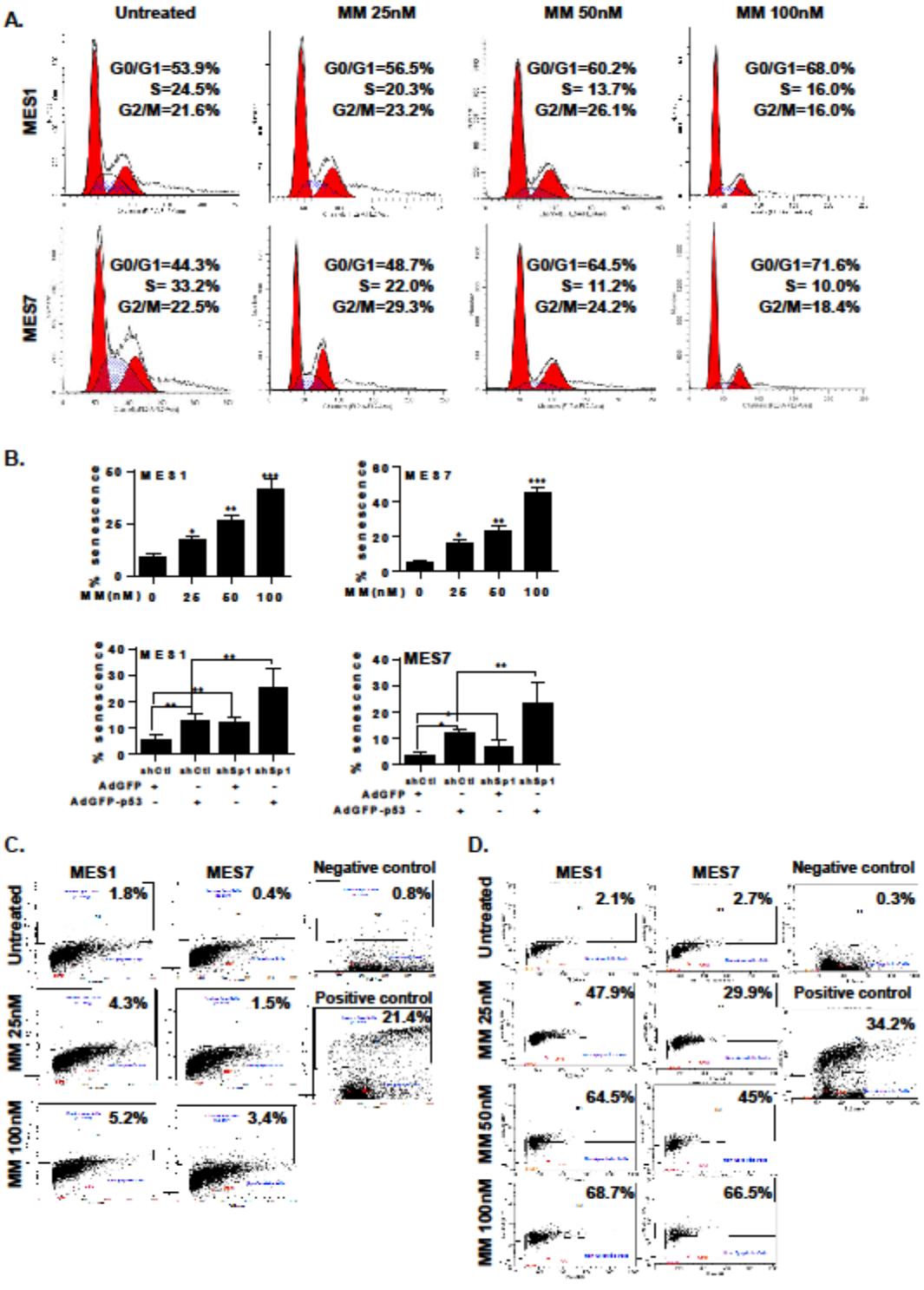
C. Left panel: Heat map revealing dose-dependent modulation of gene expression by mithramycin in tumor xenografts.

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.



14.2.5 Figure 5: Ingenuity Pathway Analysis Depicting Representative Cancer Network Targeted by Mithramycin in Tumor Xenografts Following Systemic Administration of Mithramycin in Athymic Nude Mice.

Figure 6


A.
Untreated
MM 25nM
MM 50nM
MM 100nM
MES1
MES7
G0/G1=53.9%
S=24.5%
G2/M=21.6%
G0/G1=56.5%
S=20.3%
G2/M=23.2%
G0/G1=60.2%
S= 13.7%
G2/M=26.1%
G0/G1=68.0%
S= 16.0%
G2/M=16.0%
G0/G1=44.3%
S= 33.2%
G2/M=22.5%
G0/G1=48.7%
S= 22.0%
G2/M=29.3%
G0/G1=64.5%
S= 11.2%
G2/M=24.2%
G0/G1=71.6%
S= 10.0%
G2/M=18.4%
B.
% senescence
MES1
MES7
MM(nM) 0 25 50 100
shCtl shCtl shSp1 shSp1
AdGFP + - + -
AdGFP-p53 - + - +
C.
MES1
MES7
Negative control
Positive control
Untreated
MM 25nM
MM 100nM
1.8%
0.4%
0.8%
4.3%
1.5%
21.4%
5.2%
3.4%
D.
MES1
MES7
Negative control
Untreated
MM 25nM
MM 50nM
MM 100nM
2.1%
2.7%
0.3%
47.9%
29.9%
34.2%
64.5%
45%
68.7%
66.5%

14.2.6 Figure 6: Effects of MM on Cell Cycle Progression, Senescence and Apoptosis in MPM Cells (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

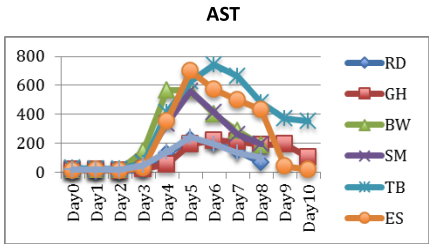
A. Propidium iodide staining demonstrating that 24 hour MM treatment induces dose dependent G0/G1 arrest in MPM cells.

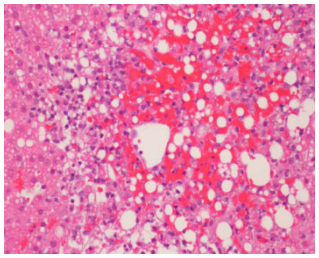
B. ß-galactosidase staining assays demonstrating that 24 hour MM treatment induces dose dependent senescence in MES1 and MES7 cells.

C. and **D.** Apo-BrdU analysis demonstrating minimal apoptosis in MPM cells immediately following 24 hour MM exposure (C), but significant dose-dependent apoptosis 48 hours following drug treatment (D).

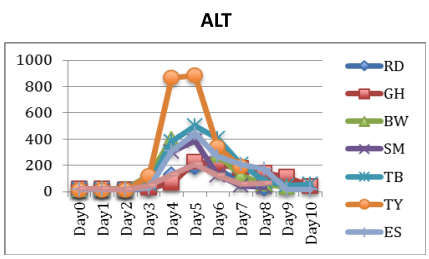
7A

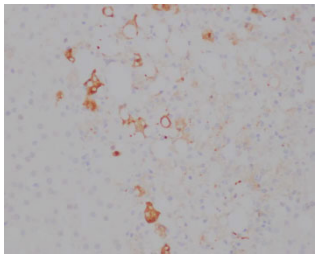
Mithramycin Induces Hepatotoxicity in Some Cancer Patients





H&E

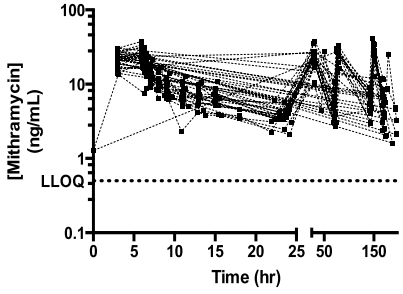


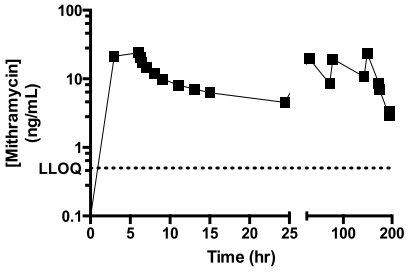


Cleaved Caspase 3

7B

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



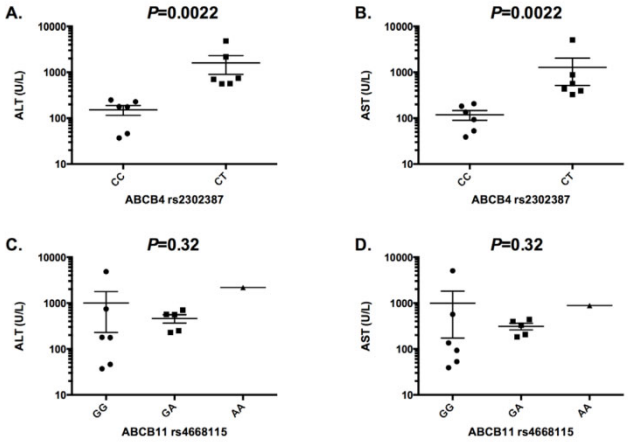


- **Peak and steady state concentrations were lower than predicted**
- **No significant differences in PK were apparent in patients with or without hepatotoxicity**

14.2.7 Figure 7: AST and ALT Levels and Representative Liver Biopsy from Patients with Mithramycin Mediated Hepatotoxicity. Transaminitis was Associated with Apoptotic Hepatocyte Death.

8A

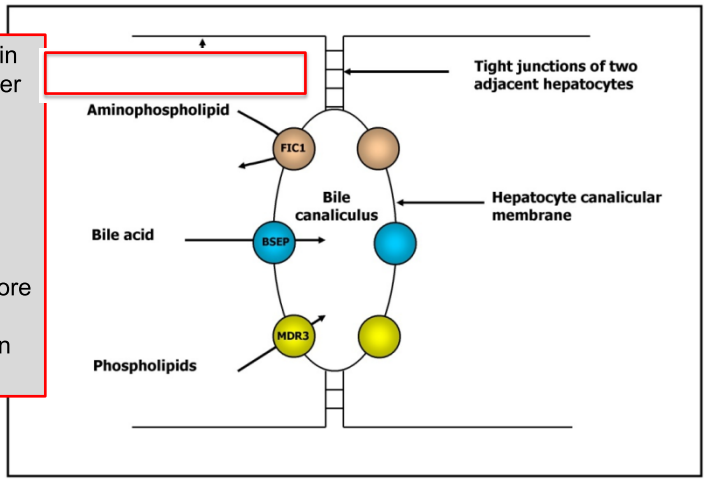
Mithramycin-Induced Hepatotoxicity Correlates with ABCB4 SNP



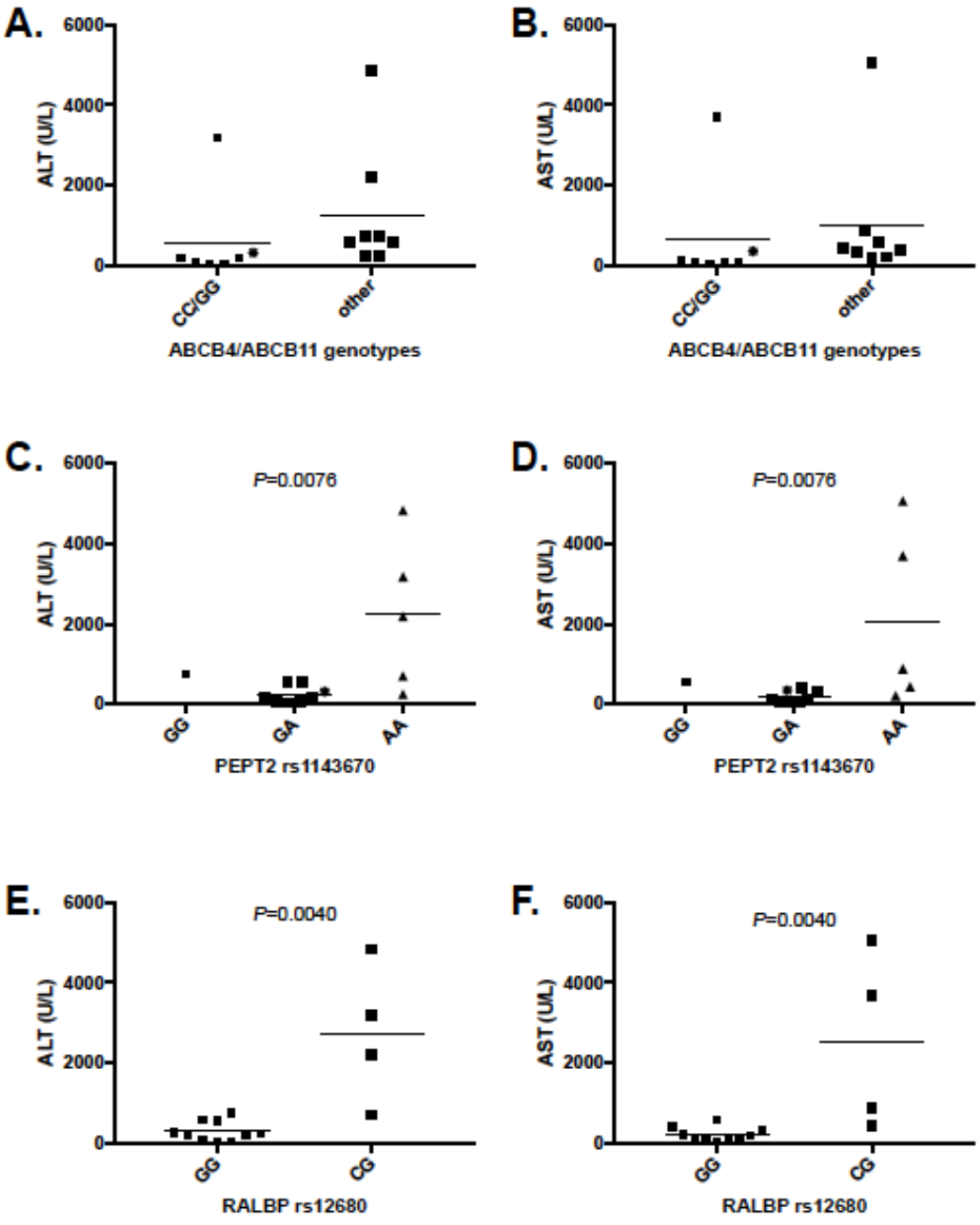
8B

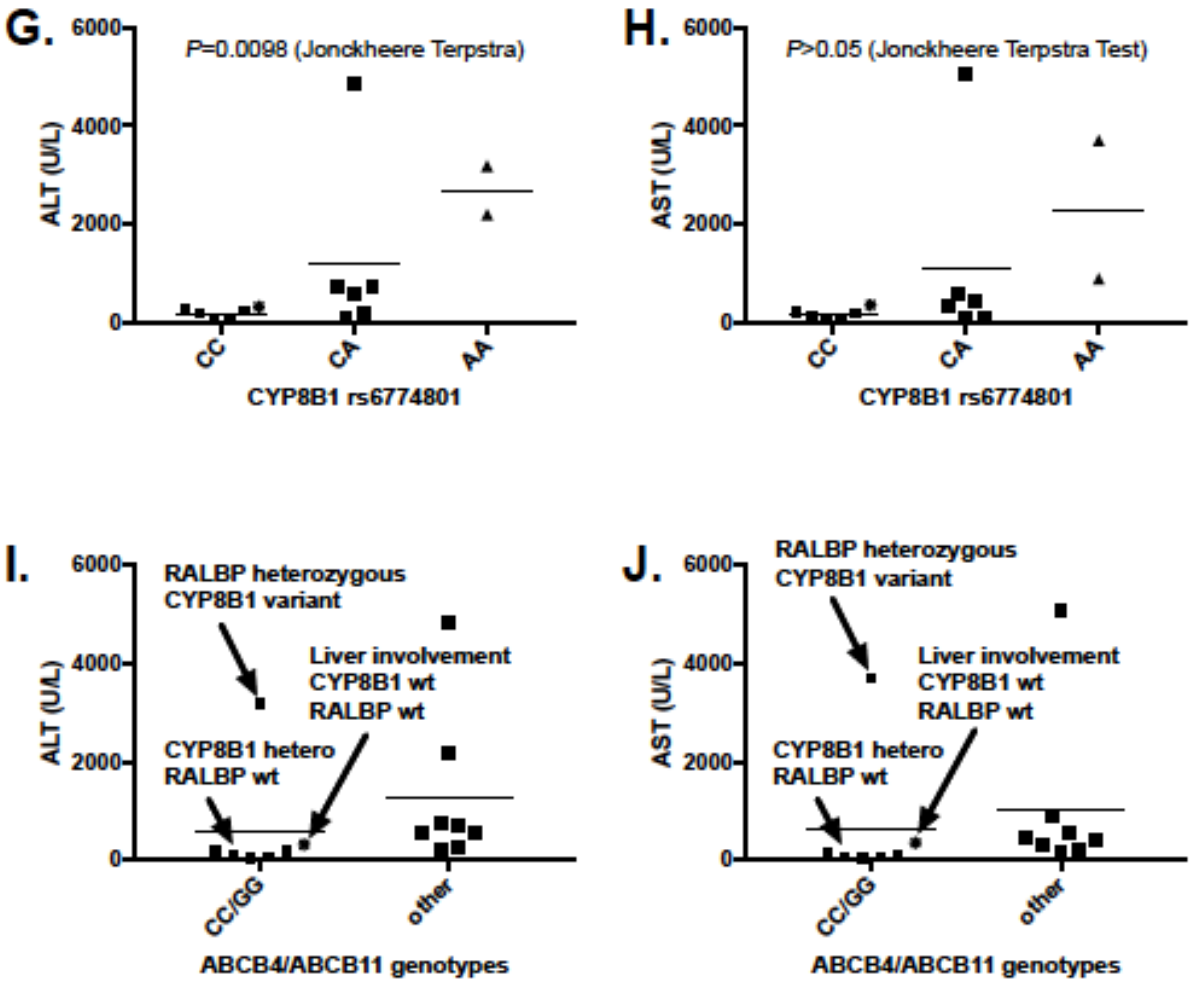
ABCB4 and *ABCB11* Regulate Bile Acid Flow

Mithramycin is a member of a rare class of drugs that impedes sinusoidal blood flow and therefore causes central vein toxicity.



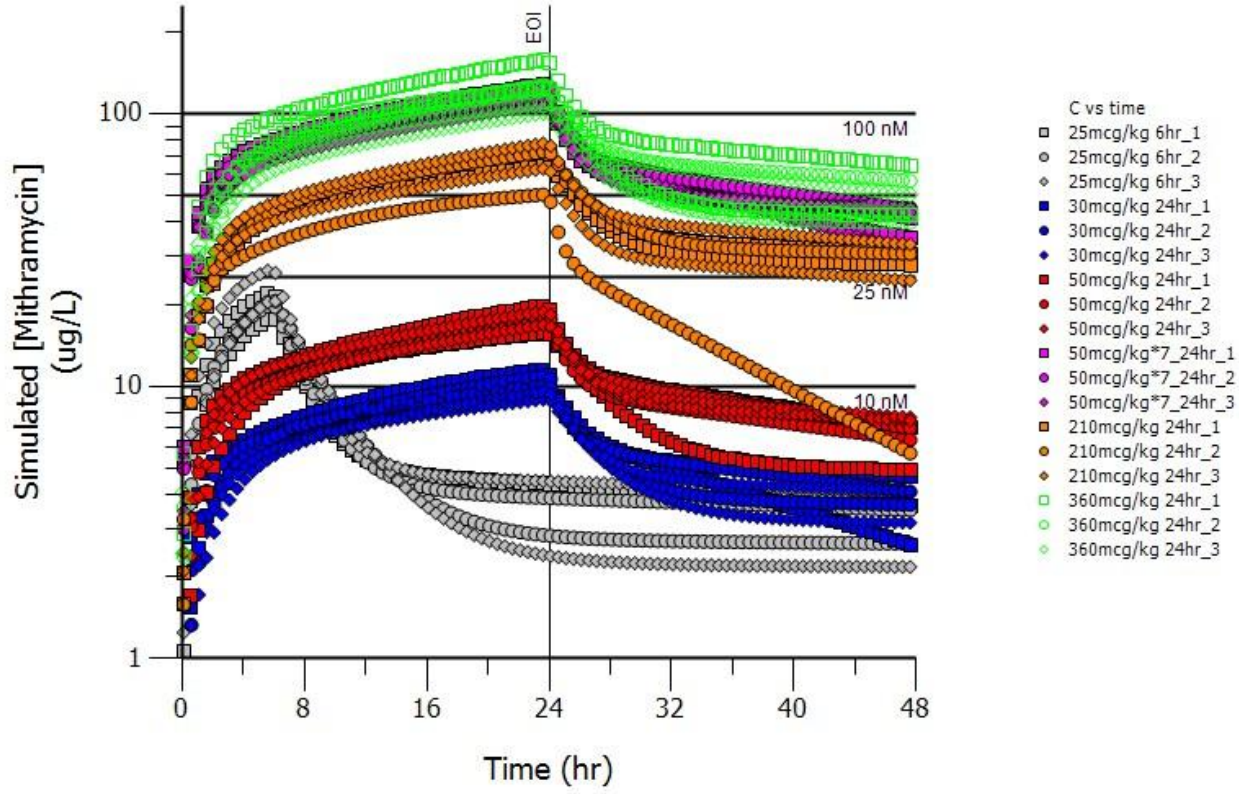
14.2.8 Figure 8: Results of DMET Analysis Demonstrating that Mithramycin Mediated Transaminitis Correlates with SNPs in ABCB4 and ABCB11.


A.
ALT (U/L)
6000
4000
2000
0
CC/GG
other
ABCB4/ABCB11 genotypes
B.
AST (U/L)
6000
4000
2000
0
CC/GG
other
ABCB4/ABCB11 genotypes
C.
P=0.0076
ALT (U/L)
6000
4000
2000
0
GG
GA
AA
PEPT2 rs1143670
D.
P=0.0076
AST (U/L)
6000
4000
2000
0
GG
GA
AA
PEPT2 rs1143670
E.
P=0.0040
ALT (U/L)
6000
4000
2000
0
GG
CG
RALBP rs12680
F.
P=0.0040
AST (U/L)
6000
4000
2000
0
GG
CG
RALBP rs12680

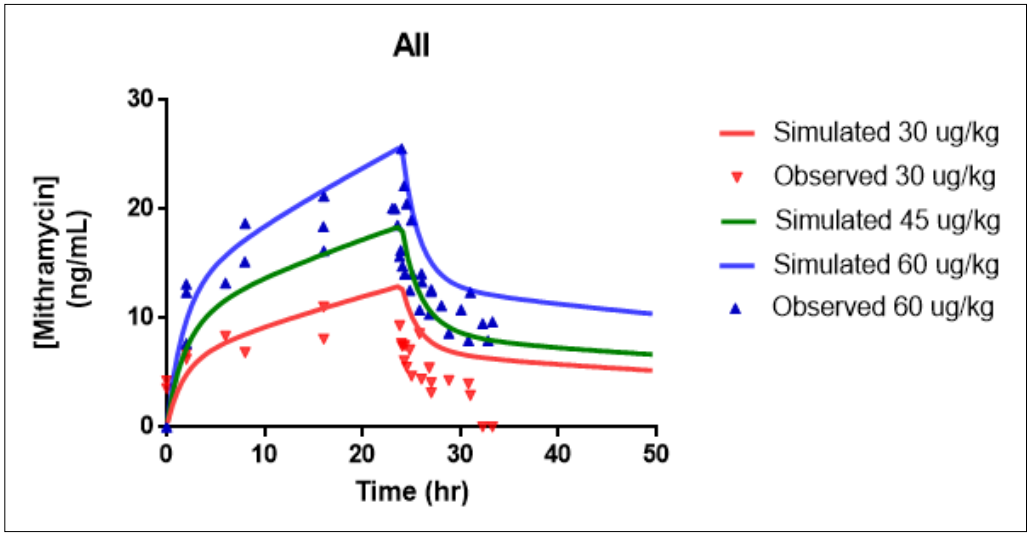


14.2.9 Figure 9: Results of DMET Analysis Depicting Associations Between AST and ALT Elevations with SNPs in ABCB4, ABCB11, PEPT2, and RALB from Patients Receiving Mithramycin.

Estimated Mithramycin Plasma Concentrations during and after 24h Infusion



14.2.10 Figure 10: Simulated Mithramycin Plasma Levels During and After 24 Hour Infusion Based on Direct Quantitation of Mithramycin Plasma Levels from the Recent Phase II Mithramycin Trial (Grey Dots).



14.2.11 Figure 11: Observed and Predicted Mithramycin Plasma Levels Following 24 Hour Mithramycin Infusions.

14.3 APPENDICES

14.3.1 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 (82)	1985	NONE								
Kofman (42) "Tx of Disseminated."	1963	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, restlessne irritability	" a surprising factor was severe cumulative toxicity -again hemorrhage on second course with PLT >50	50 ug/kg tried	
Dutcher (72)	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	1 patient removed for "hepatotoxicity" not defined	Not defined	Not defined	
Thurlimann, B (79)	1992	27-74 (median 62)	Hypercalce mia	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%			

Author	Year	Patient Age	Disease	Response Rate	Dose	Schedule	Toxicity In Cycle 1	Toxicity Subsequent Cycles	MTD?	PK
Lebbin, D (75)	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	-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.		
						B1 = twice weekly for 5 months				
Johnson, P.R.E (83)	1991	28-57	CML in blast crisis	9 Patients -1 returned to chronic phase	25 ug/kg + hydroxyurea	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 (84)	1968	42-83 (mean61)	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		

Author	Year	Patient Age	Disease	Response Rate	Dose	Schedule	Toxicity In Cycle 1	Toxicity Subsequent Cycles	MTD?	PK
Sewell, I. A.(69)	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 (71)	1965	26-64	Glioblastoma (8 patients with improvements)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			^{3}H drug crosses BBB ****
Walker (67)	1976	Median age 53 years	Anaplastic gliomas -no difference in survival	96 patients at NIH 116 total 58 got mithra -tx with surgery + randomized to mithra +/- radiation	25 ug/kg/day over 6-8 hours	Daily times 21 -then 6 wks later times 12 -then 6 wks later daily times 12 -83% got 11 doses -median is	-N/V in 58% of patients -mucositis -anemia (60%) mild to moderate -leukopenia (20%) -thrombocytopenia -LFTs-mild 30%, moderate50% in ALT/AST -hemorrhage into tumor bed in 3 patients- 2 DEATHS -chart summarizing tox			Discussion of PK data at the end of the paper -crosses BBB but should not (inhibits MDR1)
Kofman "metastatic ewing;s (73)	1973	Two reported 21 and 23 yo	ESFT	5 patients 1 durable CR	25 ug/kg/day as 8-24 hour infusion NOTE ORAL INACTIVE	-q3wks total of 6-10 courses	Thrombocytopenia Fever with infusion			

Author	Year	Patient Age	Disease	Response Rate	Dose	Schedule	Toxicity In Cycle 1	Toxicity Subsequent Cycles	MTD?	PK
Kofman, s (70)	1964	22 10 15 Much younger 3 16 yo 2 23 yo	? of redundant patients 3 ESFT reported (2 repeats)				Not systematically reported -thrombocytopenia seen			
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, bronchogenic CA, hepatpma	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
Package insert Pfizer (66)	1971	1600 patients	Testicular CA		25 ug/kg/day over 12	Q month	Contraindicated: thrombocytopenia, coagulopathy, poor bone marrow, follow PLTs, PT and bleeding time			

Author	Year	Patient Age	Disease	Response Rate	Dose	Schedule	Toxicity In Cycle 1	Toxicity Subsequent Cycles	MTD?	PK
					hours daily x 8 q month dilute in 1L of d5 and give over 4-6hrs		-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			

14.3.2 Appendix 2: Study Calendar

			Cycle 1								
	Screening Evaluation	Baseline Evaluation	Week 1							Week 2	
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 11
Mithramycin			x								
Histologic path confirmation	x										
Informed consent	x										
Medical history	x	x[k]									
Concurrent meds	x										
Physical examination	x	x[k]	x[a]								
Performance status	x	x[k]	x[a]								
CBC and chemistries[b]	x	x[k]	x	x	x	x	x				
Bile acids	x	x[k]	x[c]				x[c]				
Urinalysis, thyroid panel, ABG, PFTs	x	x[k]									
HIV, HBV, HCV serologies	x										
DMET genotyping	x										

			Cycle 1								
	Screening Evaluation	Baseline Evaluation	Week 1							Week 2	
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 11
(on study 06C0014)											
Coagulation Labs[b]	x	x[k]	x	x	x	x	x				
Pregnancy test	x	x[k]									
Cardiac function tests	x										
Disease assessment (imaging)	x	x[k]									
US liver elastography	x	x[k]				x[c]					
Adverse events			←					→			
Tumor biopsy[e]	x	x[k]				x[e]					
Research labs	x	x[k]	x			x[c]					
PK[f]			x	x							
EKG	x	x[k]	x[g]	x[j]	x	x	x				

	Cycle 2 and beyond*										Follow-up Evaluations[h]
	Week 1							Week 2			
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 11	Day 14	
Mithramycin	x										

	Cycle 2 and beyond*										Follow-up Evaluations[h]
	Week 1							Week 2			
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 11	Day 14	
Medical history											x
Concurrent meds											
Physical examination	x[a]										
Performance status	x[a]										
CBC and chemistries[b]	x	x	x	x	x						x
Bile acids	x									x[i]	
Coagulation Labs[b]	x	x	x	x	x						x
Disease assessment (imaging)										x[i]	x
US liver elastography	x[d]									x[i]	
Adverse events	←								→		
Tumor biopsy[e]											
Research labs	x										x
EKG	x[gj]	x[gj]									

[a] Within 4 days prior to study drug administration in each cycle. Cycle 1 assessment need not be completed if baseline evaluation completed in appropriate timeframe.

[b] See Section **2.1.3** for screening chemistries and coagulation labs. See Section **3.5.1** for chemistries and coagulation labs collected during the study. See Section **3.5.4** for chemistries and coagulation at follow-up.

Note: Day 1 labs can be drawn on the day prior to treatment infusion. In between treatments, monitoring lab evaluations may be drawn twice weekly (+48 hours between labs). For patients not experiencing Grade 3 or 4 toxicities these labs may be drawn once weekly in between treatments per PI discretion instead. (See Section **3.5.2**)

[c] Cycle 1 only (+/- 3 days).

	Cycle 2 and beyond*										Follow-up Evaluations[h]
	Week 1							Week 2			
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 11	Day 14	

[d] Cycle 2 and beyond, on Day 1 +/- 3 days.

[e] Baseline biopsies may be obtained at any point prior to treatment (pre-infusion) either on this protocol or from clinically indicated procedures (surgeries and/or biopsies) obtained on 06C0014. If sufficient tissue is available from a biopsy performed at a previous time at NIH or elsewhere, the baseline biopsy may be omitted.

The Cycle 1 Day 4 (+/- 3 days) biopsy is required.

If possible, an optional biopsy may be taken at treatment evaluation following Course 1. Should a patient experience a dramatic clinical response to treatment, additional optional tumor tissue biopsies may also be requested but are not required (refer to **5.2**). These biopsies will only be performed if they are easy to obtain and pose little risk to the patient.

Note: CT-guided research biopsies are not to total more than 5 in one year on this study.

[f] See **Appendix 3** for PK timepoints.

[g] Done within 2 hours pre-infusion.

[h] After completion of last treatment cycle, CBC chemistries and coagulation studies are monitored weekly until resolution of toxicities; Patients with stable disease or responding to therapy will undergo CBC, chemistries, coagulation studies, history, physical exam and tumor measurements (CT scan of the chest, abdomen and pelvis, PET/CT and brain MR without gadolinium contrast) every 2 months until off study criteria are met.

[i] End of course. 1 course = 4 cycles. Evaluation performed within +/- 2 weeks of end of course. At the end of course radiographic evaluation: CT scan (C/A/P) (with standard oral/IV contrast), PET/CT scan, and brain MR without gadolinium contrast; tumor measurements will be performed after every course of therapy (four cycles, 8 weeks +/- 2 weeks).

If the patient cannot return to the Clinical Center for end of treatment visit, a request will be made to collect required clinical labs (refer to **3.5.4**) from a local physician or laboratory. If this is not possible, patients may be assessed by telephone/or other NIH approved remote platforms for symptoms.

[j] Done within 2 hours post-infusion.

[k] Baseline assessment need not be completed if screening evaluation completed in appropriate timeframe. Baseline radiographic evaluation for assessment of disease sites within 4 weeks prior to treatment: CT scan (C/A/P) (with standard oral/IV contrast), PET/CT scan, and brain MR without gadolinium contrast.

*Patients will continue to receive treatment until criteria for removal from protocol or off-study criteria are met (see Section **3.6**).

14.3.3 Appendix 3: Sample Pharmacokinetic and EKG Worksheet for Mithramycin

14.3.3.1 Cycle 1*

TEL Research Sample # (2 10 mL red top tubes & 2 10 mL lavender top tubes)	**Coag Reference Sample # (obtain kit from TEL)**	**PK Sample # (2 3 mL lavender top tubes)**	**Hour (pre dose = within 1 hour prior to infusion)**	**Target Time**	**Actual Time**	**EKG Time**	**Initials**
C1D1	*(date)*						
R1	**C 1**	**PK 1**	pre dose				
n/a	n/a	**PK 2**	2h post start of infusion				
n/a	n/a	**PK 3**	8h post start of infusion				
n/a	n/a	**PK 4**	16h post start of infusion				
C1D2	n/a	**PK 2**	Just prior to the end of the 24 hour infusion				
n/a	n/a	**PK 3**	0.25h post-infusion				
n/a	n/a	**PK 4**	0.5h post infusion				
n/a	n/a	**PK 5**	1h post infusion				
n/a	n/a	**PK 6**	2h post infusion				
n/a	n/a	**PK 7**	3h post infusion				
n/a	n/a	**PK 8**	5h post infusion				
n/a	n/a	**PK 9**	7h post infusion				
n/a	n/a	**PK 10**	9-12h post infusion				
C1D4	*(date)*						
R2	n/a						

***Note:** If a dose modification is implemented (Section 3.3), the patient's PKs at the start of the next treatment cycle may be collected starting with Cycle 1 Day 1 of the PK calendar (refer to Section 3.5.3).

14.3.3.2 Cycle 2

TEL Research Sample # (2 10 mL red top tubes & 2 10 mL lavender top tubes)	**Coag Reference Sample # (obtain kit from TEL)**	**PK Sample # (2 3 mL lavender top tubes)**	**Hour (pre dose = within 1 hour prior to infusion)**	**Target Time**	**Actual Time**	**EKG Time**	**Initials**
C2D1	*(date)*						
R3	**C 3**	**PK 1**	pre dose				
C2D2	*(date)*	**PK 2**	Just prior to the end of the 24 hour				

14.3.3.3 Cycle 3

TEL Research Sample # (2 10 mL red top tubes & 2 10 mL lavender top tubes)	**Coag Reference Sample # (obtain kit from TEL)**	**PK Sample # (2 3 mL lavender top tubes)**	**Hour (pre dose = within 1 hour prior to infusion)**	**Target Time**	**Actual Time**	**EKG Time**	**Initials**
C3D1	*(date)*						
R4	**C 4**	**PK 1**	pre dose				
C3D2	*(date)*	**PK 2**	Just prior to the end of the 24 hour				

14.3.3.4 Cycle 4

TEL Research Sample # (2 10 mL red top tubes & 2 10 mL lavender top tubes)	**Coag Reference Sample # (obtain kit from TEL)**	**PK Sample # (2 3 mL lavender top tubes)**	**Hour (pre dose = within 1 hour prior to infusion)**	**Target Time**	**Actual Time**	**EKG Time**	**Initials**
C4D1	*(date)*						
R5	**C 5**	**PK 1**	pre dose				
C4D2	*(date)*	**PK 2**	Just prior to the end of the 24 hour				

14.3.3.5 Instructions

PK Studies (PK)

Collect 6 mL blood in 2 lavender top (3 mL) 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. Tubes should then be placed on crushed ice, and samples should be centrifuged for 15 minutes at approximately 1000 x g at 0-5 °C within 2 hours after collection. The plasma should be transferred to separate pre-labeled screw-capped polypropylene transfer tubes and stored at -80 °C until sent to the analytical site (Figg Lab).

Research Labs (R)

Samples will be collected in 2 red top (10 mL) and 2 lavender top (10 mL) tubes for PD studies and PBMC, and transported on wet ice within 1 hour to the Thoracic Epigenetics Lab (TEL).

Research Labs will be collected at the following timepoints (see also **Appendix 2: Study Calendar**):

- Baseline (once), see Section **3.5.1**;
- Within 72 h **prior to starting each infusion cycle**;
- Day 4 of Cycle 1 (+/- 3 days); and at
- Treatment evaluation.

Coagulation Studies (C)

One blue top tube transported to the TEL lab within 1 hour. Samples (1 mL of plasma) will be obtained, filtered through a 22 micron filter (Kit available in TEL) and stored at -80° C, to be saved for future clinical reference as needed.

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 Thoracic Epigenetics Lab 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, the RN (or designee) will place a label on the tube and write the sample reference # on the label on the tube. When the Thoracic Epigenetics Lab personnel or fellow processes the sample, the TEL personnel 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. TEL will retain one copy for their records and

will send the other copy with the PK samples to the Figg Lab. **Note:** Prior to sending the PK sheets to the Figg Lab, the patient name should be crossed out.

14.3.4 Appendix 4: Sample Patient Log for Reactions/Symptoms

NAME: _____ Cycle: _____

Dates of last Mithramycin infusion: _____

Reaction/Symptom	**Start Date**	**End Date**

*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:_____

14.3.5 Appendix 5: Sample Patient Log for Concomitant Medication

NAME: _____ Cycle: _____

Dates of last Mithramycin infusion: _____

Medication Taken	**Start Date**	**End Date**

*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:_____

14.3.6 Appendix 6: 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, CO2, creatinine, glucose, BUN, albumin, calcium, magnesium, LDH, phosphorus, uric acid)*
    - **LFT** *(include alk phos, ALT/AST, Total bilirubin, Direct bilirubin, Total protein)*
    - **Coagulation profiles** – *PT/PTT, fibrinogen*

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 or 240-760-6234, or email tricia.kunst@mail.nih.gov

For urgent issues, contact the Attending Physician or 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^{2+} and ionized calcium).**

Please contact the NIH page operator at 301-496-1211 and ask for the Thoracic Surgery Attending Physician 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.

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Physician Signature Print Name

NIH/NCI/TSB