



Published in final edited form as:

*Mol Cancer Ther.* 2017 May ; 16(5): 956–965. doi:10.1158/1535-7163.MCT-16-0637.

## Safe and Effective Sarcoma Therapy through Bispecific Targeting of EGFR and uPAR

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**Conflict of Interest:** The authors declare that patent “Reduction of EGFR therapeutic toxicity,” related to this work and listing JFM, DV, and AB as inventors, has been filed by the University of Minnesota Office of Technology Commercialization.

### Data and materials availability

RNAseq data for canine tumors that have not yet been published will be made available through GEO prior to publication.

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## Abstract

Sarcomas differ from carcinomas in their mesenchymal origin. Therapeutic advancements have come slowly so alternative drugs and models are urgently needed. These studies report a new drug for sarcomas that simultaneously targets both tumor and tumor neovasculature. eBAT is a bispecific angiotoxin consisting of truncated, deimmunized *Pseudomonas* exotoxin fused to epidermal growth factor (EGF) and the amino terminal fragment (ATF) of urokinase. Here, we study the drug in an *in vivo* “ontarget” companion dog trial since eBAT effectively kills canine hemangiosarcoma (HSA) and human sarcoma cells *in vitro*. We reasoned the model has value due to the common occurrence of spontaneous sarcomas in dogs and a limited lifespan allowing for rapid accrual and data collection. Splenectomized dogs with minimal residual disease were given one cycle of eBAT followed by adjuvant doxorubicin in an adaptive dose-finding, phase I–II study of 23 dogs with spontaneous, stage I–II, splenic HSA. eBAT improved 6-month survival from <40% in a comparison population to ~70% in dogs treated at a biologically active dose (50 µg/kg). Six dogs were long-term survivors, living >450 days. eBAT abated expected toxicity associated with EGFR-targeting, a finding supported by mouse studies. Urokinase plasminogen activator receptor (uPAR) and EGFR are targets for human sarcomas, so thorough evaluation is crucial for validation of the dog model. Thus, we validated these markers for human sarcoma targeting in the study of 212 human and 97 canine sarcoma samples. Our results support further translation of eBAT for human patients with sarcomas and perhaps other EGFR-expressing malignancies.

## Keywords

targeted toxin; epidermal growth factor receptor; urokinase plasminogen activator receptor; sarcoma; adaptive clinical trial; canine

## Introduction

Unlike carcinomas derived from epithelial tissues, sarcomas comprise a heterogeneous group of malignancies of mesenchymal origin [1, 2]. There are 15,000 new sarcoma cases per year in the United States, consisting of 12,000 cases of soft tissue sarcoma and 3,000 cases of bone sarcomas [1]. The 5-year overall survival rate is approximately 50–80% for sarcomas [2, 3]. Development of new targeted therapies for therapy-resistant sarcoma has suffered from the lack of widely-expressed mutations or overexpressed proteins that can be targeted therapeutically without risk of severe adverse events [2, 4–7].

eBAT, a bispecific EGF-urokinase angiotoxin, was developed as a targeted, second generation bispecific biologic drug consisting of human EGF (targeting EGFR), human amino terminal transferase (ATF is the high affinity binding moiety of human urokinase, targeting uPAR), and genetically modified *Pseudomonas* exotoxin, mutated to reduce immunogenicity and facilitate ER retention. This drug was highly efficacious in treatment of established glioma in rodent xenograft models [8]. Xenograft models are informative, but targeting human cells in “non-target” immunosuppressed mice (that do not bind human EGF

and ATF) does not yield the same clinical investigative information as studies in a large animal “ontarget” models where the drug cross-reacts with native EGFR and uPAR. Thus, we chose to undertake an “ontarget” clinical trial in companion dogs with hemangiosarcoma (HSA).

Canine HSA is a common, aggressive, incurable spontaneous sarcoma that appears to have a similar ontogenetic origin as human angiosarcoma [9, 10–12]. Canine hemangiosarcoma and human angiosarcoma are both vasoformative sarcomas with similar microscopic appearance [13] that have often metastasized by the time they are diagnosed. Humans with angiosarcoma have an expected median survival of approximately 16 months [14]; dogs with HSA have a comparable, short median survival of 4 to 6 months when treated with the standard-of-care of surgery and adjuvant chemotherapy [15, 16]. Morbidity and mortality are usually caused by metastatic spread and/or acute internal hemorrhage secondary to tumor rupture. We hypothesized that since HSA is a vascular cancer, eBAT simultaneously targeting the tumor and its vasculature rendered it an excellent therapy choice.

Expression of EGFR and PLAUR/uPAR was previously characterized in human sarcomas using conventional PCR-based assays, gene expression microarrays, and immunohistochemistry [17–20]. In this study, we confirm such expression in a variety of human sarcomas and report on EGFR and uPAR expression on canine HSA.

We showed that canine HSA tumor-initiating cells express EGFR and uPAR, and that these cells are highly sensitive to eBAT [8,21–23]. Here, we used a large “ontarget” animal study that closely parallels what could be a human clinical trial to show feasibility, safety, and efficacy of eBAT to treat sarcomas in a clinically translatable setting using spontaneous canine HSA as model, in both naive disease and minimal residual disease settings. We report on the impact of bispecific targeting on the toxicity risks associated with targeting of EGFR. Our results show that eBAT is safe and potentially effective at biologically active doses despite EGFR targeting, supporting further translation for patients with sarcomas and other EGFR-expressing malignancies. Furthermore, our findings support our belief that bispecificity reduces overall toxicity risks associated with EGFR targeting.

## Materials and Methods

### Assessment of EGFR and PLAUR/uPAR expression in human and canine tumors

EGFR and PLAUR mRNA expression was evaluated from data for 212 human sarcomas obtained through the TCGA (The Cancer Genome Atlas) Research Network (<http://cancergenome.nih.gov/>). The federal project was begun in 2005 to catalog genetic mutations responsible for cancer using genome sequencing and bioinformatics. To perform a similar analysis in dogs, we used next generation RNA sequencing (RNAseq) data from canine hemangiosarcoma and canine lymphoma samples that were reported previously [24, 25]. RNAseq for 31 canine osteosarcoma samples was performed as described [24, 26, 27]. EGFR and uPAR protein expression were evaluated in a human synovial sarcoma tissue microarray (TMA) [28]; the same methods were used to build a study-specific TMA that included tumors from 15 dogs as well as normal canine spleen, liver and kidney and spleens with nodular lymphoid hyperplasia and associated hematomas as controls. A total of 97

canine sarcoma samples were analyzed (51 HSAs and 31 osteosarcomas from independent datasets, and 15 HSAs from dogs enrolled in our clinical study). IHC methods are provided (Supplementary Methods).

### Cell lines

Hemangiosarcoma cell line Emma was derived by the Modiano laboratory in 2008 and authenticated in 2015 by the Modiano laboratory using short tandem repeat (STR) testing (DNA Diagnostic Center, Inc., Fairfield, OH). It was cultured in hemangiosarcoma medium as described [22, 29]. Human angiosarcoma cell line AS5 was obtained from Dr. Gary K. Schwartz, Columbia University Medical Center, in 2013 and was cultured in hemangiosarcoma medium. Human RD rhabdomyosarcoma cell line was obtained from The Global Bioresource Center (ATCC) in January 2015. Human U2OS osteosarcoma cell line was obtained from ATCC in June 2015. Human HPB-MLT T-cell lymphoma cell line was obtained from the Cell Resource Center for Biomedical Research, Cell Bank in October 2014. These cell lines were grown in Dulbecco's modified Eagle's Medium (DMEM) as described [30–32]. RD, U2OS and HPB-MLT were authenticated using STR profiles (DNA Diagnostics Center, Inc., Fairfield, OH) in 2016.

### eBAT production

eBAT was produced at the University of Minnesota cGMP Molecular and Cellular Therapeutics (MCT) Facility as described [8]. The construction of eBAT is illustrated in Figure 1A. Release assays were done by Pace Analytical Life Sciences, LLC (Minneapolis, MN) and/or at the MCT. Release criteria were established regarding drug purity (>95%), endotoxin (<50 Eu/mg), stability, selectivity, potency ( $IC_{50} < 1.0$  nM), sterility, and concentration. The drug was vialled and re-tested to meet critical FDA specifications.

### Laboratory Assays

Protein synthesis assays and proliferation assays measuring [ $^3$ H]leucine incorporation were used to determine the effect of eBAT on cell lines. Briefly, cells were plated in 96-well flat-bottomed plates and allowed to adhere overnight. The targeted toxins were added in triplicate at 10-fold serial dilutions and incubated for 48 hours. Wells were then pulsed with [ $^3$ H]leucine with 1  $\mu$ Ci per well and allowed to incubate for another 24 hours. Plates were then frozen to detach the cells, harvested onto glass fiber filters, washed, dried, and counted using standard scintillation methods. [ $^3$ H]leucine assays were performed using Leucine-free medium. Data are reported as the percentage of control counts.

To evaluate safety, C57BL/6 mice were administered eBAT by the intraperitoneal route twice, two days apart on days 1 and day 3, and then were observed for adverse events for 3 weeks.

### Canine clinical study

Safety and efficacy of adjuvant eBAT were assessed using a Bayesian adaptive Phase I–II trial design with pre-defined criteria of acceptable toxicity (no dose-limiting adverse events) and efficacy (>50% survival at 6 months) to guide dose finding [33]. eBAT was administered to dogs with spontaneous HSA after splenectomy and before the first of five cycles of

doxorubicin chemotherapy. Eligibility was restricted to dogs with stage-1 or stage-2 splenic HSA with no evidence of gross metastatic disease. Adverse events (AEs) were graded according to VCOG-CTCAE criteria [34]. Survival time was measured from the date of diagnosis to the time of death and was censored at the time of last contact for dogs surviving at the time of analysis.

The clinical study, called SRCBST-1 (sarcoma bispecific toxin trial-1), was conducted with approval of the University of Minnesota (UMN) Institutional Animal Care and Use Committee (IACUC Protocols 1110A06186 and 1507-32804A). Study design and implementation conformed to Consolidated Standards of Reporting Trails (CONSORT) guidelines as they apply to studies in companion animals [35]. eBAT pharmacokinetics and neutralizing antibody assays were performed for all dogs. Detailed descriptions of the comparison group, eligibility criteria and protocols for the SRCBST-1 study, pharmacokinetics and neutralizing antibody assays are provided in the Supplementary Methods.

### Statistical analysis

Univariate associations between time-to-death and gene expression, patient characteristics and tumor characteristics for the TCGA samples were assessed by cox proportional hazard regression and summarized by Kaplan-Meier curves. Associations between time-to-death and expression of EGFR or uPAR were assessed by multivariate cox regression analysis, and adjusted for each other and for patient and tumor characteristics. Associations between EGFR and uPAR expression in human and canine tumor samples were evaluated using Pearson's correlation coefficient. Cases from TCGA were censored from analysis if they had no information on survival or if they were listed as "alive" at the end of follow up (or on the date data were analyzed) in the TCGA database.

Dogs and disease characteristics were summarized using descriptive statistics. The biologically active dose was identified as specified by the design [33]. Model-based estimates of the probability of AEs and 6-month survival were obtained from the parametric model used to guide dose finding. The probability of AEs was estimated using a logistic regression model with a linear term for dose; the probability of 6-month survival was modeled using a logistic regression model with linear and quadratic terms for dose. The probability of AEs for each dose was estimated by the sample proportion with exact confidence intervals. Kaplan-Meier curves for overall survival were fit for the entire study population and only for dogs treated at the biologically active dose to obtain a non-parametric estimate of 6-month survival and median time-to-death. Dogs were censored if died of causes other than HSA or if they were alive at the time of the analysis. Associations between AEs and baseline covariates of age, weight and body condition score (BCS) were assessed using the unpaired, two-sample t-test assuming unequal variances between groups. All p-values were two-sided. All analyses were performed using R version 3.0.1 [36].

## Results

### eBAT kills canine and human sarcoma cells

To assess activity, eBAT was added to Emma cells and leucine incorporation was measured as an indication of protein synthesis activity and cell viability (Figure 1B). Emma was chosen as positive control since detectable cell surface expression of EGFR and uPAR was previously reported [22]. Emma cells were killed in a dose-dependent manner and cytotoxicity was specific since a control anti-human CD3 targeted toxin, CD3CD3KDEL, recognizing the epsilon chain of the T cell receptor did not have activity. RD human rhabdomyosarcoma cells were also killed by eBAT in a dose-dependent manner whereas BIC3, a recombinant anti-human CD3 immunotoxin had no activity. The IC<sub>50</sub> (50% inhibitory concentration for protein synthesis) for RD cells was 0.02 nM. Figure 1C shows that U-2OS human osteosarcoma cells that express high levels of EGFR and uPAR were also sensitive to eBAT and interestingly, that a bispecific targeted toxin EGF4KDEL [37, 38] that simultaneously targets EGFR and the human IL-4 receptor did not kill the human cell line as effectively as eBAT. The IC<sub>50</sub> for these cell lines was in the subnanomolar range (0.06 pM – 0.08 nM). Figure 1D shows that eBAT effectively targeted the human angiosarcoma line AS5, originating from a histologically similar tumor as canine HSA. eBAT was also tested against human HPB-MLT T-cells, which do not express EGFR or uPAR and it showed no significant cytotoxicity as expected (Figure 1E). Together, these findings indicate that eBAT is extremely potent and inhibits both protein synthesis and DNA synthesis in a highly specific manner *in vitro*.

### Human sarcomas express epidermal growth factor receptor and urokinase receptor

The most current bioinformatics TCGA database was used to explore the expression on of *EGFR* and *PLAUR* on 212 human sarcomas (Figure 2). Figure 2A shows that *EGFR* and *PLAUR* gene expression were detectable in 100% of samples regardless of sarcoma type with a variation in intensity. Supplementary Figure 1 shows Kaplan-Meier curves for time-to-death by *EGFR* expression for all subjects without subsetting. Subjects with *EGFR* expression above the median had shorter time-to-death than subjects with lower levels of *EGFR* (HR = 1.69, 95% CI: 1.02, 2.81). *EGFR* expression showed no correlation with metastasis, age, gender, sarcoma histological classification, or anatomic location. Figure 2B shows that *PLAUR* expression significantly correlated with histological classification: levels were below the median in leiomyosarcomas, synovial sarcomas, and dedifferentiated liposarcomas, whereas they were above the median in pleomorphic malignant fibrous histiocytomas (MFH), undifferentiated pleomorphic sarcomas (UPS), and myxofibrosarcomas. Expression of *EGFR* was not correlated with expression of *PLAUR* ( $R^2=0.006$ ). Yet, *EGFR* expression ( $p=0.043$ ) and *PLAUR* expression ( $p=0.058$ ) were both associated with time-to-death (Supplementary Table 1). Age, tumor volume, and presence of metastasis also were correlated with time-to-death.

Figure 2C shows expression of EGFR and uPAR proteins in human synovial sarcoma TMA. Both proteins were detectable in each of the 54 synovial sarcomas. Supplementary Table 2 shows more detailed characteristics of these patients and treatments. Neither gene was

associated with survival when assessed independently, together, or with other covariates (Supplementary Table 3).

### **Expression of epidermal growth factor receptor and urokinase receptor is conserved in canine HSAs**

In order to thoroughly evaluate *EGFR* and *PLAUR* expression in canine sarcomas, we evaluated mRNA expression in an independent dataset of 51 canine HSAs by RNAseq [24] and two additional datasets consisting of 31 canine osteosarcomas and 29 canine lymphoma tissue samples (Figures 2D, 2E, 2F) [25]. Results were similar to those in human sarcomas: expression of both *EGFR* and *PLAUR* genes was detectable in all canine sarcomas, with HSA having higher levels of *PLAUR* mRNA, and HSA and osteosarcomas having approximately equivalent levels of *EGFR* mRNA. As expected, expression of both genes was significantly lower ( $p < 2 \times 10^{-5}$ ) in canine lymphoma samples as compared to canine sarcomas (Figure 2F).

### **eBAT is safe and potentially effective in dogs with spontaneous HSA in a clinical setting**

HSA was chosen as a target disease based on its extremely poor prognosis in dogs. Immunostaining of tumor tissues from 15 dogs enrolled in the SRCBST-1 study confirmed that both eBAT targets were expressed at the protein level in all dogs examined replicating the results of immunohistochemical studies in the human synovial sarcoma TMA where both proteins were expressed almost exclusively by tumor cells. Figure 3 shows representative photomicrographs of EGFR and uPAR staining in the canine and human TMAs. Expression of both proteins was variable in non-malignant tissues. Supplementary Figure 2 shows graphical data summaries.

Table 1A summarizes baseline characteristics for all dogs by dose and Table 1B illustrates a treatment timeline for the canine study. The first dog accepted into the study was determined to have metastatic lesions to its liver upon enrollment in the trial, but it was decided to continue treatment and report results as part of the study. A CONSORT diagram showing the flow of study participants is provided in Supplementary Figure 3.

eBAT was safe and well tolerated in all dogs. When dog #23 reached the 6-month milestone, interim analysis showed that the study had reached stability at the biologically active dose of 50 $\mu$ g/kg (dose level 2 in the escalation scheme) and was unlikely to change with additional subjects so enrollment was stopped. Based on the favorable trade-off between efficacy and toxicity observed at 50 $\mu$ g/kg, this dose was identified as the biologically active dose, and was used for all subsequent cohorts.

Median survival for the 23 dogs treated with adjuvant eBAT (eBAT group) was 8.1 months (Figure 4A) compared to 4.9 months for the Comparison group of dogs treated with standard of care alone. Median survival was 8.6 months for the 17 dogs treated at the biologically active dose (Figure 4B). Overall, six-month survival rates were 65.2%, and 70.6%, and 38.7%, for the eBAT group, the group treated at the biologically active dose, and the Comparison group, respectively.

Average time from splenectomy to initiation of chemotherapy was shorter in the Comparison group (20.8 days) than in the eBAT group (43.7 days) or the group treated at the biologically active dose (46.2 days). Six (26%) of 23 dogs and five of 17 (29%) treated at the biologically active dose (dose level 2) survived one year; all six dogs surviving one year had survival of at least 450 days, and two dogs are still alive at 1245 and 963 days. Detectable levels of eBAT were achieved in the systemic circulation of dogs treated by intravenous infusion (not shown).

### **eBAT shows limited toxicity *in vivo***

For our companion canine study, the estimated probabilities of AEs by dose are shown in Table 2A, and specific information regarding AEs is shown in Table 2B. No adverse events were observed at 25 $\mu$ g/kg (dose level 1). Reversible liver toxicity was noted in two dogs treated at dose level 2, reversible hypotensive events were observed in two dogs treated at dose level 2 and two dogs treated at dose level 3. Grade 1–3 toxicities associated with subsequent doxorubicin chemotherapy were predictable and limited to 12 dogs in total. No dogs experienced cutaneous, ocular, gastrointestinal toxicity or laboratory abnormalities that have been previously associated with EGFR targeted therapies in humans [6]. Necropsy was performed in 2 of 23 dogs and showed no evidence of chronic changes attributable to eBAT. Both of these dogs died due to progressive HSA.

Since other studies have shown that EGFR-targeted therapies are associated with significant dose-limiting cutaneous and gastrointestinal toxicities [6, 7], we further examined the safety of eBAT versus EGF-toxin alone in normal C57BL/6 mice. Maximum tolerated doses were established for monospecific EGF-toxin given alone (20  $\mu$ g/kg), monospecific uPA-toxin given alone (40  $\mu$ g/kg) and both drugs administered jointly (40  $\mu$ g/kg); most deaths occurred within 7 days post-treatment. There were no deaths or gross toxicities in mice receiving up to 160  $\mu$ g/kg of eBAT (Table 2C).

### **Anti-eBAT antibody responses are sporadic and do not interfere with outcome**

eBAT contains a bacterial toxin, so immunogenicity was expected and considered as a potential barrier to bioactivity. Samples for NA measurement were available for all dogs at baseline, 19/23 dogs on Day 8, and 7/23 dogs on Day 21.

Dogs in which we could detect drug in the circulation on Day 1 had significantly better survival ( $p = 0.002$ ) than dogs in which drug was undetectable (404 days versus 172 days; hazard ratio = 0.20 (95% confidence interval = 0.07, 0.63)). Drug was detectable on Day 1 in 4 of 9 dogs with no evidence of antibody at baseline or following eBAT administration, 7 of 8 dogs with antibody formation after eBAT treatment, and 1 of 4 dogs with pre-existing antibody (this dog was treated at the highest dose). No associations were found between survival and detectable drug at days 5 or 6 ( $p = 0.542$ ), AUC at day 1 ( $p = 0.96$ ), AUC at day 5 or 6 ( $p = 0.82$ ) or the presence of neutralizing antibodies ( $p = 0.654$ ).

## **Discussion**

The major contributions of this study were the following: 1) first-time evaluation of a potent bispecific, anti-angiogenic targeted toxin in an “ontarget” large animal sarcoma model



demonstrating potential anti-sarcoma activity and long-term survival, 2) description of an EGFR-targeted therapy that is surprisingly well-tolerated, and 3) findings supporting our belief that bispecific targeting reduces toxicity risks associated with EGFR targeting.

We tested eBAT in a model of canine HSA, using an adaptive study design in the minimal residual disease setting. We identified a biologically active dose that was safe and potentially effective. The cause of the reversible hypotensive events noted in four dogs remains unclear. Hypotension was reported in a previous study investigating treatment of advanced solid tumors with immunotoxin LMB-1, occurring in some patients treated at doses greater than 75 ug/kg. Similar to our findings, these events were transient and did not require fluids or pressor agents [39]. None of the treated dogs experienced signs of capillary leak syndrome, the toxicity of greatest concern for immunotoxins [40, 41]. Furthermore, the lack of adverse events similar to those caused by EGFR-targeted therapies [6, 7] suggests that the addition of the uPAR-directed ligand enhances targeting specificity to tumors, leading to diminished toxicity, consistent with our mouse data. However, we are aware that humans are physiologically different and may provide a greater challenge.

Bispecificity is one unique aspect of eBAT, as this may permit reactivity with a wider range of cell surface markers, enhancing the ability to kill resistant tumor cell outliers. In the case of eBAT, studies showed an ability to simultaneously target uPAR on human vascular endothelial cells (HUVEC cells) and EGFR on tumor cells [8]. We believe that bispecificity contributed to the notable clinical effect. Our results are further strengthened by the design that allowed dose finding to be guided by safety and 6-month survival [33], in turn allowing us to identify a biologically active dose without having to establish a maximum tolerated dose (MTD). Dog owners participating in companion dog studies do not abide unnecessary pet mortality risk. That being said, the data suggest the biologically active dose is lower than the MTD. The favorable clinical results could be also due to testing of the drug in the minimal residual disease setting, which is a unique opportunity afforded by the canine model and is in contrast to other studies of immunotoxins in humans, where bulky, refractory, heavily pre-treated tumor loads exceed the capabilities of the test article. Canine HSA provided a setting where we could test eBAT on a targetable disease with a high probability of detecting an efficacy signal in addition to evaluating safety. This was not done with the single intent to develop a treatment specifically for hemangio/angiosarcomas, but rather provide a proof of concept to inform and optimize the design of future clinical trials in humans with a variety of targetable cancers.

Six of seven dogs had NAs on day 21, suggesting that the use of a deimmunized toxin was justified [42]. Nonetheless, the presence of NAs was not associated with survival outcomes, and there was no correlation between NAs and the dose of eBAT received or the drug PK. These findings were similar to other studies with targeted toxin where antitoxin antibody titers did not correlate with antitumor activity [43]. Our results exceeded expectations for outcome of dogs with stage-I or stage-II HSA based on our historical data and on other published data from comparable populations treated with the standard of care [44, 45]. In fact, dogs receiving eBAT had longer survival times than dogs treated with any other contemporary experimental therapy [44–47]. The most recent detection of an efficacy signal in the treatment of canine HSA prior to our study dates back to 1995 when liposome-

encapsulated muramyl tripeptide phosphatidylethanolamine ](L-MTP-PE) was used as an adjuvant to standard of care therapy [48]. The one-year survival for dogs treated with eBAT at the biologically active dose was almost 40% and the proportion of dogs living 6 months or longer nearly doubled compared to our comparison population. Five dogs were considered long-term survivors, having lived more than ~450 days.

It is intriguing that time to initiation of chemotherapy was longer in dogs treated with eBAT than in the Comparison group. It is generally assumed that a shorter time to initiation of chemotherapy would produce more favorable outcomes, but survival was longer in dogs treated with eBAT even though chemotherapy was delayed. It is unlikely that the variability in chemotherapy protocols used in the Comparison group had an impact on survival since, historically, single agent doxorubicin and combination protocols are equally effective [44, 45]. Furthermore, we found no significant difference between number of doxorubicin doses in the Comparison group versus all dogs receiving eBAT or dogs treated at the biologically active dose. The use of a Comparison group enabled us to implement a novel adaptive clinical trial design and identify an efficacy signal of eBAT, but it is important to acknowledge the potential bias associated with the lack of a contemporary control group with blinding and randomization, which would more accurately predict efficacy. Our dosing and dose schedule was chosen partly on the basis of a previous study by our group in humans with an anti-B cell cancer targeted toxin [40], and partly on laboratory animal safety data. Still, metastatic disease occurred in about half of the dogs in this eBAT study. Pharmacokinetic studies show that eBAT is metabolized quickly within a few hours. We intend to use this information to optimize dose schedule in the future. Repeat cycles could prolong remissions as has been shown in studies with targeted *Pseudomonas* exotoxin in humans [40, 49], re-treatment at relapse could prolong survival, and even the delivery methods could be improved.

The mechanism of action of eBAT remains to be fully elucidated. In this study, both eBAT targets were expressed in human sarcoma samples. Thus, our findings from the TCGA and from the synovial sarcoma TMA analysis support other reports in the literature [17–20] regarding *EGFR* and *PLAUR* expression. A recent study confirmed that uPAR was expressed in 100% (57/57) of canine HSAs tested, but only in 30% (8/26) of hemangioma samples [50]. Here, we demonstrated expression of both targets in canine HSA samples and expression was present in the tumor cells and/or in the tumor microenvironment, but they also were present in normal tissues. Taken together, our expression data indicate that these markers are excellent targets and eBAT may be highly effective in sarcoma intervention. Furthermore, our data suggest that the excellent safety profile could be due to a unique reactivity with tumor cells, although it also could be due to the extremely low dose required to control or ablate the mass of malignant cells present in the minimal residual disease setting. However, we cannot exclude the possibility that eBAT makes the microenvironment inhospitable for tumor formation. The apparent high expression of uPAR in tumor-associated mononuclear inflammatory cells, in addition to tumor cells also raises the possibility that eBAT acts through a primary immune mechanism by eliminating or attenuating this cellular compartment, which in turn removes a strong impetus for tumor formation and/or tumor progression [24, 51, 52]. The fact that EGF4KDEL was not as effective as EGFATFKDEL (eBAT) *in vitro* suggests that simultaneously targeting EGFR

and uPAR may be essential for optimal efficacy of this drug. Further studies are needed to understand how the bispecific nature of eBAT confers enhanced specificity even in an “on-target” animal model. Future imaging studies in companion animal models and humans will be required to elucidate the biodistribution of eBAT and identify sites of accumulation in tumor and non-tumor areas.

In conclusion, we demonstrated that eBAT is safe, and that the addition of a uPAR directed ligand to the EGFR targeting molecule abrogated the dose-limiting cutaneous, ocular, and gastrointestinal toxicities, or hypomagnesemia generally associated with EGFR targeting. We also showed that eBAT has biological activity in a highly metastatic, incurable canine sarcoma that carries many similarities with its human counterpart. In fact, *in vitro* testing of eBAT on the human angiosarcoma cell line AS5 showed that the drug was selective and highly effective. The strategy is not aimed at modulating EGF or uPA-dependent pathways, since neither EGFR nor uPAR appear to act as drivers of tumor progression. Rather the proteins act as “bait” for a ligand-targeted cytotoxic therapy. Given that the targets are invariably expressed in human sarcomas, our data provides a strong rationale for translation of eBAT in the treatment of human sarcomas and potentially other EGFR and uPAR-expressing tumors.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

### Funding

This work was supported by grant K01OD017242 (AB) from the Office of The Director, National Institutes of Health, grant AB15MN-002 from the National Canine Cancer Foundation (AB), a grant from the Masonic Cancer Center, University of Minnesota Sarcoma Translational Working Group (JFM, DAV, AB, JSK), grant 1889-G from the AKC Canine Health Foundation (JFM, MB, KLT), the US Public Health Service Grant R01 CA36725 awarded by the NCI and the NIAID, DHHS (DV), the Randy Shaver Cancer Research and Community Foundation (DV), Hyundai Scholar Senior Research Award, Hyundai Hope on Wheels (DV), a CETI Translational Award from the University of Minnesota Masonic Cancer Center (DV), and a grant from GREYlong (JFM). The NIH Comprehensive Cancer Center Support Grant to the Masonic Cancer Center (P30 CA077598) provided support for bioinformatics and comparative pathology services. The NIH Clinical and Translational Science Award to the University of Minnesota (UL1 TR000114) provided support for immunohistology services. RNA sequencing for osteosarcoma samples was supported by the Sobiech Osteosarcoma Fund Award from the Children’s Cancer Research Fund. The authors gratefully acknowledge generous support from the Angiosarcoma Awareness Foundation and donations to the Animal Cancer Care and Research Program of the University of Minnesota that helped support this project. JFM is supported in part by the Alvin S. and June Perlman Chair in Animal Oncology at the University of Minnesota.

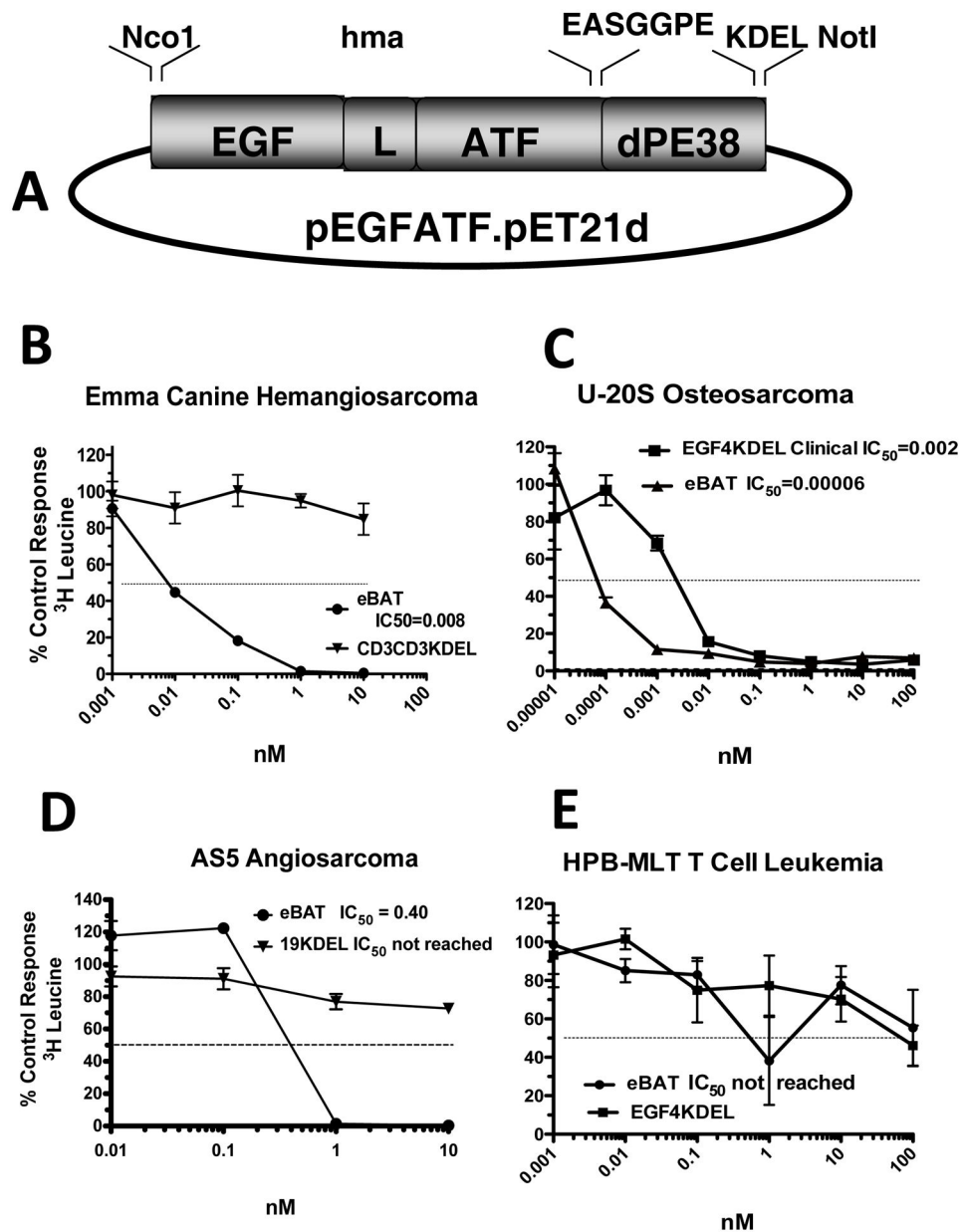
We thank Dr. Brenda Weigel for helpful discussions, Elizabeth Taras for laboratory assistance, Dr. David Largaespada and his laboratory for providing the synovial sarcoma TMAs, Dr. Gary K. Schwartz, Columbia University Medical Center, for generously providing the AS5 human angiosarcoma cell line, Drs. M. Gerard O’Sullivan, Ingrid Cornax, Ramesh Kovi, and Jill Schappa for assistance with pathological analyses; Mitzi Lewellen for sample archiving and management; the oncology clinicians and staff of the University of Minnesota Veterinary Medical Center for assistance with management of dogs participating in the study, and importantly, clients who allowed their pets to enroll in the study, and the dogs who made the study possible.

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**Figure 1. Construction and *in vitro* activity of eBAT**

Bispecific eBAT was studied for its activity against canine and human sarcoma cells. A) Expression vector for eBAT, human EGF and the high affinity amino terminal fragment of urokinase linked to a deimmunized PE<sub>38</sub>KDEL molecule. The fusion gene (from 5' end to 3' end) consisted of an NcoI restriction site, the genes for human EGF, an ATG initiation codon, the downstream 135-amino terminal fragment (ATF) from uPA linked by a 20 amino-acid segment of human muscle aldolase (HMA), the 7 amino-acid EASGGPE linker, the first 362 amino acids of the pseudomonas exotoxin (PE) molecule with KDEL at the C terminus, and a NotI restriction site at the 3' end of the construct. B) Canine EMMA cells were treated with various concentrations of eBAT and control CD3CD3KDEL and then protein synthesis was measured 3 days later using a tritiated leucine uptake assay.

Experimental variability is shown as quadruplicate samples  $\pm$  SED. C) Human U-20S Osteosarcoma cells were treated with various concentrations of eBAT tested against EGF4KDEL and then leucine incorporation was measured. D) Human AS5 angiosarcoma cells were treated with various concentrations of eBAT tested against CD19KDEL as negative control. Leucine incorporation was measured. E) eBAT was tested against HPB-MLT cells to test specificity. eBAT, EGF4KDEL and 2219KDEL showed no significant cytotoxicity.

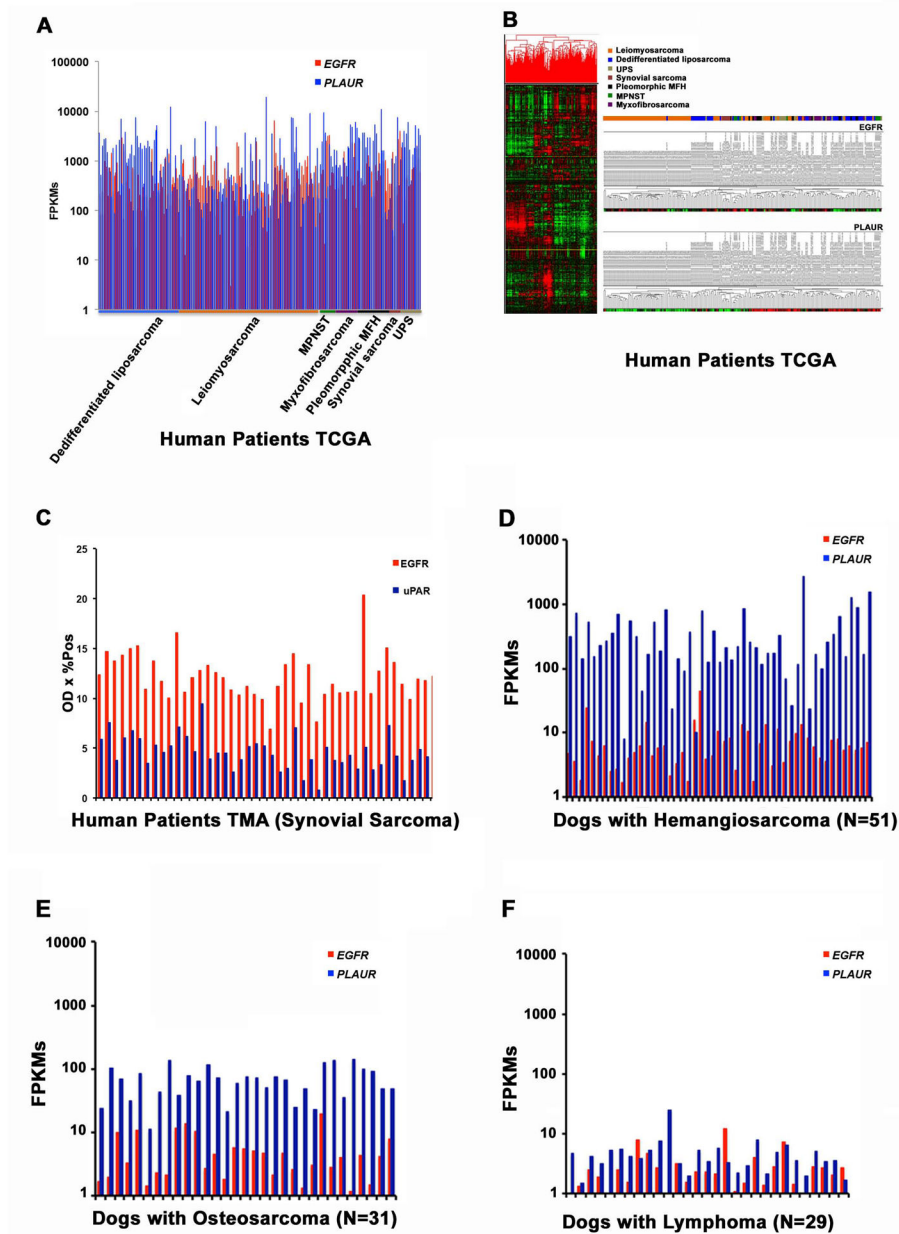
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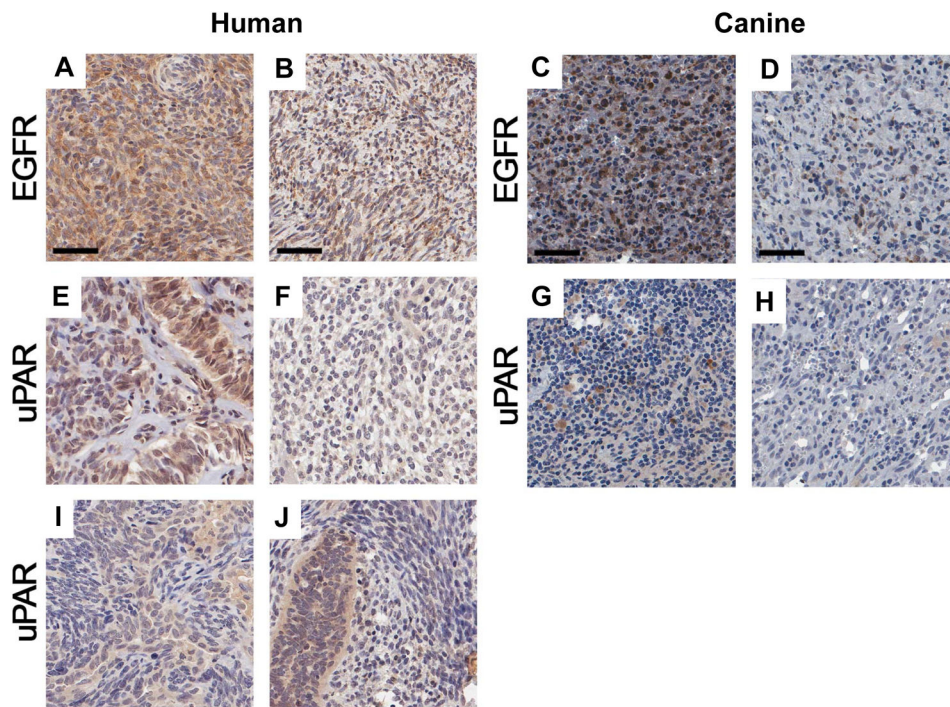




**Figure 2. *EGFR* and *PLAUR* gene expression analysis in human sarcomas and spontaneous canine tumors**

(A) *EGFR* and *PLAUR* gene expression analysis was done in 212 tumor tissue samples extracted from the TCGA database. The X-axis represents the patients supervised by tumor type and the Y-axis is the expression intensity as fragments per kilobase of transcript per million (FPKM) mapped reads. (B) Unsupervised hierarchical cluster and heat map highlighting *EGFR* and *PLAUR* expression in the human TCGA dataset. (C) *EGFR* and uPAR protein expression is shown in TMAs constructed from human synovial sarcoma tissue samples. The X-axis represents patient TMAs and the Y-axis represents optical density of *EGFR* and uPAR on immunohistochemistry. (D) *EGFR* and *PLAUR* gene

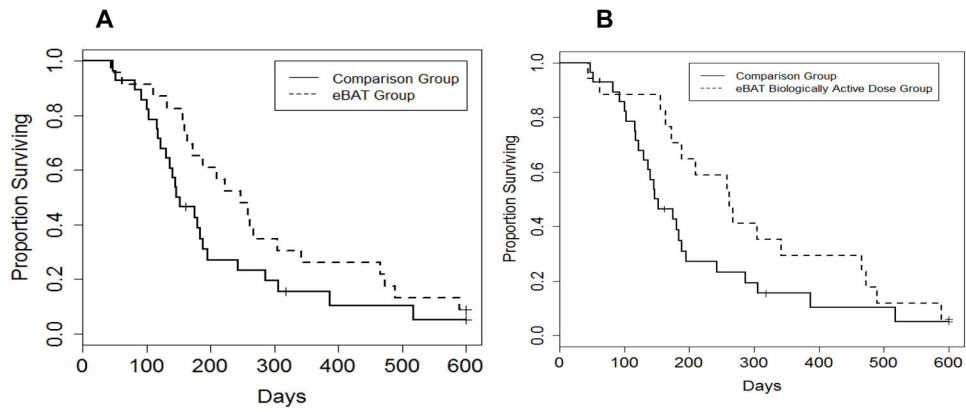
expression analysis in an independent data set of canine hemangiosarcoma samples. (E) *EGFR* and *PLAUR* gene expression analysis in canine osteosarcoma samples. (F) *EGFR* and *PLAUR* gene expression analysis in canine lymphoma samples. Tumor-bearing dogs are on the X-axis and fragments per kilobase of transcript per million mapped reads on the Y-axis, illustrating the levels of *EGFR* and *PLAUR* expression from the individual tumors. The following detailed values pertain to gene expression in TCGA samples of *EGFR* and *PLAUR*, respectively: Count: 212, 212; Mean (FPKM):653.4, 1,713; Mean (FPKM) lower confidence limit: 548.7, 1,387; Mean (FPKM) upper confidence limit: 758.0, 2,040; Variance: 600,273, 5,844,287; Standard Deviation: 774.8, 2,418; Mean Standard Error: 53.1, 165; Coefficient of Variation: 1.2, 1.4; Minimum (FPKM): 3.1, 40.9; Minimum (FPKM): 6,575.1, 19,171.7; Median (FPKM): 410.0, 757.9; Median Error: 4.56, 14.2; Percentile 25% (Q1): 215.4, 250.4; Percentile 75% (Q3): 752.9, 2,149.



**Figure 3. EGFR and uPAR expression in human synovial sarcomas and canine HSA TMA from 15 dogs in the SRCBST study**

Synovial cell sarcoma TMA spots immunohistochemically stained for EGFR and uPAR.

Representative highly and lowly stained spots for EGFR are shown (A–B human) (C–D canine). Representative highly and lowly stained spots for uPAR are shown (E–F human) (G–H canine). An example of heterogeneous expression of uPAR is shown in the human synovial TMA where uPAR expression is much higher in the glandular cells staining darkly brown and forming elongated glands, sometimes with compressed slit-like spaces between the gland cells (I). An admixture of spindled and glandular cells imparting a marbled-like appearance is also shown (J).



**Figure 4. Effect of eBAT on survival of dogs with splenic HSA treated with adjuvant doxorubicin chemotherapy**

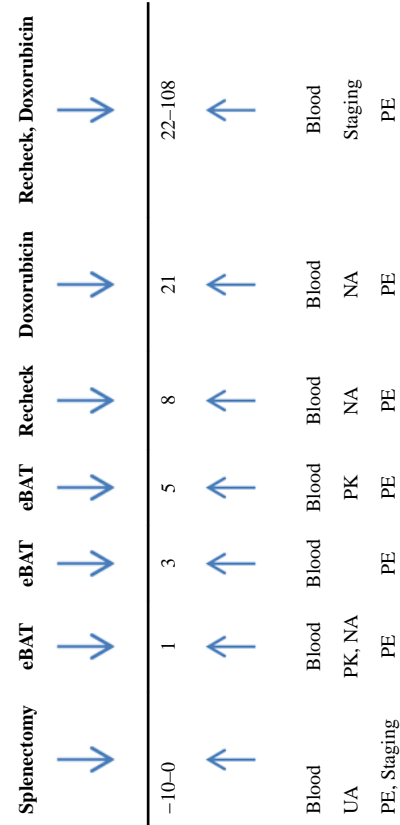
(A) Kaplan-Meier Curve for all 23 dogs in the SRCBST-1 study versus the comparison dogs.

(B) Kaplan-Meier Curve for the 17 dogs treated at the biologically active dose versus the comparison dogs. Curves illustrate prolongation of survival in dogs treated with eBAT compared to the comparison group.

**Table 1**

A. Baseline characteristics for all dogs and by dose summarized by N (%) or mean (SD)									
Variable	Level/Unit	All Dogs	Dose 1	Dose 2	Dose 3	Control	p-value <sup>1</sup>	p-value <sup>2</sup>	
Age	Years	9.4 (1.7)	9.2 (1.6)	9.5 (1.8)	8.6 (1.7)	10.5 (2.2)	0.054	0.135	
Sex	M	11 (47.8)	1 (33.3)	9 (52.9)	1 (33.3)	13 (46.4)	1	0.763	
	F	12 (52.2)	2 (66.7)	8 (47.1)	2 (66.7)	15 (53.6)			
BCS		5.7 (1)	5.7 (0.6)	5.5 (1.1)	6.3 (0.6)	5.2 (1) <sup>3</sup>	0.159	0.385	
Hemoabdomen	Y	20 (87)	2 (66.7)	15 (88.2)	3 (100)	22 (78.6)	0.487	0.69	
	N	3 (13)	1 (33.3)	2 (11.8)	0 (0)	6 (21.4)			
Stage	1	2 (8.7)	0 (0)	2 (11.8)	0 (0)	5 (17.9)			
	2	20 (87)	2 (66.7)	15 (88.2)	3 (100)	23 (82.1)			
	3	1 (4.3)	1 (33.3)	0 (0)	0 (0)	0 (0)	0.324	0.693	
Time from surgery to treatment	Days	22.9 (10.9)	15 (5.2)	25.2 (11.7)	18 (2)				
Weight	kg	24.6 (11.7)	30.1 (4.6)	22.1 (12.1)	33.3 (9.1)	27.1 (11.3)	0.445	0.178	
Time to Initiation of Chemotherapy	Days	43.7 (11.3)	35.3 (5.5)	46.2 (12.2)	38.7 (3.1)	20.8 (6.5)	0	0	
Doxorubicin	Doses	4.3 (1.4)	4.3 (1.2)	4.4 (1.5)	4 (1.7)	4.1 (1.2)	0.669	0.628	

**B. Study Protocol Timeline**



<sup>1</sup> p-value for comparison group vs. all dogs;

<sup>2</sup> p-value for comparison group vs. dose level 2;

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<sup>3</sup>BCS was missing for two control dogs;

<sup>4</sup>breeds enrolled in the study included 5 Labrador retrievers, 3 mixed breed, 2 English springer spaniels, and 1 each English setter, Brittany spaniel, Airedale terrier, Bichon Frise, Newfoundland, Vizsla, Goldendoodle, Cairn terrier, papillon, dachshund, golden retriever, rat terrier, German Shepherd Dog.

PE: Physical Examination

Blood: Complete Blood Count (CBC), Serum Biochemical Profile, Prothrombin Time (PT), Partial Thromboplastin Time (PTT)

UA: Urinalysis

eBAT: EGF Bispecific Angiotoxin

Doxorubicin: Adriamycin chemotherapy (30 mg/m<sup>2</sup>) intravenously every 3 weeks

PK: Pharmacokinetics

NA: Neutralizing Antibody

Table 2

Adverse Events (AEs) for Dogs in the SRCBST Study and Mice Treated with eBAT

A. Summary of AEs including the empirical and model-based estimated rate by treatment group			
Dose Level	N	AEs*	AE Rate – empirical (95% CI)
1 (25 ug/kg)	3	0	0% (0%, 70.8%)
2 (50 ug/kg)	17	3	17.6% (3.8%, 43.4%)
3 (100 ug/kg)	3	2	66.7% (9.4%, 99.2%)

B. Description of AEs in individual dogs, management, and outcome			
Dog ID and Breed	Dose Level	AEs	Management
MN* 11 Cairn terrier	2	Grade 3 ALT elevation after 1 <sup>st</sup> infusion Hypotensive event* during 2 <sup>nd</sup> infusion	Second eBAT infusion delayed one week IV fluid bolus 3 <sup>rd</sup> eBAT infusion not administered
MN 17 Labrador retriever	2	Hypotensive event followed by a seizure during 1 <sup>st</sup> infusion	IV fluid bolus, infusion restarted 45 minutes later with no complications
MN 22 rat terrier	2	Grade 2 ALT elevation after 1 <sup>st</sup> infusion	Monitoring
MN 07 Newfoundland	3	Hypotensive event at the end of 3 <sup>rd</sup> infusion	IV fluid bolus
MN 09 Goldendoodle	3	Hypotensive event during second infusion	IV fluid bolus, infusion not restarted
			Full recovery Full recovery
			Full recovery
			Full recovery
			Full recovery
			Full recovery

C. Summary of death events in normal mice treated with ligand specific toxins				
Treatment	Observed Deaths (%)			
	Dose (µg/kg)			
	10	20	40	80
Monospecific EGF-toxin	0/8 (0)	2/8 (25)	6/8 (75)	8/8 (100)
Monospecific uPA-toxin,	0/8 (0)	0/8 (0)	2/8 (25)	8/8 (100)
Monospecific EGF-toxin + monospecific uPA-toxin	0/7 (0)	1/7 (14)	2/7 (29)	7/7 (100)
eBAT	0/8 (0)	0/8 (0)	0/8 (0)	0/8 (0)

\* Total count of dogs experiencing AEs (not total number of AEs)

\* MN = Minnesota (institutional assignment); dogs were coded using MN followed by a number assigned sequentially based on order of enrollment

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<sup>\*\*</sup> Hypotensive events noted in 4 dogs were characterized by mean arterial pressure <60 mmHg, hind limb weakness, pale mucous membranes, weak femoral pulses, and a single vomiting episode in one dog. All other dogs had no adverse events.

\* Groups of eight C57BL/6 mice were administered monospecific EGF-toxin, monospecific uPA-toxin, monospecific EGF-toxin and monospecific uPA-toxin, and eBAT intraperitoneally twice, two days apart, on days 1 and 3 and were subsequently monitored for the occurrence of adverse events for three weeks.