

# Amendment

**Protocol Number:** 12C0135-F **Reference Number:** 364514

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**Protocol Title:** Phase I/II Trial of Mithramycin in Children and Adults with Refractory Extracranial Solid Tumors (Phase I) or Ewing Sarcoma and EWS-FLI1 Fusion Transcript (Phase II)

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\*\* I have reviewed this research project and considered the NIH Policy for Inclusion of Women and Minorities in Clinical Research. Taking into account the overall impact that the project could have on the research field involved, I feel the current plans adequately includes both sex/gender, minorities, children, and special populations, as appropriate. The current enrollment is in line with the planned enrollment report for inclusion of individuals on the basis of their sex/gender, race, and ethnicity and is appropriate and of scientific and technical merit.

**IRB Meeting Date:** Expedited

**DEC Clearance Date:** 11/21/2016

**Protocol Version Date:** 11/09/2016

**Abbreviated Title:** Ph I/II Pediatric Mithramycin

**NCI Protocol #:** 12C0135 F

**Version Date:** 11/09/2016

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**Investigational Agent:**

Drug Name:	Mithramycin
IND Number:	115272
Sponsor:	Center for Cancer Research, NCI

## **PRÉCIS**

### **Background:**

- Mithramycin, an anti-tumor antibiotic, underwent broad clinical evaluation in solid tumors and leukemias in the 1960's and demonstrated activity in some leukemias, lymphomas, and solid tumors. In particular, mithramycin was found to have activity against testicular cancers and was briefly used in the clinic for this tumor prior to the development of the currently used treatment regimen.
- The Ewing Sarcoma Family of Tumors (ESFT) is the second most common malignant bone tumor of childhood. There has been very little improvement in overall patient survival in past years, particularly for patients with high risk metastatic or relapsed disease. Therefore, there is a need for effective novel agents for the treatment of this disease.
- Multiple studies have shown that suppressing the expression of EWS-FLI1 effectively limits the tumorigenicity of ESFT cells. Laboratory studies have shown that mithramycin effectively suppresses the activity of EWS-FLI1 both *in vitro* and *in vivo*.

### **Objectives (primary):**

- Phase I portion of this study is to: determine the tolerability, toxicity, and the recommended phase II dose of mithramycin in children and adolescents with refractory extracranial solid tumors.
- Phase II portion of this trial is to: determine the objective response rate (CR and PR) of Ewing sarcoma to mithramycin in children and adults using RECIST criteria when administered at 17.5 microgram/kg over 6 hours once daily for 7 days to be repeated every 28 days until unacceptable toxicity or disease progression.
- Phase II portion of this trial to: evaluate if mithramycin inhibits NR0B1 in tumor tissue and determine changes in gene expression signature pre-treatment and at steady state on day +4 of treatment in patients  $\geq 18$  years old with Ewing sarcoma and EWS/FLI1 fusion transcript with disease amenable to percutaneous biopsy.

### **Eligibility:**

- Phase I Portion: children ( $\geq 12$  months) and adolescents ( $\leq 17$  years) with recurrent or refractory extracranial solid tumors.
- Phase II Portion in adults: adults ( $\geq 18$  years of age at enrollment) with recurrent or refractory measurable extracranial Ewing sarcoma and the EWS-FLI1 fusion transcript.
- Phase II Portion in children and adolescents: Once the adult dose is deemed safe, children ( $\geq 12$  months) and adolescents ( $\leq 17$  years) with recurrent or refractory measurable extracranial Ewing sarcoma and the EWS-FLI1 fusion transcript will begin enrollment to the Phase II portion.
- Participants must meet safety laboratory criteria and prior therapy limitations.

### **Design:**

- Phase I Portion: Mithramycin will be administered in escalating doses to children and adolescents intravenously over 6 hours once daily for 7 days to be repeated every 28 days until unacceptable toxicity or disease progression. Mithramycin will be given with

dexamethasone prophylaxis to ameliorate hepatotoxicity. The cohort at the recommended dose or MTD will be expanded up to 12 patients, and attempts will be made to enroll 6 patients that are  $\geq 12$  years of age and 6 patients that are  $< 12$  years of age to gain experience with a broad age range of patients. A maximum of 18 evaluable patients will be enrolled on the phase I portion.

- Phase II Portion: Using a Simon two stage design, mithramycin will be administered intravenously at 17.5 microgram/kg over 6 hours once daily for 7 days to be repeated every 28 days until unacceptable toxicity or disease progression to children and adults with Ewing sarcoma with EWS-FLI1 fusion transcript. Mithramycin will be given with dexamethasone prophylaxis to ameliorate hepatotoxicity. Up to 24 evaluable patients will be enrolled on the phase II portion.
- The Phase I and Phase II portions of the protocol will enroll patients simultaneously.

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## **1 INTRODUCTION**

### **1.1 STUDY OBJECTIVES**

#### 1.1.1 Primary Objectives

- 1.1.1.1 Phase I portion of this study is to: determine the tolerability, toxicity, and recommended dose of mithramycin in children and adolescents with refractory extracranial solid tumors.
- 1.1.1.2 Phase II portion of this trial is to: determine the objective response rate (CR and PR) of Ewing sarcoma with EWS-FLI1 fusion transcript to mithramycin in children and adults using RECIST criteria when administered at 17.5 microgram/kg over 6 hours once daily for 7 days to be repeated every 28 days until unacceptable toxicity or disease progression. If the pediatric phase II dose of mithramycin differs from the adult phase II dose, the protocol will be amended to address accrual and response rate to mithramycin in children in a separate stratum.
- 1.1.1.3 Evaluate if mithramycin inhibits NR0B1 in tumor tissue, and determine changes in EWS/FLI1 gene expression signature by obtaining mandatory tumor biopsies pre treatment and at steady state on day +4 of treatment in patients  $\geq 18$  years old with Ewing sarcoma and EWS/FLI1 fusion transcript and disease amenable to percutaneous biopsy (phase II).

#### 1.1.2 Secondary Objectives

- 1.1.2.1 Describe pharmacokinetic parameters for each dose level using non-compartmental methods (phase I and II)
- 1.1.2.2 Evaluate NR0B1 expression in patients with Ewing sarcoma using immunohistochemistry on archival tumor tissue, if available (phase I and II)
- 1.1.2.3 To prospectively evaluate the feasibility and utility of volumetric analysis of target lesions, and descriptively compare volumetric measurements to 1D (RECIST), and 2D (WHO) measurements (phase I and II).
- 1.1.2.4 Define preliminarily time to progression (TTP) in patients with Ewing sarcoma (phase II)

### **1.2 BACKGROUND AND RATIONALE**

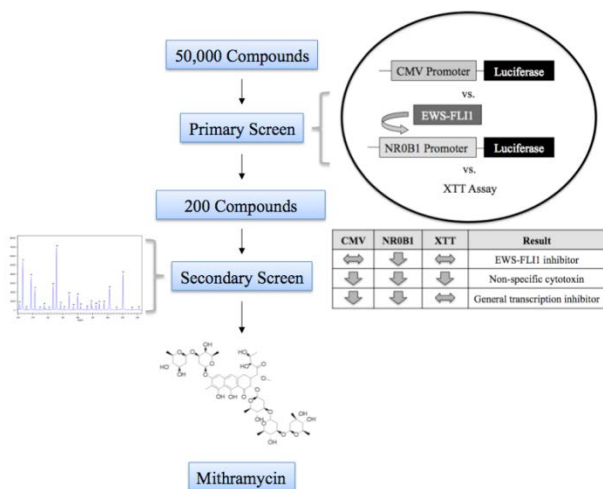
#### 1.2.1 Ewing Sarcoma Family of Tumors (ESFT)

The Ewing Sarcoma Family of Tumors (ESFT) is the second most common malignant bone tumor of childhood (1, 2). In the past 25 years since the advent of 5-drug ESFT therapy, there has been very little improvement in overall patient survival particularly for patients with high risk metastatic or relapsed disease (1). Therefore, there exists a great need for agents that target the genes this tumor depends on for survival.

All ESFT tumors have fusion oncogenic transcription factors generated by chromosomal translocations involving the EWS gene and one of the ETS (E26 transformation-specific) family of transcription factors (3, 4) These transcription factors are highly dysregulated and believed to be critical to the biology of these tumors mediating both malignant transformation and progression (5).

In 85% of cases, the fusion transcription factor is EWS-FLI1 usually generated by the t(11;22)(q24;q12) chromosomal translocation (4). This transcription factor contains the binding domain of FLI1 and the transactivating domain of EWS but loses most of the regulatory domains of both proteins (6). Therefore, this protein is constitutively active and alters the expression of more than 500 genes (7). The resulting genetic program is believed to drive malignant transformation and clearly plays a central role in mediating the oncogenic phenotype (8-10). Most notably, EWS-FLI1 has been shown to directly or indirectly mediate the evasion of apoptosis and senescence, drive proliferation and even participate in the regulation of genes involved in metastasis (11-15). In addition, multiple groups have shown that knockdown of EWS-FLI1 with antisense DNA, siRNA or dominant negative methods is incompatible with ESFT cellular survival (16). Therefore, recent preclinical efforts have been focused on developing a small molecule therapy targeting this transcription factor (17, 18). Unfortunately, previously identified small molecule inhibitors have failed to demonstrate clinical activity against ESFT (19).

We have recently completed a high throughput screen of more than 50,000 compounds to identify mithramycin as an inhibitor of EWS-FLI1. We have utilized a novel approach combining a luciferase based primary screen and a gene signature multiplex PCR secondary screen to identify compounds that block the activity of EWS-FLI1 (20). In the primary screen, we required compounds to suppress the expression of luciferase driven by the promoter of a well- characterized EWS-FLI1 downstream target, NR0B1 without changing the expression of a constitutively active CMV driven promoter or decreasing cell viability as measured by an XTT assay (Figure 1). By performing these three screens in parallel, we were able to control for non-specific cytotoxic agents and general transcription inhibitors. We subsequently required the compounds to reverse a gene signature of more than 10 downstream targets of EWS-FLI1 utilizing a novel multiplex PCR assay that we developed to sort and prioritize compounds for further validation.



The compound that most effectively suppressed EWS-FLI1 activity was mithramycin (20). We showed that mithramycin suppresses the mRNA expression of our gene signature of EWS-FLI1 targets as well as an independent published gene signature of 50 different EWS-FLI1 targets. In addition, the suppression of EWS-FLI1 downstream targets also occurs at the protein level and leads to the induction of apoptosis in ESFT cells. These effects translate into a significant reduction of growth of two different xenograft models of Ewing sarcoma with one xenograft virtually eliminated from the mouse with mithramycin treatment (20). Finally, we found evidence of activity of mithramycin in

patients with Ewing sarcoma. Five patients with advanced, widely metastatic Ewing sarcoma were treated with mithramycin in the 1960s and at least one durable complete response was reported (21). The findings in our screen combined with the preclinical and clinical activity of mithramycin detailed below provide the rationale for the proposed phase I/II trial of mithramycin in children

and young adults with refractory solid tumors and Ewing sarcoma and EWS-FLI1 fusion transcript.

### 1.2.2 Preclinical studies

Mithramycin effectively suppresses the activity of EWS-FLI1 both *in vitro* and *in vivo*. We have shown by multiplex PCR, quantitative real time PCR and microarray expression profiling that mithramycin reverses the induction of two independently generated gene signatures of downstream targets of EWS-FLI1 (20). In addition, we have demonstrated that mithramycin suppresses the protein expression of two well-characterized downstream targets of EWS-FLI1, ID2 and NR0B1 in two different ESFT cell lines *in vitro* at nanomolar concentrations (22-24). The timing of this suppression correlates well with the induction of apoptosis and translates to an IC<sub>50</sub> of 10-15 nM in a panel of ESFT cell lines.

Finally, we used a xenograft model to show suppression of growth of two different ESFT xenografts. The TC32 xenograft model showed a regression of tumor in the first week of treatment that persisted long after every mouse in the control had to be sacrificed for progression. The TC71 xenograft also showed an impressive suppression in growth that translated into a doubling in survival for the affected mice. In contrast, despite relatively similar *in vitro* IC<sub>50</sub>s, an osteosarcoma MNNG-HOS xenograft showed minimal suppression in growth and no improvement in survival with mithramycin treatment. In addition, we were able to recapitulate the biochemistry observed *in vitro* by utilizing immunohistochemistry to demonstrate suppression of NR0B1, an EWS-FLI1 downstream target, in the mithramycin treated but not the control tumor tissue (20).

In summary, ESFT depends on the continued expression of EWS-FLI1 for cell survival. We have completed a high throughput screen to identify mithramycin as an inhibitor of EWS-FLI1 and shown suppression of activity both *in vitro* and *in vivo*. As predicted, blockade of EWS-FLI1 causes the induction of apoptosis, leads to a nM *in vitro* IC<sub>50</sub> and suppresses growth of ESFT xenografts.

#### 1.2.2.1 Preclinical toxicology

In mice, the LD<sub>50</sub> was determined to be about 2000 mcg/kg (6000 mcg/m<sup>2</sup>) of body weight. This dose is higher than the 100 mcg/kg of body weight that was found to be lethal in some dogs (2000 mcg/m<sup>2</sup>) and monkeys (1200 mcg/m<sup>2</sup>) (25). However, the drug was found to be essentially non-toxic in dogs and monkeys administered at a dose of 24-50 mcg/kg/day (480-1000 mcg/m<sup>2</sup> dog, 288-600 µg/m<sup>2</sup> monkey) intravenously for 24 consecutive days (25). The most frequently encountered toxicities at higher doses were bleeding, anorexia, vomiting, elevated liver function tests, electrolyte abnormalities, bone marrow suppression and azotemia (25).

In our experience in mice, doses of 1 mg/kg/day three times a week for 6 weeks delivered via intraperitoneal injection was essentially non-toxic to the mice. At necropsy, the mice treated at this dose and schedule had mild hypoalbuminemia, slightly increased ALT/AST with some evidence of liver toxicity described as mild to moderate and minimal thrombocytopenia.

It is notable that we were able to show that the drug interferes with the expression of the EWS-FLI1 downstream target NR0B1 in our mouse models at this 1 mg/kg/day dose. However, it is not clear if the dose of 25 mcg/kg/dose is high enough to interfere with EWS-FLI1 activity in patients. Therefore, one of the primary objectives will be to establish the inhibition of NR0B1 expression through mandatory tumor biopsies when feasible (see Section **Error! Reference source not found.**). These results will be critical for the interpretation of patient response to treatment, as

well as the future clinical development of mithramycin and the preclinical development of mithramycin analogs.

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 (26). Preliminary pharmacokinetic parameters of <sup>125</sup>I-plicamycin (mithramycin) in human plasma using a radioimmunoassay were described (27). Three patients were studied and received mithramycin at a dose ranging from 0.85 to 1.0 mg/m<sup>2</sup> IV over 2 hours. Drug elimination was biphasic, with a mean elimination half-life of 10.6 hours (±1.7), and a clearance rate of 11.1± 0.4 ml/min/m<sup>2</sup>.

### 1.2.3 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. In particular, mithramycin was found to have activity against testicular cancer and was briefly used in the clinic for this tumor prior to the development of the currently used treatment regimen. However, a series of case reports in the literature suggest a particular sensitivity of Ewing sarcoma for mithramycin treatment. Prior clinical trials are summarized in Appendix 1.

The clinical experience with mithramycin is extensive as more than 1500 patients treated with mithramycin are reported in the literature. A formal Maximum Tolerated Dose (MTD) has not been established. However, the earliest reports of the drug efficacy at 50 mcg/kg/dose and therefore established this dose daily times 5 as the optimal treatment regimen (28). Subsequent to this report, a follow-up study of 58 patients found significant toxicity consisting of anorexia, nausea and vomiting at this dose and schedule in 70% of patients (29). As a result, the dose of drug used in most studies was 25 mcg/kg/day on various schedules. The recommended dose for the treatment of testicular cancer from the package insert from Pfizer was 25 mcg/kg/day daily times 7 (25). In addition, it was reported that significant toxicity is dose related and occurs at doses above 30 mcg/kg/dose (25). It is notable that the dose that led to the responses in the Ewing patients was 25 mcg/kg/day as a 8 to 24 hour infusion for 5-7 days (21).

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 (25)). Unfortunately, there was no systematic investigation of these toxicities and it is not clear the amount of supportive care these patients received. For example, nausea and vomiting occurred quite frequently, however, in at least two reports, the nausea responded to treatment with phenothiazines or other anti-emetics (30-32).

The most profound toxicity seen was hemorrhage. The tendency for bleeding occurred infrequently and in early reports was attributed to thrombocytopenia and accounted for 3 deaths out of 84 patients treated (33). Subsequent studies showed that bleeding occurred early in treatment at about day 4 and was dose related occurring in 5.4% of 1150 patients treated at 30 mcg/kg/day or less (25). This time frame is significantly shorter than what is typically seen for chemotherapy induced thrombocytopenia. In addition, it appears as though the platelet count remained >20,000/mcL in most of the cases of hemorrhage in the literature. Subsequent to these studies, it was determined that mithramycin treatment caused a decrease in coagulation factors II, V, VII and X (34). In addition, subsequent studies revealed that mithramycin causes abnormal platelet adhesion in the setting of a normal platelet count consistent with a platelet adhesion

molecule deficiency (35). It is notable, that in addition to inhibiting EWS-FLI1, mithramycin is known to inhibit the SP1 transcription which is known to drive the biosynthesis of coagulation factors as well as platelet adhesion glycoproteins. Nevertheless, 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, and how effective the blood product support was in that era of treatment. In particular, it is not clear if patients received platelets in the setting of a normal platelet count as would be required for an acquired platelet adhesion defect. Regardless of the etiology, this side effect must be approached with extreme caution.

Another rare but significant side effect described in the literature is the report of toxic epidermal necrolysis that has been associated with mithramycin treatment (36, 37). This side effect is reported in two separate case reports but was omitted in the package insert from Pfizer (37) and appears to begin with plethora and non-specific skin findings. The incidence of this side effect is unclear, however due to the substantial nature of this reaction, all skin eruptions should be approached with significant caution.

One report describes potentiation of the cardiotoxicity of anthracyclines with mithramycin treatment (38). However, this report provides no details of the cardiotoxicity observed and refers to mitomycin. No other mention of cardiotoxicity has been found in the literature, package insert or IND that was previously filed.

Finally, no report or investigation of cumulative toxicity could be found in the literature. The closest report is one of a long term follow-up of patients with testicular carcinoma treated with mithramycin (39). In this report 10 patients with metastatic testicular carcinoma out of 62 treated with mithramycin were available for long term follow up at 23 years. No long-term side effect of the drug is reported.

#### 1.2.4 Clinical activity

In 1960, the first evaluation of more than 36 patients with a variety of tumor histologies found an overall response rate of just under 20% defined as tumor shrinkage, improvement in performance status or a leveling off of body weight (28). Subsequent to this, mithramycin was evaluated in a number of reports and activity was found in a variety of histologies although patient numbers and standard methods of evaluating responses were limited (31, 40-42). The tumor type that seemed to show a consistent response was testicular carcinoma. In one series of 305 patients with stage III testicular CA, 10% of patients achieved a complete response and an additional 25% showed some evidence of tumor regression (25). Many of these cases were durable responses in patients with widely metastatic disease. However, the overall response rate was low and the widespread use of the drug in this tumor, likely was limited by the development of the successful “Einhorn regimen” for the treatment of this cancer.

In addition to testicular carcinoma, mithramycin was used for the treatment of malignant hypercalcemia with 45% of patients responding to a single dose of mithramycin in one study (43). However, in the same report, the bisphosphonate pamidronate was far superior and ultimately replaced mithramycin in this setting. For similar reasons, mithramycin was investigated for the treatment of Paget’s disease of bone where it was found to have good activity in the management of this disorder of osteoclasts. In one report, 10/10 patients with Paget’s disease reported improvements in pain and an increase in overall activity with treatment (32).

The activity of mithramycin in Ewing sarcoma was impressive in some of the earliest reports of this drug in the clinic (33). A total of 5 patients with ESFT have been treated with mithramycin and reported in the literature (21). Of those 5 patients, 2 patients experienced widespread regressions of metastatic disease with mithramycin treatment as a single agent. One of the two patients had a durable completed response and was followed for more than 7 years (21). It is not clear why the drug was not further investigated in ESFT. It is likely that the advent of anthracycline based therapies and the evolution of combination therapies supplanted the interest of the community in alternative single agent therapies.

#### 1.2.5 Clinical studies in children

No formal pediatric studies of mithramycin in children have been reported. Based on the literature available, only 5 patients under the age of 18 received mithramycin (40). In this published report of 26 patients, 5 children (ages 10-16 years of age) with embryonal carcinoma of the testis, malignant teratoma testis or Ewing sarcoma received total mithramycin doses of 185-250 mg/kg over 24 hours. No toxicity data was reported on these five study participants. One subject experienced a mixed response after the first course, one had regression of small metastases but no change in larger tumors, and one subject experienced transient improvement in pain reports. It is notable in this report that the patient with Ewing sarcoma that achieved a complete response was a 22 year-old adult.

#### 1.2.6 Clinical Protocol 12C0135

Two adult patients with Ewing Sarcoma have been enrolled on this trial and treated at the 25mcg/kg dose level. Following the second dose, both patients experienced grade 4, dose-limiting AST and ALT elevation and also non dose-limiting increased LDH, GGT, and fever. Bilirubin remained normal and synthetic function was minimally abnormal transiently. Both patients also had a fever after the 2nd dose and very mild nausea. No abdominal pain or other symptoms were observed. In both cases, LFTs recovered fairly quickly after holding drug. Mithramycin was held after the 2nd dose for both patients. One of the patients was retreated at a lower dose of 17.5 mg/kg per protocol. Dexamethasone prophylaxis was administered in a similar fashion to what is done for other anti-cancer agents, which can result in hepatotoxicity, such as trabectedin. The aforementioned patient was able to receive all 7 doses of mithramycin with transient elevation in LFTs peaking at about 200 U/L, and recovery to grade 1 or baseline 12 days after the last dose of mithramycin.

Based on the data noted above, we are amending the protocol to lower the phase II dose to 17.5 mcg/kg and the phase I starting dose to 13 mcg/kg and to add dexamethasone prophylaxis with the goal to ameliorate hepatotoxicity.

### 1.3 RATIONALE SUMMARY

Our high throughput screen of more than 50,000 compounds identified mithramycin as an inhibitor of EWS-FLI1. ESFT has a strong dependence on EWS-FLI1 for cellular survival and we have shown that mithramycin interferes with this activity *in vitro* and *in vivo* and markedly suppresses ESFT xenograft growth. Responses to single agent mithramycin have also been described in patients with refractory ESFT, but the objective response rate of mithramycin in ESFTs has not been determined. These findings provide a strong rationale for the development of mithramycin in patients with EWS-FLI1 positive Ewing sarcoma.



In addition, mithramycin demonstrated activity in other adult solid tumors, and our preclinical data suggest that other pediatric solid tumors might also be sensitive to mithramycin treatment.

We therefore propose a broad phase I trial of mithramycin for children and adolescents with refractory solid tumors to confirm tolerability of mithramycin at the recommended dose for adults established in the 1960s. There will be a limited dose escalation to the adult recommended dose. At the adult recommended dose (or MTD) up to six children  $\leq 12$  years of age and six children  $\geq 12$  years of age will be enrolled to gain experience with the toxicities of mithramycin over a broad age range.

Simultaneous to enrolling children in the phase I portion of this trial, adults with EWS-FLI1 positive Ewing sarcoma will enroll on the phase II portion of the trial, which has the goal to determine the activity of mithramycin in EWS-FLI1 positive Ewing sarcoma. Initiating the phase II study in adults while determining the optimal dose in children, is based on extensive previous safety and dosing information obtained from clinical trials conducted in adults and the anecdotal reports of activity in patients with Ewing sarcoma treated in the past with mithramycin. The Phase II portion will be implemented in adults using the previously approved mithramycin dose of 25 microgram/kg administered over six hours once daily for 7 days to be repeated every 28 days.

Once the adult phase II dose has been determined safe in children and adolescents, children will also be accrued to the phase II portion. Should the pediatric phase II dose of mithramycin differ from the adult phase II dose, the protocol will be amended to address accrual and response rate to mithramycin in children in a separate stratum. The objective response rate of mithramycin will be determined in EWS-FLI1 positive Ewing sarcoma using a 2-stage design and targeting an objective response rate (RECIST PR and CR) of 25%.

## **2 ELIGIBILITY ASSESSMENT AND ENROLLMENT**

### **2.1 ELIGIBILITY CRITERIA**

#### **2.1.1 Inclusion Criteria**

##### **2.1.1.1 Diagnosis**

Patient's current disease state must be one for which there is no known curative therapy or therapy proven to prolong survival with an acceptable quality of life.

Phase I Portion: Measurable or evaluable refractory or recurrent extracranial solid tumors, excluding brain tumors and cerebral metastases.

Phase II Portion adults and children: Refractory or recurrent extracranial Ewing sarcoma with EWS-FLI1 fusion transcript. Patients enrolled to this cohort must have measurable disease. Presence of the transcript will be determined during histologic confirmation of disease with a CLIA approved EWS-FLI paraffin assay in the Laboratory of Pathology CCR, NCI, unless a pathology report documenting presence of the transcript using a CLIA approved assay is obtained from the referring institution.

**2.1.1.2 Histologic confirmation of disease in the Laboratory of Pathology, CCR, NCI, NIH.**

##### **2.1.1.3 Age**

Phase I Portion:  $\geq 12$  months to  $\leq 17$  years

Phase II Portion in adults initially:  $\geq 18$  years

Phase II Portion expanded in pediatrics after determination of phase II dose in children will include children  $\geq 12$  months to  $\leq 17$  years

2.1.1.4 Performance Score: Karnofsky ( $> 10$ -17 years old) or Lansky ( $\leq 10$  years old)  $\geq 50\%$ , or ECOG 1 or 2 (adults) (See Appendix 2)

2.1.1.5 Prior therapy

$\geq 2$  weeks must have elapsed since local palliative XRT (small port) or other substantial BM radiation;

$\geq 24$  weeks must have elapsed since prior TBI, craniospinal XRT, or if  $\geq 50\%$  radiation of pelvis;

$\geq 12$  weeks must have elapsed since stem cell transplant or infusion without TBI and no active graft vs. host disease;

$\geq 3$  weeks must have elapsed from last dose of myelosuppressive chemotherapy (six weeks for nitrosoureas);

at least 3 half-lives must have elapsed since monoclonal antibody;

(<https://members.childrensoncologygroup.org/Disc/devtherapeutics/default.asp> for listing of monoclonal antibody half-lives.)

$\geq 7$  days must have elapsed from the last dose of biologic agents.

$\geq 7$  days since the completion of therapy with a growth factor

2.1.1.6 Recovered from acute toxicities of prior therapy to  $\leq$  Grade 1; specifically

a) Hematologic and Coagulation Parameters

- i. Peripheral ANC  $\geq 1000/\text{mcL}$
- ii. Platelets  $\geq 75,000/\text{mcL}$  (transfusion independent)
- iii. Hemoglobin  $\geq 8 \text{ g/dL}$  (PRBC transfusions permitted)
- iv. Normal PT/PTT with the exception of a lupus anti-coagulant, which is permitted, may be corrected with Vitamin K administration or transfusion. Fibrinogen  $\geq$  the lower limit of normal.

b) Hepatic Function

- i. Bilirubin (total)  $\leq 1.5 \text{ X}$  upper limit of normal (ULN)
- ii. ALT (SGPT)  $\leq 2.5 \text{ X}$  ULN
- iii. Albumin  $> 2 \text{ g/dL}$

c) Renal Function

- i. Creatinine clearance  $\geq 60 \text{ mL/min/1.73 m}^2$ , or serum creatinine base on age and gender as follows:

Age (years)	Maximum Serum Creatinine (mg/dL)	
2 to $< 6$	0.8	0.8
6 to $< 10$	1	1

10 to < 13	1.2	1.2
13 to < 16	1.5	1.4
≥ 16	1.7	1.4

- Normal calcium, magnesium and phosphorus (can be on oral supplementation)
- 2.1.1.7 Cardiac Function: Left ventricular ejection fraction (EF) within normal institutional limits by Echocardiogram MUGA or cardiac MRI.
- 2.1.1.8 Ability to give informed consent. For patients <18 years of age their legal guardian must give informed consent. Pediatric patients will be included in age-appropriate discussion in order to obtain verbal assent.
- 2.1.1.9 Female and male patients (and when relevant their partners) must be willing to practice birth control (including abstinence) during and for two months after treatment, if of childbearing potential during sexual contact with a female of childbearing potential.
- 2.1.1.10 A durable power of attorney (DPA) will be offered to all patients ≥ 18 years old.
- 2.1.1.11 Eligibility criteria for mandatory serial tumor biopsies
- Age: ≥ 18 years old
  - Ewing sarcoma with EWS-FLI1 fusion transcript
  - Hematologic and coagulation parameters within 2 days prior to each biopsy: Normal PT/PTT with exception of lupus anticoagulant, platelets ≥75,000/mcL, peripheral ANC ≥750/mcL
  - Willing to undergo biopsies, which will only be performed on tumors amenable to percutaneous biopsy ([Error! Reference source not found.](#))
- 2.1.2 Exclusion Criteria
- 2.1.2.1 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.
- 2.1.2.2 Patients with a history intracranial Ewing sarcoma including cerebral metastases
- 2.1.2.3 Patients with evidence of active bleeding, intratumoral hemorrhage or history of bleeding diatheses
- 2.1.2.4 Patients who are receiving anticoagulants other than prophylactic anticoagulation of venous or arterial access devices, provided that requirements for PT, PTT and fibrinogen are met, as described in Section [Error! Reference source not found.](#).
- 2.1.2.5 Investigational Drugs: Patients who are currently receiving another investigational drug
- 2.1.2.6 Patients who are concurrently receiving agents, which may increase the risk for mithramycin related toxicities, such as hemorrhage including:
- Thrombolytic agents

- Anti-inflammatory drugs, nonsteroidal (NSAIDs) or aspirin or salicylate-containing products, which may increase risk of hemorrhage
- Dextran
- Dipyridamole
- Sulfinpyrazone
- Valproic acid

- 2.1.2.7 Anti-cancer Agents: Patients who are currently receiving other anti-cancer agents
- 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 plicamycin (mithramycin)
- 2.1.2.11 Requirement for any of the contraindicated medications: nonsteroidal anti-inflammatory drugs, aspirin, dextran or other iron containing solutions (due to incompatibility), dipyridamole, sulfinpyrazone or valproic acid
- 2.1.2.12 Patients who in the opinion of the investigator may not be able to comply with the safety monitoring requirements of the study.
- 2.1.2.13 Patients receiving concurrently other therapies directed at their cancer.
- 2.1.3 Recruitment Strategies

The following recruitment strategies will be employed to elicit potential candidates for this trial:

1. Listed on clinical trials.gov;
2. Listed in PDQ;
3. In addition, sarcoma patient advocacy and research groups such as the Children's Oncology Group, the Sarcoma Alliance for Research through Collaboration (SARC), The Sarcoma Foundation of America, The Liddy Shriver Sarcoma Initiative, the Quad W foundation will be notified.

Prior to distribution of any recruitment materials, such materials will be submitted to the NCI IRB for review.

## **2.2 SCREENING EVALUATION**

### **2.2.1 General**

Pre-treatment blood test should be performed within 7 days and imaging studies within 2 weeks prior to enrollment on the trial unless otherwise stated. The evaluations required prior to starting treatment are listed in table form in Appendix 3 Study Calendar.

HISTORY AND PHYSICAL EXAMINATION: including documentation of height and weight (average of 3 measurements on the same day), measurable disease, vital signs (blood pressure, heart rate, respiratory rate, temperature), performance status, history of bleeding disorder, HIV, HBV, HCV, and signs and symptoms within one week of enrollment.

### **2.2.2 Laboratory**

2.2.2.1 ROUTINE LABS: complete blood count, differential, LDH, SGPT (ALT), SGOT (AST), alkaline phosphatase, bilirubin (total and direct), BUN, creatinine, amylase, lipase, electrolytes, calcium, magnesium, phosphorus, uric acid, albumin and thyroid panel.

2.2.2.2 Urinalysis.

2.2.2.3 URINE PREGNANCY TEST: required for females of childbearing potential

2.2.2.4 Coagulation Studies: PT/PTT/Thrombin Time/Fibrinogen, PFA-100, VWF antigen, VWF activity, Factor VIII activity, D-dimer, Fibrin degradation products and FXIII activity. In addition, 1 mL of plasma will be obtained, filtered through a 0.22 micron filter, frozen and saved for future clinical reference if needed ([Error! Reference source not found.](#) and [Error! Reference source not found.](#)).

2.2.3 Radiographic Evaluation

Assessment of disease sites by appropriate radiological evaluation. This could include a CT scan of chest, abdomen and pelvis and primary tumor, and bone scan, FDG-PET or MRI as indicated. A consistent method of disease evaluation for each patient will be used throughout the study.

Imaging of the head/brain to rule out intracranial disease including brain metastases is not required in all patients, but should be performed if there is clinical concern for intracranial disease/brain metastases

2.2.4 Cardiac Function Studies

Assessment of cardiac function (ejection fraction/shortening fraction) must be performed within 4 weeks of enrollment by MUGA or Echocardiogram to rule out cardiomyopathy. In addition, a 12-lead EKG must be performed.

2.2.5 Pathologic/Tissue Evaluation

Histologic confirmation of tumor will be performed on all patients by the NCI Laboratory of Clinical Pathology, NIH. For participants in the Phase II portion: the presence of the transcript with a CLIA approved EWS-FLI paraffin assay will be determined on samples from all sites in the Laboratory of Pathology CCR, NCI (See [Error! Reference source not found.](#)); unless a pathology report documenting presence of the transcript using a CLIA approved assay is obtained from the referring institution.

## **2.3 REGISTRATION PROCEDURES**

Patients must be registered by contacting the research nurse in the Pediatric Oncology Branch (POB) (When registering a patient, information about all entry criteria (e.g., laboratory results) must be available to allow for verification of eligibility. The principal investigator or an Associate Investigator must also be contacted to discuss the patient prior to entry on study. Research nurse for this trial is:

Donna M. Bernstein, R.N.  
Pediatric Oncology Branch  
Building 10, Room 13C101  
10 Center Drive  
Bethesda, Maryland 20892  
Phone: (301) 435-7804  
Email: [db302w@nih.gov](mailto:db302w@nih.gov)

### 2.3.1 Registration at NCI

Authorized staff must register an eligible candidate with NCI Central Registration Office (CRO) within 24 hours of signing consent. A registration Eligibility Checklist from the web site (<http://home.ccr.cancer.gov/intra/eligibility/welcome.htm>) must be completed and faxed to 301-480-0757. After confirmation of eligibility at Central Registration Office, CRO staff will call pharmacy to advise them of the acceptance of the patient on the protocol prior to the release of any investigational agents. Verification of Registration will be forwarded electronically via e-mail to the research team. A recorder is available during non-working hours.

## 3 STUDY IMPLEMENTATION

### 3.1 STUDY DESIGN

This study will be conducted as a single institution, open label Phase I/II trial of mithramycin. The Phase I portion will be conducted in children and adolescents (12 months to  $\leq 17$  years of age) with refractory extracranial solid tumors. The cohort at the recommended dose or MTD will be expanded up to 12 patients, and attempts will be made to enroll at least 6 patients that are  $\geq 12$  years of age and 6 patients that are  $< 12$  years of age to gain experience with a broad age range of patients.

In the phase II portion of the trial the adult dose (17.5 mcg/kg/dose) will be administered to patients with measurable refractory or recurrent Ewing sarcoma and EWS-FLI1 fusion transcript to determine the objective response rate (CR and PR) to mithramycin using RECIST criteria. Initially this portion of the trial will be open to eligible participants  $\geq 18$  years of age. Enrollment will be expanded to include children and adolescents once safety at this dose is determined. If the pediatric phase II dose of mithramycin differs from the adult phase II dose, the protocol will be amended to address accrual and response rate to mithramycin in children in a separate stratum.

The Phase I and Phase II portions of the protocol will enroll patients simultaneously.

Patients with ESFT enrolled on this study (phase II portion) who meet eligibility criteria for tumor biopsies will undergo mandatory tumor biopsies to assess EWS-FLI1 target blockade by mithramycin (see [Appendix 7: Studies to be performed on tumor tissue](#)).

Mithramycin will be administered intravenously over 6 hours once daily for 7 days to be repeated every 28 days. Patients may continue to receive mithramycin for as long as they tolerate drug (Sections [3.1.1](#) and [3.4](#)) and do not have progressive disease (Section [6.2](#)).

Patients on study will be closely monitored for the development of toxicity including regular physical examinations, blood pressure monitoring, laboratory evaluations, and physical examinations with particular attention to the development of any signs of bleeding. Patients will be admitted to the hospital for the first treatment cycle during administration of mithramycin in order to allow for close monitoring of toxicities.

Disease will be monitored after every even treatment cycle using appropriate imaging studies using RECIST criteria.

Correlative studies are described in Section [5](#).

### 3.1.1 Dose Limiting Toxicity

Dose limiting toxicity (DLT) will be defined as non-hematologic toxicity and hematologic toxicity. Toxicities observed during the first treatment cycle will be used to determine the MTD. For the Phase II portion of the study, the below toxicities will be considered treatment limiting.

- **Non-hematologic DLT** will be defined as any grade  $\geq 3$  non-hematological toxicity possibly, probably, or definitely related to mithramycin with the exception of:
  - Grade 3 nausea that is controlled by symptomatic treatment with anti-emetics within 72 hours.
  - Grade 3 vomiting that is controlled by symptomatic treatment with anti-emetics within 72 hours.
  - Grade 3 diarrhea that is controlled by symptomatic treatment within 72 hours.
  - Asymptomatic grade 3 elevation of serum transaminases that return to  $\leq$  grade 1 within 14 days of completing mithramycin administration during a cycle.
  - Asymptomatic grade 3 electrolyte abnormalities that are correctable to grade 2 or less within 48 hours.
- **Hematologic DLT** will be defined as:
  - Grade 4 neutropenia (ANC  $< 500/\text{mcL}$ ) on two consecutive blood counts drawn at least 72 hours apart in patients receiving prophylactic growth factor support (See Section 3.4) or
  - Grade 4 thrombocytopenia ( $< 25,000/\text{mcL}$ ) refractory to platelet transfusion\*.
  - Failure to recover platelet count to  $\geq 75,000/\text{mcL}$  by day 42 of starting a treatment cycle.
- Any grade 2 bleeding not promptly (within 6 hours of appropriate intervention) corrected with blood product support will be considered dose-limiting.
- Bleeding that does not stop within 6 hours of administration of appropriate treatment including platelets, will be considered DLT and mithramycin will be discontinued.
- Any grade 2 mithramycin related toxicities of  $\geq 7$  days may be considered dose limiting if they are intolerable to the patient and cannot be controlled with standard supportive measures.
- Failure to recover from toxicity within 42 days of starting a treatment cycle to the level required for initiating subsequent cycles (Section 3.4) will be considered dose-limiting

\*Thrombocytopenia will be allowed in this trial provided that that patient is not refractory to platelet transfusions and that the bone marrow recovers from the previous cycle. This toxicity will be tolerated because of the theoretical possibility that it reflects mithramycin inhibiting the target. It is known that FLI1 plays a prominent role in megakaryocyte development. Patients with haploinsufficiency of FLI1 are known to have congenital thrombocytopenia (Paris-Trousseau Syndrome) and megakaryocyte maturation arrest (44). Furthermore, mithramycin is believed to work by blocking binding of FLI1 since that portion of the EWS-FLI1 gene fusion protein is responsible for DNA binding. In addition, patients treated with mithramycin in the 1960s

experienced isolated thrombocytopenia. Therefore, it follows that if mithramycin blocks the action of EWS-FLI1, it may also affect FLI1 and therefore cause thrombocytopenia.

Details regarding the management of thrombocytopenia and bleeding are provided in Section: 4.1

Details regarding retreatment of patients experiencing neutropenia are provided in Section 3.3.

### 3.1.2 Dose Escalation in Phase I Portion

The initial dose of mithramycin administered in the Phase I portion is 74% of the phase II dose which equals 13 mcg/kg/dose, to be repeated every 28 days. Once safety of the starting dose is established, mithramycin will be escalated to the dose of 17.5 mcg/kg/dose according to the following table.

This limited proposed dose escalation is shown below:

Dose Level	Dose (mcg/kg/dose)	Percent change
-1	9.0	-31%
1	13	0
2	17.5	35%

All patients will receive dexamethasone as described in Section 4.3.

Cohorts of 3 to 6 patients will be treated at each dose level. Toxicity will be graded using version 4.0 of the NCI Common Toxicity Criteria, and the MTD will be defined based on toxicities observed during the first treatment cycle. When a minimum of 3 patients who are evaluable for toxicity have completed 1 cycle of therapy without evidence of DLT, subsequent patients may be enrolled at the next higher dose level. If DLT is observed in 1 patient from the initial cohort of 3 patients, up to an additional 3 patients will be entered at that dose level. If none of these additional patients experiences a DLT (1/6 with DLT), the dose will be escalated. If  $\geq 1$  of the additional patients experience a DLT ( $\geq 2/6$  with DLT), the MTD has been exceeded. If the MTD has been exceeded at the first dose level, then the subsequent cohort of patients will be treated at dose level -1. If  $\geq 2/6$  patients experience DLT at the -1 dose (9.0 mcg/kg/dose), no further patients will be enrolled until completion of discussions with the sponsor, FDA and NCI IRB and approval of a protocol amendment.

The MTD will be the maximum dose at which fewer than one-third of patients experience DLT during cycle 1 of therapy.

### 3.1.3 Phase II Portion

Using a Simon two stage design (see Section 8), mithramycin will be administered intravenously at the recommended dose and schedule up to 24 patients evaluable for response. All patients will receive dexamethasone as described in Section 4.3. Initially adults ( $\geq 18$  years of age) with Ewing sarcoma and EWS-FLI1 fusion transcript will be enrolled in order to determine the objective response rate (PR and CR) using RECIST. Once adult dose is deemed safe, children ( $\geq 12$  months) and adolescents ( $\leq 17$  years) with Ewing sarcoma and EWS-FLI1 fusion transcript will begin enrollment to the Phase II portion. Should the pediatric phase II dose of mithramycin differ from the adult phase II dose, the protocol will be amended to address accrual and response rate to mithramycin in children in a separate stratum.



Assessment of NR0B1 in serial tumor biopsies: Should less than 12 patients with evaluable tumor biopsies be available at the time response objectives are met, enrollment on this trial will continue for patients undergoing tumor biopsies until a minimum of 12 patients with tumor biopsies evaluable for analysis of NR0B1 inhibition have been enrolled ([Appendix 7: Studies to be performed on tumor tissue](#)).

### **3.2 DRUG ADMINISTRATION**

Mithramycin will be administered intravenously during hospitalization for the first cycle. The dose of mithramycin will be diluted in appropriate fluids and will be infused over 6 hours daily for 7 days to be repeated every 28 days (+/- 2 days) ([Appendix 3: Study Calendar](#)). In the phase II portion, mithramycin will be administered at a dose of 17.5 mcg/kg/dose.

Intravenous hydration should be provided as clinically indicated.

### **3.3 DOSE REDUCTION OR MODIFICATIONS**

Patients who experience a DLT (as defined in Section [3.1.1](#)) possibly, probably or definitely related to mithramycin, will have mithramycin held.

Patients may continue on study at a reduced dose (reduced one dose level as defined in Section [3.1.2](#)), after having recovered from the experienced DLT within  $\leq 42$  days of starting the treatment cycle.

If a patient experiences any of the following events, mithramycin will be held and no further mithramycin will be administered:

- grade 3 or 4 anaphylaxis or allergic reaction
- grade 4 DIC
- grade 4 erythema multiforme, toxic epidermal necrosis (TEN), or Stevens-Johnson syndrome
- grade 4 diarrhea
- grade 3 hemorrhage if endoscopic or operative intervention required or any grade 4 hemorrhage
- grade 2 or greater bleeding that does not resolve within 6 hours of administration of appropriate treatment
- CNS hemorrhage of any grade

For the Phase 1 portion only: Patients who initiate treatment at the -1 dose level and experience a DLT, will have a ~30% dose reduction for subsequent cycles.

For both Phase 1 and Phase 2 portions: If another DLT is observed on the reduced dose, the patient will discontinue protocol therapy permanently unless the patient is receiving benefit from mithramycin as evidenced by disease stabilization or tumor shrinkage, in which case a further dose reduction will be permitted.

Patients who experience Grade 4 neutropenia ( $<500/\text{mcL}$ ) on two consecutive blood counts drawn at least 72 hours apart, who recover to meet criteria for additional cycles may receive mithramycin at full dose in the next cycle with growth factor support. If grade 4 neutropenia ( $<500/\text{mcL}$ ) on two consecutive blood counts drawn at least 72 hours apart recurs with growth factor support, this will be dose-limiting (Section [3.1.1](#)), and dosing may continue at a reduced dose of mithramycin as described above with growth factor support.

Patients who do not recover from toxicity within 42 days of starting a treatment cycle to the level required for initiating subsequent cycles (Section 3.4) will be permanently removed from treatment with mithramycin.

For the Phase 2 portion only: If 2 or more of the initial 6 patients enrolled at the 17.5 mcg/kg dose experience dose-limiting hepatotoxicity, enrollment will be put on hold until a discussion with the sponsor and IRB and possibly FDA have been held.

### **3.4 CRITERIA FOR ADDITIONAL CYCLES**

Patients will be eligible to continue to receive mithramycin for as long as they do not experience clinical or radiographic disease progression and tolerate mithramycin (defined as not meeting off treatment criteria). If a patient experiences DLT on a reduced dose of mithramycin (as defined in Section 3.3), no further mithramycin will be administered.

Patients may receive the next cycle at the same dose level (or reduced level as above) if:

- 1) patient has stable or responding disease;
- 2) has a platelet count  $\geq 75,000/\text{mcL}$ , and an ANC  $\geq 1000/\text{mcL}$ , and meets other laboratory parameters defined in the eligibility section; and
- 3) has not met any criteria for removal from treatment or off study (Section 3.7)

### **3.5 On Study Protocol Evaluation ([APPENDIX 3: STUDY CALENDAR](#))**

#### **3.5.1 Monitoring During Active Treatment:**

3.5.1.1 History and physical exam: at baseline and weekly.

3.5.1.2 Weight: at baseline and recommended daily days 2 to 7 each cycle

3.5.1.3 A 12-lead EKG will be performed day 1 and 7 of each cycle, and day 4 of cycle 1. All EKGs should be done prior to drug infusion on that day and as close as possible to the time the trough PK is drawn. Document the time the EKG is performed on the PK sheet ([Appendix 4: Pharmacokinetic Worksheet for Mithramycin \(Phase I and Phase II\) For nursing staff](#))

3.5.1.4 Laboratory evaluation:

CBC with differential count daily (starting on day 2 to day 7) during mithramycin administration; then twice weekly during cycles 1-3. During subsequent cycles: CBC with differential count twice weekly during administration, then weekly.

Chemistry: sodium, potassium, chloride, CO<sub>2</sub>, creatinine, glucose, BUN, albumin, calcium, magnesium, daily (starting on from day 2 to day 7) during drug administration; then twice weekly during cycles 1-3. During subsequent cycles chemistries are performed weekly.

Liver Function Tests: alkaline phosphatase, ALT/GPT, AST/GOT, total bilirubin, total protein, daily (starting on day 2 to day 7) during drug administration; then twice weekly during cycles 1-3. During subsequent cycles chemistries are performed weekly.

Coagulation Studies: PT/PTT/Thrombin Time, Fibrinogen prior to and once on days 3, 4 or 5 and days 7, 8, or 9 of every cycle. In addition, prior to cycle 1 and 2, 1 mL of plasma will be obtained, filtered through a 0.22 micron filter, frozen and saved for future clinical reference if needed (See [Appendix 8: Plasma samples for clinical purpose](#)).

Pregnancy test for women of child-bearing age: prior to each cycle.

Urinalysis: prior to each cycle.

#### 3.5.1.5 Tumor Measurements:

Disease evaluations will be performed after every EVEN numbered treatment cycle, i.e. cycle 2, cycle 4, etc, and when removed from treatment, when feasible: Assessment of disease sites will be performed using the most appropriate radiological evaluation. This could include a CT scan of chest, abdomen and pelvis and primary tumor, bone scan, FDG-PET or MRI as indicated. A consistent method of disease evaluation will be used throughout the study. Imaging studies used for response evaluation will be sent to the NCI POB for volumetric analysis as secondary endpoint (See [Appendix 9: Imaging studies for volumetric analysis of tumor burden](#)).

#### 3.5.2 Monitoring after completion of treatment with mithramycin:

After completion of therapy, patients will be monitored until recovered from toxicity to baseline or stabilized.

##### 3.5.2.1 History and physical exam weekly

##### 3.5.2.2 Laboratory evaluation: CBC, Acute, Hepatic and Mineral panels, uric acid, LDH, PT/PTT weekly

#### 3.5.3 Off Study Evaluation (at least 30 days after last dose)

##### 3.5.3.1 History and physical exam

##### 3.5.3.2 12-lead EKG

##### 3.5.3.3 Laboratory evaluation: CBC, Acute, Hepatic and Mineral panels, uric acid, LDH, PT/PTT

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

### 3.6 CONCURRENT THERAPIES

Other cancer chemotherapy, radiation, immunotherapy, biologic therapy, or investigational agents cannot be administered to patient while receiving mithramycin.

### 3.7 CRITERIA FOR REMOVAL FROM PROTOCOL THERAPY AND OFF STUDY CRITERIA

#### 3.7.1 Criteria for removal from protocol therapy

1. A patient may be removed from the protocol for the following non-medical or administrative reasons:
  - a. Patient refusal of further treatments (reasons must be noted on the patient's CRF).
  - b. It is deemed in the best interest of the patient. In this instance the PI should be notified and the reasons of withdrawal should be noted in the CRF.
  - c. Serious protocol violation as determined by the PI.
2. Any patient who develops DLT and does not recover from the toxicity by day 42 after the start of the cycle (Section [3.1.1](#) and Section [3.4](#))
3. Any patient who develops any one of the following:
  - a. grade 3 or 4 anaphylaxis or allergic reaction;
  - b. grade 4 DIC;

- c. grade 4 erythema multiforme, toxic epidermal necrosis (TEN), or Stevens-Johnson syndrome);
  - d. grade 4 diarrhea;
  - e. grade 3 hemorrhage if endoscopic or operative intervention required or any grade 4 hemorrhage;
  - f. grade 2 or greater bleeding that does not resolve within 6 hours of administration of appropriate treatment;
  - g. CNS hemorrhage of any grade.
4. Any patient who does not recover from the toxicity by day 42 to meet criteria to start additional cycles (Section 3.4).
  5. Any patient who develops DLT after a dose reduction for DLT (Section 3.3) unless the patient continues to receive benefit from mithramycin. Patients who received initial treatment at 25mcg/kg will be allowed two dose reductions prior to coming off treatment.
  6. Any patient with clinical or radiographic evidence of progressive disease on treatment, as defined in Section 6.2, following any treatment cycle will be removed from the treatment.
  7. A patient who develops a concurrent serious medical condition that might preclude or contraindicate the further administration of mithramycin will be removed from treatment.
  8. A patient who becomes pregnant will be immediately taken off therapy.
  9. Patients who are taken off protocol therapy due to toxicity are to be followed, if feasible, until toxicity resolves to baseline or grade 1 or less. Follow-up data will be collected until off study criteria are met unless consent is withdrawn.

### 3.7.2 Criteria for removal from Study

1. Death
2. Lost to follow-up
3. Withdrawal of consent for any further data submission
4. Entry onto another therapeutic study
5. Patients who are taken off treatment for toxicity will be followed until resolution of toxicity and then taken off study (at least 30 days after the last dose of mithramycin).
6. The PI deems it is in the best interest of the patient

### 3.7.3 Off Study Procedure

Authorized staff must notify Central Registration Office (CRO) when a subject is taken off-study. An off-study form from the web site (<http://home.ccr.cancer.gov/intra/eligibility/welcome.htm>) main page must be completed and faxed to 301-480-0757.

## 4 CONCOMITANT MEDICATIONS AND SUPPORTIVE MEASURES

### 4.1 MANAGEMENT OF BLEEDING, HEMATOLOGIC AND BLOOD PRODUCT SUPPORT

Blood product support should be provided to maintain platelets  $\geq 25,000$  cells/mcl and as clinically indicated.

Growth factor support to patients who experience grade 4 neutropenia (ANC  $<500/\mu\text{L}$ ) will not be administered prophylactically, but may be administered as defined in Section 3.3. In addition,

growth factor support may be administered to patients who develop grade 4 neutropenia if clinically indicated, for example in patients who develop sepsis.

If patients experience any signs of bleeding ( $\leq$  Grade 2) including, epistaxis, hematuria, hematemesis or blood in the stool, the patient will immediately undergo a comprehensive clinical evaluation, coagulation profile including PT/PTT/fibrinogen, thrombin time, PFA-100, D-dimer, and comprehensive metabolic panel including  $\text{Ca}^{2+}$  and ionized calcium. Any abnormality will be corrected as clinically indicated with blood product support with guidance by hematology (NIH CC hematology consult). If a patient experiences bleeding in the setting of a normal platelet count, platelet function tests will be performed and the patient should be transfused with platelets even in the presence of a normal platelet count while these studies are pending. Mithramycin has also been known to block SP1, and SP1 has been shown to play a role in the expression of GPIIb. Therefore, mithramycin may cause a deficiency in this protein in mature platelets theoretically mimicking the bleeding diathesis, Glanzmanns thrombasthenia (45).

#### **4.2 ANTIEMETIC THERAPY**

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

#### **4.3 HEPATOTOXICITY PROPHYLAXIS USING DEXAMETHASONE**

Patients will receive 2.5 mg dexamethasone/ $\text{m}^2$  BSA PO or IV starting 12-16 hours before the first dose of mithramycin, then prior to the first dose of mithramycin and then twice daily until 24 hours after the last dose of mithramycin (46, 47).

#### **4.4 ANTIDIARRHEALS**

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

#### **4.5 ELECTROLYTE REPLACEMENT**

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

#### **4.6 QTc PROLONGATION**

If grade 3 or 4 QTc prolongation is noted on a 12-lead EKG, then repeat the 12-lead EKG to confirm finding and consult cardiology. Hold next dose until cardiology consult is obtained.

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

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

Detailed plasma pharmacokinetic (PK) sampling of mithramycin will be performed prior to and at the completion of the first dose of mithramycin during cycle 1 on the phase I and phase II portion of the trial. In addition, trough and end of infusion samples will be obtained with the day 2, 4 and 7 doses to assess mithramycin accumulation, and samples will be obtained 24 and 48 hours after the day 7 dose of mithramycin on days 8 and 9, respectively. Deviations in the timing of the PK samples will be recorded on the PK worksheets, but not reported as a protocol deviation. In addition, the omission of individual PK samples will be recorded, but not be reported as deviation

to the NCI IRB. PK analysis will be performed at the NCI, POB, PETS. Refer to details in [Appendix 4: Pharmacokinetic Worksheet for Mithramycin \(Phase I and Phase II\) For nursing staff](#) and [Appendix 5: Pharmacokinetic Worksheet for Mithramycin \(Phase I and Phase II\) For Laboratory Personnel and Research Nurses](#).

## **5.2 STUDIES ON TUMOR TISSUE**

### **5.2.1 Evaluation of NR0B1 expression on archival tumor tissue**

In all patients with Ewing sarcoma and archival tumor tissue available, evaluation of NR0B1 as target of mithramycin will be evaluated using immunohistochemistry in the Laboratory of Pathology by Dr. Maria Tsokos and Dr. Mark Raffeld (See [Appendix 7: Studies to be performed on tumor tissue](#) for details).

### **5.2.2 Confirmation of EWS/FLI1 fusion transcript for eligibility for phase II portion:**

Presence of the EWS-FLI1 fusion transcript will be determined during histologic confirmation of disease with a CLIA approved EWS-FLI1 paraffin assay in the Laboratory of Pathology CCR, NCI, unless a pathology report documenting presence of the transcript using a CLIA approved assay is obtained from the referring institution (See [Appendix 7: Studies to be performed on tumor tissue for details](#)). If archival tumor tissue is not available in paraffin blocks, 5 consecutive unstained slides and an H& E slide may be used.

### **5.2.3 Evaluation of NR0B1 expression from mandatory tumor biopsies**

In patients with Ewing sarcoma and EWS-FLI1 fusion transcript  $\geq 18$  years old, who have disease amenable to percutaneous biopsy, and who meet other eligibility criteria for biopsy, a biopsy will be obtained prior to treatment and at steady state on treatment. Patients who meet these criteria will have to agree to the biopsies in order to being able to participate in the trial.

Tissue will be collected and immediately sectioned, a portion will be separated and frozen on dry ice for RNA collection for microarray and/or gene signature evaluation of EWS-FLI1 downstream target expression (see Section 5.2.3.1). Another portion will be imbedded in paraffin and sectioned. This tissue will be stained using immunohistochemistry for NR0B1 and if tissue remains, proteins in the apoptotic pathway will be evaluated including survivin (BIRC5), XIAP, Caspase-8 (CASP8), and BCL-XL (BCL2L1) (See [Appendix 7: Studies to be performed on tumor tissue](#) for details).

### **5.2.4 Other Investigations (See [Appendix 7: Studies to be performed on tumor tissue](#))**

#### **5.2.4.1 RNA for EWS-FLI1 gene signature**

When remaining tissue is available from tumor biopsies pre treatment and at steady state, EWS-FLI1 gene signature analysis will be performed on the fluidigm platform.

#### **5.2.4.2 Microarray expression profiling**

When tissue is available, microarray expression profiling using standard Affymetrix HU.133 platform.

#### **5.2.4.3 Establishment of Cell Lines**

Finally, if adequate tissue is available and participants agree, efforts will be made to establish cell lines.

### 5.2.5 Future Research Studies

- 5.2.5.1 Serum samples for potential biomarker analysis: One serum sample will be obtained prior to cycle 1, and then at **the** same time of restaging studies after every even cycle. Details regarding sample handling and time points are provided in **Appendix 6: Serum Sample For Potential Biomarker (Phase I and Phase II)**.

## 5.3 SAMPLE STORAGE, TRACKING AND DISPOSITION

### 5.3.1 Pediatric Research Samples: *Blood Drawing Limits for Research Purposes*

Research blood sample aliquot size will be minimized for patients < 18 years of age and the total amount restricted to a maximum of 3 ml/kg per draw and 7 ml/kg per 6-week period (maximum 450 ml). If the volume of blood needed for research samples exceeds the sample limits described here, pharmacokinetic samples have priority over the serum samples for future biomarker analysis.

### 5.3.2 NIH Policy on Research Use of Stored Human Samples, Specimens, or Data

This study is coordinated by the Pediatric Oncology Branch, NCI. Sample collection and initial processing will be conducted as outlined in Appendices 4 through 8. Samples will only be labeled with study identification numbers; no personally identifiable information should be included.

Storage of pharmacokinetic and pharmacodynamic samples will occur in designated monitored freezers (-80°C) in the Pharmacology & Experimental Therapeutics Section (PETS) Laboratory, POB, NCI on 1 West. Imaging studies will be stored at the PETS laboratory on password protected computers. All samples obtained on this study will be tracked using LabMatrix. Pharmacokinetic samples will be analyzed at the NCI PETS. Samples will be identified and tracked using unique identifiers linked to each subject's unique patient number (study number). Codes linking personal identifiable information to the unique identifier will be stored in secure, computer servers with limited coded access or locked file cabinets in POB with access limited to the PI or study coordinator. Archival tumor tissue (blocks or unstained slides will be stained for NR0B1 and other markers at the Laboratory of Pathology by Dr. Tsokos. Unused material will be returned to the submitting institution upon their request. Mandatory tumor samples will be analyzed for NR0B1 expression at the Laboratory of Pathology. Gene expression signatures will be analyzed in Dr. Helman's laboratory. Samples will be stored at -80°C in the laboratory of Dr. Lee Helman. Samples will be tracked and considered the responsibility of the principal investigator. The study will remain open and status reported to the NCI IRB until all samples have been analyzed, reported or destroyed. Unintentional loss or destruction of any samples will be reported to the NCI IRB as part of annual continuing reviews. Any use of samples not outlined in Section 5 will require prospective NCI IRB review and approval.

At the termination of this protocol, if additional studies are to be performed on any samples retaining patient identifiers, obtained during the conduct of this trial, a Request to Conduct Research for Stored Human Samples Specimens, or Data Collected in a Terminated NCI-IRB Protocol will be submitted. Otherwise, specimens will be disposed of in accordance with the environmental protection laws, regulations and guidelines of the Federal Government and the State of Maryland.

## **6 DATA COLLECTION AND EVALUATION**

### **6.1 DATA COLLECTION**

The PI will be responsible for overseeing entry of data into an in-house password protected electronic system 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.

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

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

Patients will complete a patient log for reactions and symptoms related to mithramycin infusion (**Appendix 10: Patient Log for Reactions/Symptoms**), and a concomitant medication log (**Appendix 11: Patient Log for Concomitant Medication**). Physicians or care providers, who perform on study evaluations between cycles will receive instructions regarding required on study evaluations (**Appendix 12: Monitoring Toxicities Between Mithramycin Cycles with Local Physician**).

#### **6.1.1 Source Documents**

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

### **6.2 RESPONSE CRITERIA**

Objective response and progression will be evaluated in this study using the new international criteria proposed by the Response Evaluation Criteria in Solid Tumors (RECIST) Committee [version 1.1 (48)].

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.

#### **6.2.1 Response Criteria for Radiographic Studies**

##### **6.2.1.1 Measuring of Soft Tissue Disease**

Response and progression will be evaluated in this study using the new international criteria proposed by the revised Response Evaluation Criteria in Solid Tumors (RECIST) guideline [version 1.1 (48)]. Changes in the largest diameter (unidimensional measurement) of the tumor lesions and the shortest diameter in the case of malignant lymph nodes are used in the RECIST criteria.



a. Evaluation of Target Lesions

**Complete Response (CR)**

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

**Partial Response (PR)**

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

**Progressive Disease (PD)**

At least a 20% increase in the sum of the diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. (Note: the appearance of one or more new lesions is also considered progressions)

**Stable Disease (SD)**

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

b. Evaluation of Non-Target Lesions

**Complete Response (CR)**

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

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

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

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

**Progressive Disease (PD)**

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

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

c. Evaluation of Best Overall Response

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for progressive disease the smallest measurements recorded since the treatment started). The patient's best

response assignment will depend on the achievement of both measurement and confirmation criteria.

Target Lesions	Non-Target Lesions	New Lesions	Overall Response	Best Overall Response when Confirmation is Required*
CR	CR	No	CR	≥4 wks. Confirmation**
CR	Non-CR Non-PD	No	PR	≥4 wks. Confirmation**
CR	Not evaluated	No	PR	
PR	Non-CR Non-PD Not evaluated	No	PR	
SD	Non-CR Non-PD Not evaluated	No	SD	Documented at least once ≥4 wks. from baseline**
PD	Any	Yes or No	PD	No prior SD, PR or CR
Any	PD***	Yes or No	PD	
Any	Any	Yes	PD	
<p>*See RECIST 1.1 manuscript for further details on what is evidence of a new lesion.  **Only for non-randomized trials with response as primary endpoint.  ***In exceptional circumstances, unequivocal progression in non-target lesions may be accepted as disease progression</p>				

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

#### 6.2.2 Confirmatory Measurement/Duration of Response

##### a. 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. Objective response measures will be confirmed at the Coordinating Center. The participating site should forward staging studies of participants who have achieved PR or CR status to the Coordinating Center for confirmation.

##### b. 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.

### 6.2.3 Measurable Disease

Measurable lesions are defined as those that can be accurately measured in at least one dimension (longest diameter to be recorded) as  $\geq 20$  mm by chest x-ray, as  $\geq 10$  mm with CT scan, or  $\geq 10$  mm with calipers by clinical exam. All tumor measurements must be recorded in millimeters (or decimal fractions of centimeters).

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

### 6.2.4 Malignant Lymph Nodes

To be considered pathologically enlarged and measurable, a lymph node must be  $\geq 20$  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.

### 6.2.5 Non-Measurable Disease

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

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

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

### 6.2.6 Target Lesions

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

Progressive disease by RECIST criteria [1] noted after the first re-staging scan may represent disease that was not detected on the pre-study scan, and a confirmatory scan will be required at the next scheduled re-staging evaluation unless clinically not indicated. If confirmed, progression should be dated by the initial time when the lesions are first detected. If progressive disease by RECIST criteria is seen after cycle 2, but not confirmed on subsequent restaging scan, the scans from after cycle 2 would serve as the baseline scan to evaluate for disease progression (49).

#### 6.2.7 Non-Target Lesions

All other lesions (or sites of disease) including any measurable lesions over and above the 5 target lesions should be identified as **non-target lesions** and should also be recorded at baseline. Measurements of these lesions are not required, but the presence, absence, or in rare cases unequivocal progression of each should be noted throughout follow-up.

#### 6.2.8 Metastatic Bone Lesions

Disease progression is considered if a minimum of two new lesions is observed on bone scan. New lesions seen by the end of cycle 2 or before cycle 3 (with the first re-staging bone scan) may represent disease that was not detected on the pre-study scan, and a confirmatory scan will be required at the next scheduled re-staging bone scan unless clinically not indicated. If confirmed, progression should be dated by the initial time when the lesions are first detected. If new lesions are seen after cycle 2, but no additional lesions are seen on confirmatory scans, the scans from post-cycle 2 would serve as the baseline scan to evaluate for disease progression (49).

#### Clinical Lesions

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

#### 6.2.9 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.

#### 6.2.10 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.

### 6.2.11 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. All sites will submit baseline and restaging studies to NCI for volumetric analysis. (See [Error! Reference source not found.](#) for details).

## 6.3 TOXICITY CRITERIA

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

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

### 7.1 DEFINITIONS

#### 7.1.1 Adverse Event

An adverse event is defined as any reaction, side effect, or untoward event that occurs during the course of the clinical trial, whether or not the event is considered related to the treatment or clinically significant. For this study, AEs will include events reported by the patient, as well as clinically significant abnormal findings on physical examination or laboratory evaluation. A new illness, symptom, sign or clinically significant laboratory abnormality or worsening of a pre-existing condition or abnormality is considered an AE. All AEs must be recorded on the AE case report form.

All AEs, including clinically significant abnormal findings on laboratory evaluations, regardless of severity, will be followed until satisfactory resolution. AEs should be reported up to 30 days after the last administration of investigational agent/intervention and have an attribution of at least possibly related to the agent/intervention should be recorded and reported as per sections [7.2](#), [7.3](#), and [7.4](#).

An abnormal laboratory value will be considered an AE 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.

#### 7.1.2 Suspected adverse reaction

Suspected adverse reaction means any adverse event for which there is a reasonable possibility that the drug caused the adverse event. For the purposes of IND safety reporting, 'reasonable

possibility' means there is evidence to suggest a causal relationship between the drug and the adverse event. A suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug.

#### 7.1.3 Unexpected adverse reaction

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

#### 7.1.4 Serious

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

#### 7.1.5 Serious Adverse Event (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 drug experience
- Inpatient hospitalization or prolongation of existing hospitalization
- Persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
- A congenital anomaly/birth defect
- Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse drug experience when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition

#### 7.1.6 Disability

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

#### 7.1.7 Life-threatening adverse drug experience

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

#### 7.1.8 Protocol Deviation (NIH Definition)

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

#### 7.1.9 Non-Compliance (NIH Definition)

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

#### 7.1.10 Unanticipated Problem

Any incident, experience, or outcome that:

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

## 7.2 NCI-IRB AND NCI CLINICAL DIRECTOR REPORTING

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

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

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

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

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

The protocol PI will report to the NCI-IRB:

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

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

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

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

### 7.2.4 Request for waiver from IRB reporting: Anticipated PD and Expedited Non-UP AEs

Protocol evaluations and initiation of mithramycin treatment for subsequent cycles, which are performed within a window of +/- 7 days of the protocol required schedule will not be considered a deviation, and not reported to the IRB.

## 7.3 IND SPONSOR REPORTING CRITERIA

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

An investigator must immediately report to the sponsor, using the mandatory MedWatch form 3500a, any serious adverse event, whether or not considered drug related, including those listed in the protocol or investigator brochure and must include an assessment of whether there is a reasonable possibility that the drug caused the event.

- All Grade 5 (fatal) events (except death due to progressive disease) must be reported via email within 24 hours. A complete report must be submitted within one business day.
- All other serious adverse events including deaths due to progressive disease must be reported within one business day

Study endpoints that are serious adverse events (e.g. all-cause mortality) must be reported in accordance with the protocol unless there is evidence suggesting a causal relationship between the drug and the event (e.g. death from anaphylaxis). In that case, the investigator must immediately report the death to the sponsor.

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

### 7.3.2 Annual Reporting of AEs to the Sponsor

## 7.4 FDA REPORTING CRITERIA

### 7.4.1 IND Safety Reports to the FDA (Refer to 21 CFR 312.32)

The Sponsor will notify the FDA of any unexpected fatal or life-threatening suspected adverse reactions as soon as possible but no later than 7 calendar days of initial receipt of the information using the MedWatch Form 3500a.

The Sponsor is also responsible for reporting:

- any suspected adverse reaction that is both serious and unexpected;
- any findings from clinical, epidemiological, or pooled analysis of multiple studies or any findings from animal or in vitro testing that suggest a significant risk in humans exposed to the drug;
- clinically important increase in the rate of a serious suspected adverse reaction over that listed in the protocol or investigator brochure;

to the FDA and to all investigators no later than 15 calendar days after determining that the information qualifies for reporting using the MedWatch Form 3500a. If FDA requests any



additional data or information, the sponsor must submit it to the FDA as soon as possible, but no later than 15 calendar days after receiving the request.

#### 7.4.2 FDA Annual Reports (Refer to 21 CFR 312.33)

The study Sponsor will submit a brief report annually of the progress of the trial within 60 days of the anniversary date that the IND went into effect as indicated in 21CFR 312.33, and any associated FDA correspondences regarding the IND annual report.

### **7.5 DATA AND SAFETY MONITORING PLAN**

#### 7.5.1 Principal Investigator/Research Team

The clinical research team will meet on a regular basis, biweekly when patients are being actively treated on the trial to discuss each patient in detail. The PI will meet regularly with participating site investigators during study enrollment and treatment. Decisions about dose level enrollment and dose escalation during the phase I portion will be made based on the toxicity data from prior patients.

All data will be collected in a timely manner, entered into C3D and reviewed by the principal investigator or a lead associate investigator in a timely manner. Adverse events will be reported as required above. Any safety concerns, new information that might affect either the ethical and or scientific conduct of the trial, or protocol deviations will be immediately reported to the IRB using iRIS and to the Sponsor.

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

#### 7.5.2 Sponsor Monitoring Plan

This trial will be monitored by personnel employed by Harris Technical Services on contract to the NCI, NIH. 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.

At least 25% of enrolled patients will be randomly selected and monitored at least biannually, based on accrual rate. The patients selected will have 100% source document verification done. Additional monitoring activities will include: adherence to protocol specified study eligibility, treatment plans, data collection for safety and efficacy, reporting and time frames of adverse events to the NCI IRB and FDA, and informed consent requirements. Written reports will be generated in response to the monitoring activities and submitted to the Principal investigator and Clinical Director or Deputy Clinical Director, CCR, NCI.

## **8 STATISTICAL CONSIDERATIONS**

### **8.1 PHASE I PORTION**

The phase I portion of this study will enroll patients in a standard 3 + 3 design as described in Section [Error! Reference source not found.](#). The cohort at the recommended dose or MTD will be expanded to 12 patients, and attempts will be made to enroll at least 6 patients that are  $\geq 12$  years of age and 6 patients that are  $< 12$  years of age to gain experience with a broad age range of patients.

The maximum number of evaluable patients for this phase is 18 (6 patients at 2 dose levels and 6 to expand MTD to 12).

## 8.2 PHASE II PORTION

The primary endpoint will be objective response by RECIST criteria v 1.1. The best response of disease to mithramycin will be examined. The following outlines the Simon optimal two stage design that will be used:

	Cumulative Number of Responses	Decision
Stage 1: Enter 9 patients	0	Terminate the trial: agent ineffective
	1 or more	Inconclusive result, continue trial (proceed to Stage 2)
Stage 2: Enter 15 additional patients	2 or fewer	Terminate the trial: agent ineffective
	3 or more	Terminate the trial: agent effective

We will consider mithramycin not of sufficient interest for further evaluation in a disease category if the true response rate is 5% ( $p_0=0.05$ ) and of sufficient activity if the true response rate is 25% ( $p_1=0.25$ ). As summarized in the above table, with  $\alpha=0.10$  (10% probability of accepting an ineffective treatment) and  $\beta=0.10$  (10% probability of rejecting an effective treatment), the study will initially enroll 9 patients and if 0 of 9 have a response, then no further patients will be enrolled. If 1 or more respond, accrual will continue until a total of 24 evaluable patients have been enrolled. After 24 patients have been enrolled and evaluated, 1-2 with a response will be considered insufficient while if 3 or more respond in 24 patients, this will be considered sufficient activity for further investigation. Under the null hypothesis, the probability of early termination is 63%.

The two patients that were enrolled in the 25 mcg/kg dose of the Phase II portion will be evaluated for response but not be counted as evaluable for the design above, as they received mithramycin at a higher single dose (25 mcg/kg) than the new phase II dose (17.5 mcg/dose) determined with amendment B. The accrual ceiling will be increased by two patients to allow for these non-evaluable patients.

If the pediatric phase II dose of mithramycin determined on this trial differs from the adult phase II dose, the protocol will be amended to address accrual and response rate to mithramycin in children in a separate stratum.

### 8.2.1 Methods of Analysis

Patients who are considered evaluable for response will be included in an analysis of time to progression. Time to progression will be estimated using the product-limit method of Kaplan and Meier. The probability of progression free survival at 6 months will be summarized. Response rates will be calculated as the percent of patients whose best response is a CR or PR, and the fraction of responses obtained will have a 95% confidence interval, which takes into consideration the two-stage nature of the design (50). Toxicity information recorded will include the type,

severity, time of onset, time of resolution, and the probable association with the study regimen. Tables will be constructed to summarize the observed incidence by severity and type of toxicity.

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

### **8.3 EVALUATION OF RESPONSE**

Any patient who is enrolled and receives at least one dose 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 cycle and the tumor is not removed surgically prior to the time complete response or partial response is confirmed; or (3) the patient demonstrates a complete or partial response as confirmed according to protocol criteria. Patients who electively terminate therapy before receiving all 7 doses of mithramycin during the first treatment cycle and do not expire within 28 days from start of treatment will be replaced. Patients who demonstrate a complete or partial response will be considered to have experienced a response. The evaluation period for determination of the best response will be 6 treatment cycles. All other patients will be considered non-responders.

### **8.4 INHIBITION OF NR0B1 IN TUMOR TISSUE AND CHANGES IN GENE EXPRESSION SIGNATURE**

It will be important to determine if mithramycin inhibits NR0B1 in at least a modest fraction of patients in order to confirm that the agent is correctly meeting its intended target. This will require that biopsies be obtained on subjects 18 years or older with accessible biopsy sites, at both pre-treatment as well as on treatment. Given that no more than 42 subjects are expected to enroll onto this study as a maximum, the accrual target for this endpoint will be based on no more than 50% of the maximum enrollment projected. This portion of the study will be conducted as a pilot using up to 20 evaluable subjects. With 20 subjects, there would be 90% power to detect a difference between an unacceptably low fraction with inhibition of 5% and a more modestly acceptable fraction of 25% using a one-sided 0.10 alpha level exact test for a binomial proportion. If only 12 patients are able to provide biopsy results at both times, there would be 90% power to detect a difference between 5% and 45% with inhibition. Thus, while up to 20 patients with paired biopsies would be desirable, as few as 12 patients with the complete evaluation would be adequate to address this to some degree.

Should less than 12 patients with evaluable tumor biopsies be available at the time the toxicity and response objectives are met, enrollment on this trial will continue for patients undergoing tumor biopsies until a minimum of 12 patients with tumor biopsies evaluable for analysis of NR0B1 inhibition have been enrolled. In order to be evaluable for analysis of NR0B1 inhibition, NR0B1 expression has to be present on the tumor biopsy obtained at baseline.

### **8.5 SECONDARY ENDPOINTS**

Pharmacokinetic analysis will be conducted using non-compartmental methods and estimated pharmacokinetic parameters will be presented for each dose level using summary statistics. When

pharmacodynamic studies are analyzed, descriptive statistics for each dose level will be calculated for each surrogate endpoint.

The associations and differences between tumor burden and change in tumor burden as measured by RECIST, WHO, and volumetric analysis will be summarized with scatter plots and correlation coefficients. The results of these descriptive analyses will be used to design further studies.

## **8.6 NUMBER AND RATE OF ACCRUAL**

If a maximum of 18 patients are required to complete the phase I portion, and 9 to 24 patients are required to complete the phase II portion, the maximum number of evaluable patients required to complete both portions of this trial is 42.

With an estimated monthly accrual of 1-2 patients, the projected duration of this study is 2 to 3.5 years.

## **9 HUMAN SUBJECTS PROTECTIONS**

### **9.1 RATIONALE FOR SUBJECT SELECTION**

The Ewing sarcoma family of tumors (ESFT) is the second most common malignant bone tumor of childhood. In the past 25 years since the advent of 5 drug ESFT therapy, there has been very little improvement in overall patient survival particularly for patients with high risk metastatic or relapsed disease. Laboratory research has demonstrated potential efficacy of mithramycin in Ewing Sarcoma Family of Tumors with EWS-FLI1 fusion transcript.

No groups, in regards to gender, and racial and ethnic groups, are being excluded from participation in the trial. Females who are pregnant or breastfeeding will not be eligible for the trial due to risks of fetal and teratogenic adverse events as seen animal studies.

### **9.2 PARTICIPATION OF CHILDREN**

This study is designed to define the toxicities and maximum tolerated dose of mithramycin in children and adolescents with solid tumors. It will also evaluate the activity of mithramycin in the treatment of children and adolescents, as well as adults with Ewing Sarcoma Family of Tumors with EWS-FLI1 fusion transcript. Therefore children and adolescents will be entered onto this research study. Adults ( $\geq 18$  yrs) who are cognitively impaired prior to study entry and who have not previously assigned DPA to a family member or friend will not be eligible for the trial, because they cannot give informed consent. Pediatric and adult oncologists who have extensive experience in performing investigational drug trials in children will conduct the trial. Patients will be cared for in the POB outpatient pediatric oncology clinic or outpatient setting and if patients require hospital admission, they will be cared for on the pediatric unit of the CRC by the POB staff. Patients  $> 35$  years old will be cared for by adult oncologists.

### **9.3 EVALUATION OF BENEFITS AND RISKS/DISCOMFORTS**

Patients under the age of 18 years will be entered on this trial. The primary risk to patients participating in this research study is from toxicity of mithramycin, an investigational agent in this patient population. The protocol provides for detailed and careful monitoring of all patients to assess for toxicity and the dose escalation scheme is very conservative. Toxicity data from the current dose level will be collected and reviewed to ensure that there were no severe (dose-limiting) toxicities prior to escalating to the next higher dose level. Although the primary objective

of the phase I portion of this trial is to define the qualitative toxicities and maximum tolerated dose of the drug, all patients entered on the trial will have refractory or recurrent disease, which this agent may benefit. Patients enrolled on the phase II portion of the study will be diagnosed with Ewing Sarcoma Family of Tumors with EWS-FLI1 fusion transcript, for which laboratory data suggested this agent may be of benefit. Mithramycin offers a potential for direct benefit although the likelihood of this may be small. The potential benefits from this therapy are disease stabilization or shrinkage and a reduction in symptoms caused by the tumor. Therefore, this protocol involves greater than minimal risk, but presents the potential for direct benefit to individual subjects.

Mandatory serial tumor biopsies will be obtained in participants with Ewing sarcoma with EWS-FLI1 fusion transcript who are 18 years of age or older, who have disease amenable to biopsy as outlined in Section **Error! Reference source not found.** Risks include risk of bleeding, bruising, pain or rarely, infection.

#### **9.4 CONSENT AND ASSENT PROCESS AND DOCUMENTATION**

The investigational nature and research objectives of this trial, the procedures and treatments involved and their attendant risks and discomforts and benefits, and potential alternative therapies will be carefully explained to the patient or the patient's parents or guardian if he/she is a child, and a signed informed consent document will be obtained prior to entry onto the study. The investigators are requesting a waiver from the IRB to allow only one parent to sign the informed consent to enter a child on the protocol. Because many patients must travel to the NIH from long distances at substantial expense, requiring both parents to be present for the consent process could be a financial hardship for many families. The PI or an associate investigator of the trial will obtain consent. Where deemed appropriate by the clinician and the child's parents or guardian, the child will also be included in all discussions about the trial and verbal assent will be obtained. The parent or guardian will sign the designated line on the informed consent attesting to the fact that the child has given assent.

### **10 PHARMACEUTICAL INFORMATION**

#### **10.1 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.

##### 10.1.1 Source

Mithramycin was supplied by Fermentek Ltd (Jerusalem, Israel) to the NCI under an IND held by the CCR. The NIH Clinical Center Pharmacy will be testing and vialing the investigational agent.

Drug will be shipped to participating centers upon receipt of IRB approval.

##### 10.1.2 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 <http://www.drugs.com/mmx/mithracin.html - citec00152801>
- Gastrointestinal bleeding (bloody or black, tarry stools; vomiting of blood)
- hepatotoxicity (yellow eyes or skin) ALT and AST (severe) and GGT elevation
- LDH elevation
- 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.

10.1.3 Formulation and preparation

Mithramycin is supplied as 2 mg/vial, freeze dried powder for injection. To prepare the initial dilution of 500 mcg 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
100-249	100

All dilutions will be infused over 6 hours.

#### 10.1.4 Stability and Storage

Prior to reconstitution, store between 2 and 8 °C (36 and 46 °F). Store in a light-resistant container.

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

#### 10.1.5 Administration procedures

Infuse intravenously over 6 hours using chemotherapy precautions. Infusion bags have to be protected from light from the time of preparation through completion of the infusion. Observe frequently for signs of extravasation. Should extravasation occur, the infusion should be terminated at that site and reinstated at another site. Moderate heat should be applied to the site of extravasation to disperse the drug and minimize local tissue irritation and discomfort. No in-line filter should be used for mithramycin administration.

#### 10.1.6 Contraindications

The following medications are contraindicated:

- Anticoagulants, coumarin- or indandione-derivatives
- Heparin other than heparin flushes
- Thrombolytic agents
- Anti-inflammatory drugs, nonsteroidal (NSAIDs) or aspirin or salicylate-containing products, which may increase risk of hemorrhage
- Dextran
- Dipyridamole
- Sulfinpyrazone
- Valproic acid

## **10.2 DEXAMETHASONE**

### 10.2.1 Source:

Dexamethasone will be provided from commercial sources by the NIH Clinical Center Pharmacy Department.

### 10.2.2 Toxicity:

#### Common

Cardiovascular: Hypertension

Dermatologic: Atrophic condition of skin, Finding of skin healing, Impaired

Endocrine metabolic: Cushing's syndrome, Decreased body growth

Gastrointestinal: Disorders of gastrointestinal tract

Immunologic: At risk for infection

Musculoskeletal: Osteoporosis

Ophthalmic: Cataract (5%), Raised intraocular pressure (25%)

Psychiatric: Depression, Euphoria

Respiratory: Pulmonary tuberculosis

#### Serious

Endocrine metabolic: Hyperglycemia, Primary adrenocortical insufficiency

Ophthalmic: Conjunctival hemorrhage (22%), Glaucoma, Vitreous detachment (2%)

Musculoskeletal and connective tissue disorders: Avascular Necrosis

### 10.2.3 Formulation and preparation:

Oral Tablet (Scored): 4 mg

Injection, solution, as sodium phosphate: 4 mg/mL (1 mL, 5 mL, 30 mL);

### 10.2.4 Administration procedures:

Oral: Administer with meals to decrease GI upset.

I.V.: Administer intravenously over 10 minutes.

### 10.2.5 Incompatibilities

Contraindicated: Praziquantel (theoretical), Rotavirus Vaccine, Live (established)

Major: Aldesleukin (theoretical), Bupropion (theoretical), Darunavir (theoretical), Dasatinib (theoretical), Etravirine (theoretical), Fosamprenavir (theoretical), Imatinib (theoretical), Ixabepilone (theoretical), Lapatinib (theoretical), Nilotinib (theoretical), Quetiapine (probable), Romidepsin (theoretical), Sunitinib (theoretical), Temsirolimus (theoretical), Thalidomide (probable)



## 11 APPENDICES

### 11.1 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
F Fraisse (51)	1980	26	Hypercalcaemia in pregnancy	-	25 mcg/kg	daily x4	Day 4 fever 40° C, shock, SGOT 1400 mUI/L, SGPT 1600 mUI/L, plt 52,000, hemothorax, diffuse hemorrhage, hepatic necrosis, death	-	-	-
Donaldson(52)	1985	NONE								
Kofman (33) "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, restlessness, irritability "Hepatic and renal damage not major factors except in patients who received very large doses or who had poor hepatic or renal function at start"	" a surprising factor was severe cumulative toxicity -again hemorrhage on second course with PLT >50	50 ug/kg tried	
Dutcher (42)	1997	35-74 (median 51)	CML (mithra + interferon)	13 pts 2SD 3PR 1CR	25 ug/kg over 2-4 hrs	M, W,F for 2 weeks then one dose Qmonth	1 patient removed for "hepatotoxicity" not defined	Not defined	Not defined	
Thurlimann, B (43)	1992	27-74 (median 62)	Hypercalcaemia	N/A	25 ug/kg	Day 1	-Vomiting Moderate 18% Severe 5% -Phlebitis 11% -Fever 9% -Mean 2x AST/ALT -DEATH arterial bleed from duodenal ulcer 1 Patient *thrombocytopenia 5%			
Lebbin, D (32)	1974	47-74 yrs	10 patients with Paget's	10/10 with improved pain and increased activity	25 ug/kg	Daily x 10 for cycle 1 Then A = Daily x5 every	-N/V – responded to anti-emetics NO dose reductions -uremia- 7/10 -thrombocytopenia 1/10 (<150k)	-no evidence of cumulative hepatotox.		

				(all regimens effective)		week for a month Vs B = Q week for 14 months Vs B1 = twice weekly for 5 months	NO hemorrhage-withheld drug if PLT<150k -Isocitrate Dehydrogenase elevation 1/10			
Johnson, P.R.E (53)	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 (54)	1968	42-83 (mean 61)	Various -1 fibrosarcoma	32 patients 12- no change 7 minor regression 1 major regression (rectal CA)	25 ug/kg over 12 hrs	Daily x 8	11 patients tolerated 8 day course -15 4-7 days -6 < 4days -none-10 -Anorexia/nausea 9 pts -Severe vomiting 5 pts -thrombocytopenia 4 pts -diarrhea 3 pts -CNS symptoms 3 patients -thrombophlebitis 3 pts SGOT, SGPT, LDH, and AP were carefully watched. Occasional patient developed a marked rise in LDH in absence of other changes in LFTs.	Only one patient got a second course separated by a month -stopped with vomiting		
Sewell, I. A. (31)	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 (41)	1965	26-64	Glioblastoma	14	25 ug/kg over 8 hours	Daily times 8	2 required held drug for thrombocytopenia, N/V -otherwise limited toxicity			<sup>3</sup> H drug crosses BBB

			(8 patients with improvements) 3 (3+), 2 (2+), 2 (2+)	-tx with surgery + radiation +mithra						****
Walker (26)	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 daily times 12 -then 6 wks later daily times 12 -83% got 11 doses -median is 21 doses	-N/V in 58% of patients -mucositis -anemia (60%) mild to moderate -leukopenia (20%) -thrombocytopenia <25,000/ $\mu$ L only 2 pts. -LFTs-mild 30%, moderate-severe 50% in ALT/AST LDH elevation of 18 pts 83 % >400 units  -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 (21)	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			
Kofman, s (40)	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(28)	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 LFT changes common at doses >50 mcg/kg/day (increase AP, GOT, decrease PT); 2 pts SGOT of 800 U		50 $\mu$ g/dose	

Spear (29)	1963	58 patients (only 29 with performance status >50)	Variety of adult melanoma, bronchogenic CA, hepatoma	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
Dube (55)		7 pts adults	Advanced metastatic cancer		25 to 30 mcg/kg/day as cont. infusion	For 5-13 days until toxicity	In all pts hematologic and hepatic toxicity: decrease WBC and plt, increase SGOT			
Package insert Pfizer (25)	1971	1600 patients	Testicular CA		25 ug/kg/day over 12 hours daily x 8 q month  dilute in 1L of d5 and give over 4-6hrs	Q month	Contraindicated: thrombocytopenia, coagulopathy, poor bone marrow, follow PLTs, PT and bleeding time -hemorrhagic syndrome is dose related if 30 ug/kg/day or less 10 or fewer bleeds -drug mortality rate is 1.6% IF >30 12% bleeding with mortality rate of 5.7% COMMON: Anorexia, N/V, stomatitis LESS COMMON: Fever, drowsiness, lethargy, malaise, H/A, depression, phlebitis, flushing, skin rash LABS: Thrombocytopenia, leucopenia (6%), increased clotting, bleeding time, abnormal clot retraction, LFTs, AP, LDH, bili Renal, Increased BUN, CR and proteinuria Hypocalcemia, hypophosphatemia, hypokalemia			

**11.2 APPENDIX 2: PERFORMANCE STATUS SCALES/SCORES**

<b>PERFORMANCE STATUS CRITERIA</b> <i>Karnofsky and Lansky performance scores are intended to be multiples of 10.</i>					
<b>ECOG (Zubrod)</b>		<b>Karnofsky</b>		<b>Lansky</b>	
<b>Score</b>	<b>Description</b>	<b>Score</b>	<b>Description</b>	<b>Score</b>	<b>Description</b>
0	Fully active, able to carry on all pre-disease performance without restriction.	100%	Normal, no complaints, no evidence of disease.	100%	Fully active, normal.
		90%	Able to carry on normal activity; minor signs of symptoms of disease.	90%	Minor restrictions in physically strenuous activity.
1	Restricted in physically strenuous activity but ambulatory, able to carry out light or sedentary work, e.g., light housework, office work.	80%	Able to carry on normal activity with effort; some signs or symptoms of disease.	80%	Active, but tires more quickly.
		70%	Cares for self, unable to carry on normal activity or do active work.	70%	Both greater restriction of, and less time spent in, play activities.
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours.	60%	Requires occasional assistance but is able to care for most of own needs.	60%	Up and around, but minimal active play; keeps busy with quieter activities.
		50%	Requires considerable assistance and frequent medical care.	50%	Gets dressed, but lies around much of the day; no active play; able to participate in quiet play and activities.
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours	40%	Disabled; requires special care and assistance.	40%	Mostly in bed; participates in quiet activities.
		30%	Severely disabled; hospitalization indicated, although death not imminent.	30%	In bed; needs assistance even for quiet play.
4	Completely disabled. Cannot carry on any self-care. Totally confined to a bed or chair	20%	Very ill; hospitalization necessary; active supportive treatment required.	20%	Often sleeping; play entirely limited to very passive activities.
		10%	Moribund, fatal process progressing rapidly	10%	No play; does not get out of bed
5	Dead	0%	Patient expired	0%	Unresponsive; Dead

### 11.3 APPENDIX 3: STUDY CALENDAR

Procedure <sup>P</sup>	Screening/ Baseline <sup>**</sup>	Cycle 1 (28 Days) and subsequent cycles														Post Therapy Follow-up
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 14	Day 21	Day 28	At least 30 days after last mithramycin dose <sup>m</sup>	
History, PE <sup>a</sup>	X	X <sup>a</sup>						X <sup>a</sup>				X <sup>a</sup>	X <sup>a</sup>	X <sup>a</sup>	X <sup>a</sup>	
Weight <sup>aa</sup>	X		X	X	X	X	X	X								
Height (baseline only)	X															
Vital signs (BP, HR, RR, temp)	X	X	X	X	X	X	X	X								
Performance Score	X													X	X	
Histologic Path confirmation	X															
<ul style="list-style-type: none"> <li>CBC, differential</li> <li>sodium, potassium, chloride, CO2, creatinine, glucose, BUN, albumin, calcium, magnesium, phosphorus, uric acid LDH and (amylase, lipase, baseline only)</li> <li>alk phos, ALT/AST, T bilirubin, Direct bilirubin, T protein</li> </ul>	X		X <sup>b</sup>	X <sup>b</sup>	X <sup>b</sup>	X <sup>b</sup>	X <sup>b</sup>	X <sup>b</sup>		X <sup>b</sup>	X <sup>b</sup>	X <sup>b</sup>	X <sup>b</sup>	X <sup>b</sup>	X	
<ul style="list-style-type: none"> <li>Plasma sample (1mL)<sup>c</sup></li> </ul>		X <sup>c</sup>														
<ul style="list-style-type: none"> <li>PT/PTT, Thrombin Time, Fibrinogen<sup>d</sup></li> </ul>	X <sup>d</sup>	X <sup>d</sup>		X <sup>d</sup>	X <sup>d</sup>	X <sup>d</sup>		X <sup>d</sup>	X <sup>d</sup>	X <sup>d</sup>					X	
<ul style="list-style-type: none"> <li>HIV, HBV, HCV<sup>e</sup></li> </ul>	X <sup>e</sup>															
<ul style="list-style-type: none"> <li>Pregnancy Test (urine)<sup>f</sup></li> </ul>	X <sup>f</sup>	X <sup>f</sup>														

Procedure <sup>p</sup>	Screening/ Baseline <sup>**</sup>	Cycle 1 (28 Days) and subsequent cycles														Post Therapy Follow-up
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 14	Day 21	Day 28	At least 30 days after last mithramycin dose <sup>m</sup>	
• Urinalysis	X <sup>ff</sup>	X <sup>ff</sup>														
• Coags: , PFA-100, VWF antigen, VWF activity, Factor VIII activity, D-dimer, fibrin degradation products, FXIII activity	X															
Mithramycin infusion		X	X	X	X	X	X	X								
Receive oral/IV dexamethasone <sup>o</sup>		X	X	X	X	X	X	X	X							
MUGA or echocardiogram	X															
12-lead EKG <sup>n</sup>	X	X			X <sup>n</sup>			X								X
Radiological Assessments: CT, MRI, FDG-PET, Bone scan <sup>g</sup>	X													X <sup>g</sup>		X
Pharmacokinetics <sup>h</sup>		X <sup>h</sup>	X <sup>h</sup>		X <sup>h</sup>			X <sup>h</sup>	X <sup>h</sup> (and D9)							
Serum Pharmacodynamic Marker <sup>i</sup>		X <sup>i</sup>														
Mandatory biopsy for NR0B1 expression <sup>j</sup>	X <sup>j</sup>				X <sup>j</sup>											
Adverse Events <sup>k</sup>		X	—————→											X		
Concomitant Medications <sup>l</sup>		X	—————→											X		

<sup>\*\*</sup>Blood tests within 7 days prior to enrollment; imaging studies within 2 weeks prior to enrollment (UNLESS otherwise stated), histological path evaluation is recommended to be completed prior to arrival

<sup>a</sup>Weekly during active treatment, and weekly until recovered from toxicity.

<sup>aa</sup>Daily weight recommended for days 2-7 of mithramycin infusion.

<sup>b</sup>Daily on days 2 through 7, then twice weekly days 9 to 28 of cycles 1 through 3

<sup>c</sup>1 mL of plasma will be obtained, filtered through a 0.22 micron filter, frozen and saved by hematology pathology for future reference if needed, prior to receiving drug on day 1 of cycle 1 and 2 only. See **Error! Reference source not found.** for details.

<sup>d</sup>Coagulation studies: drawn on days 1, (3, 4 or 5) and (7, 8 or 9) or as clinically indicated. If screening coagulation studies drawn within 1 week of starting drug, day 1 can be omitted for cycle 1 only.

<sup>e</sup>Excluded by history only. No laboratory testing of HIV, HBV or HCV is required.

<sup>f</sup>required at screening/prior to cycle 1 and prior to each subsequent cycle on females of childbearing potential

<sup>g</sup>required at screening/prior to cycle 1 and prior to each subsequent cycle

<sup>g</sup>performed after every EVEN numbered treatment cycle, i.e. cycle 2, cycle 4, etc until off therapy and then every two months until off study or as indicated by clinical response criteria see (section 6.2). Imaging studies will be sent to NCI POB for volumetric analysis (see **Error! Reference source not found.** for details).

<sup>h</sup>PK samples: prior to and at the completion of the first infusion, then trough and end of infusion samples with day 4 and 7 doses, and 24 and 48 hours after day 7 dose. Samples will therefore be drawn on days 1, 2, 4, 7, 8, 9 as indicated in **Error! Reference source not found.** and **Error! Reference source not found.**

<sup>i</sup>A serum sample for potential biomarker analysis will be obtained prior to the first mithramycin injection, and then at the time of each restaging studies. See **Error! Reference source not found.** for details.

<sup>j</sup>Eligibility and timing for mandatory biopsy is defined in **Error! Reference source not found.**

<sup>k</sup>See **Error! Reference source not found.** to obtain sheet to record adverse events at home

<sup>l</sup>See **Error! Reference source not found.** to record all medications taken at home

<sup>m</sup>Post therapy evaluations will be completed until at least 30 days after the last dose of mithramycin and until resolution of toxicity.

<sup>n</sup>All EKGs should be done prior to infusion. EKG on day 4 to be done on cycle 1 only.

<sup>o</sup>2.5 mg dexamethasone/m<sup>2</sup> BSA PO or IV starting 12 hours before the first dose of mithramycin, then prior to the first dose, and then twice daily for 24 hours after the last dose of mithramycin

<sup>p</sup>For patients who have discontinued mithramycin infusions during any cycle prior to completion of the 7 daily infusions, the required laboratory studies and other evaluations can be adjusted by the principal investigator or associate investigator as necessary.



**11.4 APPENDIX 4: PHARMACOKINETIC WORKSHEET FOR MITHRAMYCIN (PHASE I AND PHASE II) FOR NURSING STAFF**

**DAY 1**

Patient ID		Weight (kg), height (cm), BSA (m <sup>2</sup> )	
Dose level (mg/kg)		Total dose (mg)	
Date:		Infusion start time	
		Infusion stop time	
Pre-dose EKG time:		Signature of person completing EKG:	

Sample #	Hour	Target Time	Actual Time	Signature
1	pre dose			
2	3 h post-start of infusion			
3	Just prior to the end of the 6 hour infusion			
4	0.25 h post-infusion			
5	0.5			
6	1			
7	2			
8	3			
9	5			
10	7			
11*	9-12			

\*If feasible

Collect 3 mL blood in a lavender top tube. 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 hr after collection. The plasma should be transferred to separate pre-labeled screw-capped polypropylene transfer tubes and stored at -80°C until shipped to the analytical site.

Patient ID		Weight (kg), height (cm), BSA (m <sup>2</sup> )	
Dose level (mg/kg)		Total dose (mg)	

**Day 2 Date:** \_\_\_\_\_ **Infusion start time:** \_\_\_\_\_ **Stop time:** \_\_\_\_\_

Sample #	Hours	Target Time	Actual Time	Signature
1	pre dose			
2	End of infusion			

**Day 4 Date:** \_\_\_\_\_ **Infusion start time:** \_\_\_\_\_ **Stop time:** \_\_\_\_\_

**Pre-dose EKG Time:** \_\_\_\_\_ **Signature of person completing EKG:** \_\_\_\_\_

Sample #	Hours	Target Time	Actual Time	Signature
1	pre dose			
2	End of infusion			

**Day 7 Date:** \_\_\_\_\_ **Infusion start time:** \_\_\_\_\_ **Stop time:** \_\_\_\_\_

**Pre-dose EKG Time:** \_\_\_\_\_ **Signature of person completing EKG:** \_\_\_\_\_

Sample #	Hours	Target Time	Actual Time	Signature
1	pre dose			
2	End of infusion			

**Day 8\* Date:**

Sample #	Hours	Target Time	Actual Time	Signature
1	24 hours after day 7 dose			

**Abbreviated Title:** Ph I/II Pediatric Mithramycin  
**Version Date:** 11/09/2016

2	24.5 hours after day 7 dose			
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**Day 9\* Date:**

Sample #	Hours	Target Time	Actual Time	Signature
*1	48 hours after day 7 dose			
*2	48.5 hours after day 7 dose			

\*If feasible

Collect 3 mL blood in a lavender top tube. 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 hr after collection. The plasma should be transferred to separate pre-labeled screw-capped polypropylene transfer tubes and stored at -80°C. Samples will be transported on dry ice to:

Diane Cole  
 Pharmacology and Experimental Therapeutics Section  
 Pediatric Oncology Branch, NCI  
 10 Center Drive, Building 10, room 1-5750.  
 Bethesda, MD 201892-1101.  
 Phone: 301-496-1757  
 E-mail: [coled@mail.nih.gov](mailto:coled@mail.nih.gov)

Please notify Ms. Cole by e-mail or phone prior to transport of the samples.

**11.5 APPENDIX 5: PHARMACOKINETIC WORKSHEET FOR MITHRAMYCIN (PHASE I AND PHASE II) FOR LABORATORY PERSONNEL AND RESEARCH NURSES**

**DAY 1**

Patient ID		Weight (kg), height (cm), BSA (m <sup>2</sup> )	
Dose level (mg/kg)		Total dose (mg)	
Date:		Infusion start time	
		Infusion stop time	

Sample #	Hour	Time Specimen in Lab	Time in Centrifuge	Time in Freezer	Signature
1	pre dose				
2	3 h post-start of infusion				
3	Just prior to the end of the 6 hour infusion				
4	0.25 h post-infusion				
5	0.5				
6	1				
7	2				
8	3				
9	5				
10	7				
11*	9-12				

\*If feasible

Collect 3 mL blood in a lavender top tube. 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 hr after collection. The plasma should be transferred to separate pre-labeled screw-capped polypropylene transfer tubes and stored at -80°C until shipped to the analytical site.

Patient ID		Weight (kg), height (cm), BSA (m <sup>2</sup> )	
Dose level (mg/kg)		Total dose (mg)	

**Day 2 Date:**                      **Infusion start time:**                      **Stop time:**

Sample #	Hours	Time Specimen in Lab	Time in Centrifuge	Time in Freezer	Signature
1	pre dose				
2	End of infusion				

**Day 4 Date:**                      **Infusion start time:**                      **Stop time:**

Sample #	Hours	Time Specimen in Lab	Time in Centrifuge	Time in Freezer	Signature
1	pre dose				
2	End of infusion				

**Day 7 Date:**                      **Infusion start time:**                      **Stop time:**

Sample #	Hours	Time Specimen in Lab	Time in Centrifuge	Time in Freezer	Signature
1	pre dose				
2	End of infusion				

**Day 8\* Date:**

Sample #	Hours	Time Specimen in Lab	Time in Centrifuge	Time in Freezer	Signature
1	24 hours after day 7 dose				

2	24.5 hours after day 7 dose				
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**Day 9\* Date:**

Sample #	Hours	Time Specimen in Lab	Time in Centrifuge	Time in Freezer	Signature
*1	48 hours after day 7 dose				
*2	48.5 hours after day 7 dose				

\*If feasible

Collect 3 mL blood in a lavender top tube. 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 hr after collection. The plasma should be transferred to separate pre-labeled screw-capped polypropylene transfer tubes and stored at -80°C. Samples will be transported on dry ice to:

Diane Cole  
 Pharmacology and Experimental Therapeutics Section  
 Pediatric Oncology Branch, NCI  
 10 Center Drive, Building 10, room 1-5750.  
 Bethesda, MD 201892-1101.  
 Phone: 301-496-1757  
 E-mail: [coled@mail.nih.gov](mailto:coled@mail.nih.gov)

Please notify Ms. Cole by e-mail or phone prior to transport of the samples.

## 11.6 APPENDIX 6: SERUM SAMPLE FOR POTENTIAL BIOMARKER (PHASE I AND PHASE II)

### Materials required:

- Red top glass, no additive, no clot activator with uncoated interior, vacutainer tubes (examples: BD366397, BD366442, BD366441).
- Polypropylene screw top freezer tubes, 2 ml (examples: Nalgene LX23633 or Nunc 377267).

### Procedure:

- 1) Obtain a peripheral blood sample. Fill red top tube approximately three-fourths full (the tube will automatically stop filling when the vacuum is gone).
- 2) The tube of whole blood should be allowed to sit for 40 - 50 minutes to allow clotting to complete.
- 3) Centrifuge the tube at 3000rpm for 10 minutes.
- 4) Using a transfer pipette, remove the serum from the tube and dispense into the freezer tubes provided in aliquots of 1 mL.
- 5) Label the freezer tube with the study ID number patient ID number, and date. Complete information below:

Sample #	Patient ID number:	Treatment	Date of sample collection:
1		Prior to treatment	
2		Pre cycle 3	
3		Pre cycle 5	
4		Pre cycle 7	
5		Pre cycle 9	
6		Pre cycle__*	

\*Subsequent samples prior to each restaging cycles.

- 6) Freeze at  $-40^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$  within 2 hours of phlebotomy. Freeze the tubes in an upright position.
- 7) Store the frozen samples at  $-40^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$ .
- 8) Deliver samples on dry ice to

Brigitte Widemann, MD  
NCI, POB  
10 Center Drive, Building 10 CRC  
Room 1-5750, MSC 1101  
Bethesda, MD 20892  
Phone: 301-496-7387

## **11.7 APPENDIX 7: STUDIES TO BE PERFORMED ON TUMOR TISSUE**

### **Archival tumor tissue**

a) Archival paraffin embedded tumor tissue for confirmation of EWS/FLI1 fusion transcript to determine eligibility for phase II portion of trial, and for evaluation of NR0B1 expression in patients with Ewing sarcoma

Archival paraffin embedded tissue, or if paraffin embedded tissue is not available, 5 consecutive unstained slides, should be sent via express carrier to:

Brigitte Widemann, MD  
NCI, POB  
10 Center Drive, Building 10 CRC  
Room 1-5750, MSC 1101  
Bethesda, MD 20892  
Phone: 301-496-7387

Please notify Dr. Widemann by e-mail ([widemanb@mail.nih.gov](mailto:widemanb@mail.nih.gov)) prior to shipment.

Dr. Widemann will submit these samples to the Laboratory of Pathology for testing.

### **Tumor tissue from Mandatory tumor biopsies for NR0B1 expression and gene signature (Phase II)**

In patients with Ewing sarcoma and EWS-FLI1 fusion transcript  $\geq 18$  years old, who have disease amenable to percutaneous biopsy, and who meet other eligibility criteria for biopsy, a biopsy will be obtained prior to treatment and at steady state on treatment during cycle 1. Patients who meet these criteria will have to agree to the biopsies in order to being able to participate in the trial.

#### ***Tumor Biopsies***

Willingness to undergo tumor biopsies is required for patients with disease amenable to percutaneous biopsy on this protocol because modulation of molecular targets in tumor biopsies after drug treatment is an important objective of this study.

Baseline biopsies will be performed following patient enrolling on study. If an initial attempt at percutaneous biopsy is unsuccessful, the patient will be given an option to proceed with a repeated attempt at percutaneous biopsy. A separate consent form must be signed for each biopsy procedure, so patients may choose not to undergo subsequent biopsies. If the baseline biopsy is unsuccessful or the patient refuses to undergo subsequent biopsies, no further biopsies will be performed, but the patient will remain on study, receive study medication, and other correlative studies will be performed. Similarly, patients who miss the first dose of drug will not subsequently be biopsied, but will remain on study.

#### ***Timing of tumor biopsies***

For NR0B1 expression and gene expression signature tumor one tumor biopsy each will be obtained prior to treatment, and day +4 (+/- 1 day) during cycle 1 at steady state on treatment with mithramycin.

#### ***Biopsy procedure***



No more than 2 tumor biopsy procedures per patient will be performed by Interventional Radiology. It is preferred that two core biopsies not less than 18-gauge in diameter and at least 1 cm in length are obtained. Only percutaneous biopsies will be performed. No biopsy of lung metastases will be attempted.

It is estimated that there will be between 2-5 million cells from each biopsy. If a site is deemed appropriate for biopsy with minimal risk to the participant by agreement between the investigators and Interventional Radiology, an attempt at biopsy will be made. Determination of a disease site amenable to biopsy will be determined on an individual case basis after discussion with an interventional radiologist. The biopsy procedure to be used in this protocol is described below. Such biopsies can be safely performed as evidenced by literature reports (56) and Medical Oncology Branch experience at the Clinical Center. Risks of the procedure include, but are not limited to, bleeding, infection, pain, and scarring. We will follow Clinical Center Interventional Radiology SOPs for coagulant panel and platelets.

All biopsies will be by percutaneous approach. No biopsy by an invasive (endoscopic, laparoscopic, or surgical) procedure will be performed. Only cutaneous, subcutaneous, or easily accessible parenchymal lesion core biopsies will be performed; however, there will be no core biopsies of lung lesions.

The use of imaging to facilitate biopsies will be decided by members of the Interventional Radiology team and may include ultrasound, CT scan, or MRI. Should CT scan be needed for biopsy, the number of scans for each procedure will be limited to the minimum number needed to safely obtain a biopsy. All cases will be carefully reviewed with the interventional radiologists at NIH who have extensive experience in performing such procedures. Only if the procedure is considered to be low risk will we proceed with tumor biopsy in a given participant.

***Biopsy processing:***

Tissue will be collected and immediately sectioned, a portion will be separated and frozen on dry ice for RNA collection for microarray and/or gene signature evaluation of EWS-FLI1 downstream target expression. Another portion will be imbedded in paraffin and sectioned. This tissue will be stained using immunohistochemistry for NR0B1 and if tissue remains, proteins in the apoptotic pathway will be evaluated including survivin (BIRC5), XIAP, Caspase-8 (CASP8), and BCL-XL (BCL2L1). The remaining tissue will be distributed with the goal of establishing cell lines or performing basic laboratory investigations.

Biopsy specimens will be collected by Dr. Brigitte Widemann and distributed to Dr. Helman's laboratory and the Laboratory of Pathology for analysis.

Brigitte Widemann, MD  
NCI, POB  
10 Center Drive, Building 10 CRC  
Room 1-5750, MSC 1101  
Bethesda, MD 20892  
Phone: 301-496-7387

## **11.8 APPENDIX 8: PLASMA SAMPLES FOR CLINICAL PURPOSE**

One mL of plasma will be obtained prior to initiating treatment with mithramycin on cycle 1 and prior to cycle 2 for future clinical reference in patients who experience bleeding, if needed.

Obtain 2 mL of blood, separate plasma by centrifugation, and filter plasma through a 0.22 micron filter. Freeze the filtered plasma at -80°C for future clinical reference if needed.

Samples will be stored in PETS laboratory freezers.

Plasma sample pre treatment with mithramycin cycle 1: Date obtained: \_\_\_\_\_

Plasma sample pre cycle 2 of mithramycin: Date obtained: \_\_\_\_\_

Specific Instructions:

Obtain blood in 6 ml purple top tube.

**SPIN AT 4000 RPM X 10 Minutes**

**OBTAIN FILTERED AND NON-FILTERED SAMPLES**

- 1) Non-Filtered sample – pipette out 1 ml from purple top tube and put into nuc, label as non-filtered, and freeze.
- 2) Filtered samples –
  - a) pipette out greater than 1 ml from purple top tube and put into temporary nuc.
  - b) Attach MILLEX GV 0.22 um filter to 5 cc syringe
  - c) Attach large bore needle to exposed end of filter
  - d) Extract 1 ml from nuc, discard temporary nuc
  - e) Transfer from syringe (with filter and needle still attached) to a clean nuc, label as FILTERED, and freeze.

Supplies:

Labels

3 1.8 ml nucs

5 ml syringe

large bore needle

Millex GV 0.22 um filter

Pipettes

Specimen bags

## **11.9 APPENDIX 9: IMAGING STUDIES FOR VOLUMETRIC ANALYSIS OF TUMOR BURDEN**

As a secondary endpoint, measurable disease will be quantified using volumetric MRI analysis. This analysis will be performed at the PETS laboratory.

Imaging studies will be analyzed by Dr. Eva Dombi. :

Eva Dombi, MD  
NCI, POB  
10 Center Drive, Building 10 CRC  
Room 1-5750, MSC 1101  
Bethesda, MD 20892  
Phone: 301-451-7023

**11.10 APPENDIX 10: PATIENT LOG FOR REACTIONS/SYMPTOMS**

Patient ID: \_\_\_\_\_ Cycle: \_\_\_\_\_

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

<u>Reaction/Symptom</u>	<u>Start Date</u>	<u>End Date</u>

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

Patient/Parent signature: \_\_\_\_\_ Date: \_\_\_\_\_

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

**11.11 APPENDIX 11: PATIENT LOG FOR CONCOMITANT MEDICATION**

Patient ID: \_\_\_\_\_ Cycle: \_\_\_\_\_  
Dates of last Mithramycin infusion: \_\_\_\_\_

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

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

Patient/Parent signature: \_\_\_\_\_ Date: \_\_\_\_\_

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

**11.12 APPENDIX 12: MONITORING TOXICITIES BETWEEN MITHRAMYCIN CYCLES WITH LOCAL PHYSICIAN**

Patient ID: \_\_\_\_\_

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

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

The patient named above is currently participating on a Phase I-II Mithramycin study conducted at the NCI, and requires the following lab tests and evaluations between \_\_\_\_\_ (date) and \_\_\_\_\_ (date)

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

**CBC/Diff**

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

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

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

Please FAX all lab results and medical summaries to: \_\_\_\_\_

For any questions, please call:

NAME OF STUDY CONTACT: \_\_\_\_\_ Phone: \_\_\_\_\_

For urgent issues, contact the referring investigator at the following phone number: \_\_\_\_\_  
(Physician Phone #  
HERE)

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

**Please contact the number above with any clinically significant bleed and support with appropriate blood product support. Bleeding that does not stop within 6 hours with appropriate treatment will be considered a dose limiting toxicity and further dosing of mithramycin will be discontinued.**

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

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

Date: \_\_\_\_\_

## 12 REFERENCES

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2. Gurney JG, Swensen AR, Bulterys M. *Malignant Bone Tumors.* Bethesda: National Cancer Institute, 1999.
3. Delattre O, Zucman J, Melot T, Garau XS, Zucker JM, Lenoir GM, et al. The Ewing family of tumors--a subgroup of small-round-cell tumors defined by specific chimeric transcripts. *N Engl J Med.* 1994;331(5):294-9.
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