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Preclinical validation of the utility of BLZ-100 in providing fluorescence contrast for imaging canine spontaneous solid tumors

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Abstract

There is a need in surgical oncology for contrast agents that can enable real-time intraoperative visualization of solid tumors that can enable complete resections while sparing normal surrounding tissues. The Tumor Paint™ agent BLZ-100 is a peptide-fluorophore conjugate that can specifically bind solid tumors and fluoresce in the near-infrared range, minimizing light scatter and signal attenuation. In this study, we provide a preclinical proof of concept for use of this imaging contrast agent as administered before surgery to dogs with a variety of naturally occurring spontaneous tumors. Imaging was performed on excised tissues as well as intraoperatively in a subset of cases. Actionable contrast was achieved between tumor tissue and surrounding normal tissues in adenocarcinomas, squamous cell carcinomas, mast cell tumors and soft tissue sarcomas. Subcutaneous soft tissue sarcomas were labeled with the highest fluorescence intensity and greatest tumor-to-background signal ratio. Our results establish a foundation that rationalizes clinical studies in humans with soft tissue sarcoma, an indication with a notably high unmet need.

Keywords

tumor imaging; dogs; sarcomas; surgery; near-infrared; fluorescence

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Introduction

Surgery is a primary treatment modality for many types of cancer, with extent of surgical resection often directly related to survival. Complete resection significantly influences overall survival in many tumor types, including brain tumors [1-5], prostate cancer [6, 7], and soft tissue sarcomas [8-10]. The surgical goal of complete resection must be balanced with the need to spare surrounding normal tissue. This is particularly true of brain tumors, since removal of additional tissue can unnecessarily increase the extent of cognitive and functional impairment.

It can be difficult to distinguish cancer tissue from normal tissue during surgery. Technologies such as MRI and PET provide static images taken before surgery, which assists in localizing the tumor but does not provide real-time guidance during resection. Improvement in intraoperative tumor visualization would benefit any solid tumor resection, as it would enable surgeons to better determine the extent of local invasion or regional tumor spread. Helping determine which patients are candidates for sphincter-sparing surgery in colorectal cancer [11], for neurovascular bundle preservation in radical prostatectomy [12], or who would respond well to limb-sparing approaches for soft tissue sarcoma [13], are just a few examples of how intraoperative visualization of cancer tissue would be helpful in making surgical decisions.

Fluorescence-guided resection is an emerging technology that promises to revolutionize cancer surgery by providing optical contrast between tumor tissue and surrounding normal tissues [14-18]. Research and development has been most active with imaging agents and detection devices that work within the near-infrared (NIR) wavelengths, due to the minimal absorption and scattering of NIR light by blood and other tissues [17, 19], enabling detection of fluorescence through intervening non-fluorescent tissue [20]. The NIR fluorophore indocyanine green (ICG) has been in clinical use for decades, primarily for vascular imaging and more recently to support identification of sentinel lymph nodes [21]. Additional NIR fluorophores are in development [22-25]. There are marketed devices for intraoperative detection of NIR fluorescence, and more in development [15, 26-29].

Chlorotoxin (CTX) is a 36-amino acid peptide that has proven useful for tumor targeting [30]. CTX binds specifically to many types of tumor cells, allowing for potential broad applicability to a wide range of cancers. Biotinylated CTX bound to 131 out of 138 primary human brain and other neuroectodermal tumor samples in a study of primary human tumor samples [31]. Normal control tissues were consistently negative for staining. Preclinical [27, 32] and clinical [33-35] studies using CTX labeled with fluorescent or radioactive tags support the utility of CTX conjugates in targeting and imaging a broad range of solid tumors. CTX binds directly to Annexin A2, and knockdown of Annexin A2 expression in cultured cells causes loss of CTX binding [36]. Annexin A2 associates with S100A10 to form a heterotetramer called Calpactin I or AIIIt [37]. Over-expression of Annexin A2 in many types of cancer, knockdown and blocking experiments, and the known roles of many of the Annexin A2 and/or AIIIt ligands in tumorigenesis, angiogenesis, and metastasis, all support a strong role for Annexin A2 in cancer cell biology [38].

A modified form of the CTX peptide covalently bound to ICG is being developed to provide an intraoperative fluorescent imaging agent that will specifically label tumor tissue and enable more complete and precise surgical resection. This new imaging agent, BLZ-100, has demonstrated imaging efficacy in mouse tumor models [27]. To facilitate clinical translation of BLZ-100, a feasibility study was conducted in canine patients with spontaneously occurring solid tumors. Dogs with spontaneously arising tumors provide valuable information for development of cancer therapies, since their cancers have features similar to those seen in human tumors [39, 40]. Many types of canine tumors resemble human disease, including sarcomas, mammary and lung cancers, squamous cell cancers, and gliomas. The value of comparative oncology studies in drug development is well-recognized, leading to the emergence of groups such as the NCI's Comparative Oncology Trials Consortium [40] that are designed to facilitate these studies. Comparative oncology studies can also benefit animals, since agents developed for use in humans are frequently also used in veterinary oncology [39]. Here we show that BLZ-100 can be safely used in dogs at doses that result in effective labeling of tumor tissue, and that intraoperative imaging of the resulting fluorescence is feasible.

Materials and Methods

BLZ-100 dose preparation

BLZ-100 was provided by Blaze Bioscience, Inc. Briefly, an amine reactive ester form of ICG was conjugated to a modified CTX peptide using standard amine reactive chemistry. The reaction was allowed to proceed in the dark at room temperature and monitored by reversed-phase HPLC to confirm the reaction went to completion. Free dye was removed by reversed-phase HPLC and fractions containing conjugate were pooled and lyophilized. Lyophilized conjugate was formulated in 10 mM Tris, 5% mannitol, (pH 7.2) to a final concentration of 2 mg/mL, filtered through a 0.2 μ m syringe filter, and vialled under aseptic conditions. Vials were stored at -20°C .

Clinical protocol

Dogs were enrolled that presented to the Washington State University Veterinary Teaching Hospital with solid tumors amenable to surgical removal as part of standard therapy. The protocol was approved by the Hospital's IACUC, and adhered to the NIH Guide for the Care and Use of Laboratory Animals. Dogs were staged according to WHO guidelines and all were negative for metastasis to distant sites. Two patients (2 and 20) had disease present in regional draining lymph nodes, which were removed as part of their surgical treatment. Dogs were premedicated with 1 mg/kg diphenhydramine and BLZ-100 was injected through an intravenous catheter. Single dose administration was utilized to model the anticipated clinical dosing. The dose escalation strategy was modeled after a typical 3+3 clinical trial design. The starting dose was a fraction of the standard imaging dose in mice, adjusted for body size. Diphenhydramine was administered up to two additional doses after BLZ-100 injection to control pseudoallergic symptoms, as deemed appropriate by the supervising veterinarian. Safety was assessed by monitoring general clinical parameters during and after drug administration and by analysis of blood and urine samples obtained before administration of BLZ-100 and just prior to surgery. Laboratory tests included complete

blood count, serum biochemistry (ALT, ALP, BUN, creatinine, total protein, albumin, globulin, calcium, phosphorus, bilirubin, sodium, potassium, chloride), and urinalysis (specific gravity, protein, pH, bilirubin, RBC). Surgery was performed 24 hours (23 dogs) or 48 hours (5 dogs) post-dosing. Various anesthetic protocols and post-operative pain management therapies were used as dictated by individual patient status and disease state. Following excision, margins of interest were marked on the gross, intact specimen prior to sectioning. The specimen was then sutured to a cardboard support to maintain anatomic integrity. A longitudinal section through the center of the long axis of the specimen was excised, full thickness, 0.5 cm wide for fluorescence imaging analysis. A similar section was made perpendicular to the first through the widest portion of the narrower diameter of the specimen, also for fluorescence imaging analysis. The remaining 4 mass quadrants were submitted for histologic analysis. Tissues were formalin fixed and protected from light until analysis.

Quantification of fluorescence in tumors

Formalin fixed tissues were rinsed in phosphate - buffered saline prior to gross imaging. Samples were then incubated in increasing concentrations of sucrose and embedded in O.C.T. (Tissue-Tek) freezing compound to preserve fluorescence during sectioning. Embedded tissues were sliced into 30 micron sections using a cryostat, and placed on gelatin subbed slides (Southern Biotech). Adjacent sections were stained with hematoxylin and eosin using standard histology methods. Quantitative *ex vivo* imaging was performed using the Odyssey[®] CLx NIR scanner (LI-COR Biosciences), 800 nm channel at 21 micron resolution. For optimal dose evaluation, fluorescence signal was quantified using Image Studio software (LI-COR) by measuring signal in a 110368 pixel region of interest (ROI) within the tumor. For tumor to background ratio (TBR) evaluation, fluorescent signal was quantified using ROIs in tumor and non-tumor areas of gross tissue images. Comparison with histology was used to ensure that ROIs were within areas of tumor.

Data analysis and statistics

Fluorescence intensity was quantified using Image Studio software to analyze raw data from Odyssey scans. TBRs were calculated using ROI signal measurements from gross tumor and normal adjacent tissue. Body surface area was calculated using the formula $K_m \times BW(kg)^{0.67} / 100$, where K_m for dogs = 10.1. Linear regression analysis (GraphPad Prism) was used to define the relationship between study variables and fluorescence signal.

Intraoperative imaging

A prototype intraoperative fluorescence imaging device [41] became available partway through the study, and was used for dogs 17 through 28. The prototype, a semi portable, self-standing unit with two sCMOS cameras (one for fluorescence and one for color) contained in the moveable head, allowed *in vivo* and immediate *ex vivo* tumor and tumor bed imaging. This instrument includes a channel designed for imaging ICG wavelengths that are spectrally separated from ambient light. Fluorescence excitation light generated by LEDs (6.3 W, 745 nm peak wavelength; 730-750 nm bandpass excitation filter) was reprojected by a lens to illuminate the imaging field. Emission light was captured through collection optics

(760-841 nm bandpass filter). The system has a 10 cm field of view at a working distance of 75 cm. A pulsing scheme was implemented to prevent ambient light from biasing the fluorescent image. Briefly, a foreground frame was acquired with the excitation LEDs on and a background frame was acquired immediately afterward with the excitation LEDs off. A 10 msec exposure time was used for both images. The background frame was then subtracted from the foreground frame and presented as the image. Additional technical information is available online [42]. When feasible, the imaging head remained over the surgical site to collect continuous images during surgery. When continual imaging was not feasible (e.g. because of interference between the imaging head and other equipment), images were taken after skin and muscle were retracted and tumor exposed, and after tumor removal to look for residual signal in the tumor bed. For tumors removed *en bloc*, images were taken *ex vivo* of exposed tumor during tissue processing. Excised tumors and surrounding tissue were imaged prior to and after they were sectioned for further imaging and histologic analysis. ROI analyses and line profile plots were performed using the software provided with the instrument to provide a semi-quantitative method of interpreting the tumor boundary based on the fluorescence images. All ROI analysis was performed using monochrome NIR images captured at 40 msec integration time.

Results

Patients, dose administration, and safety

Canine patients with any solid tumor type were eligible for the study, and dose escalation was done to determine an efficacious and safe dose. Escalation was not continued beyond the efficacious dose, as definition of a maximum safe dose was not a goal. Twenty-eight dogs were enrolled, with tumor types including soft tissue sarcoma; oral and cutaneous squamous cell carcinomas; mast cell tumors; adenocarcinomas including lung, mammary, and thyroid; and meningioma. The study protocol was an add-on to standard-of-care patient management. All dogs received surgery with intent to cure or control local disease. Patient and tumor characteristics, and actual doses administered (normalized to body surface area), are summarized in Table 1.

BLZ-100 was given intravenously 24 hours prior to surgery, except patients 13 – 17 who were dosed 48 hours prior to surgery in order to explore the effect of time on signal and tumor to background ratio (TBR). Since no consistent improvement in results was observed in the 48 hour group (Table 1), the remaining patients had surgery 24 hours after dosing for scheduling convenience. Complete blood count, serum chemistry, and urinalysis data from prior to injection and just prior to surgery (24 or 48 hours post-injection) had changes that occurred infrequently and without a clear pattern of events related to time of dosing or dose level. There were no clinically significant laboratory abnormalities.

Nearly all dogs experienced an immediate pseudoallergy/hypersensitivity reaction within 10 minutes of dosing, characterized by erythema, pruritus, and less commonly swelling of the muzzle and distal extremities. The severity of the reaction was not related to dose level or rate of administration. The reactions were self-limiting, ameliorated by diphenhydramine, and completely resolved within 4 hours in all cases. These observations are consistent with a systemic release of histamine, which has been reported for a wide variety of drugs following

administration to canines [43, 44]. Some dogs (mast cell tumor and CNS tumor patients) were receiving corticosteroids as part of standard management, but corticosteroids were not required for management of reactions in any patient. No other systemic changes were identified. All dogs tolerated anesthesia and surgery normally. There were no apparent surgical complications associated with BLZ-100.

Ex vivo image analysis and optimal dose

As a first step in determining the effective dose range of BLZ-100 in dogs, fluorescence was measured in gross resected tissues using the Odyssey[®] CLx NIR scanner. Regions of interest (ROI) were analyzed using Image Studio software provided with the instrument. The Odyssey is a light-tight flatbed scanner with a fixed-output laser, which generates quantitative fluorescence intensity data. To ensure comparability across the data set, all samples were scanned on one instrument, using the same instrument settings. Gross tissues were used for this analysis so that the whole sample could be included; this better represents how the product will be used clinically. Total fluorescence within each tumor ROI was plotted as a function of dose (Figure 1A). Regions of tumor and non-tumor were verified by comparison with histology (Figure 1B, C). Due to the large variation in body size, doses were normalized to body surface area (m²) for analysis. At doses up to 0.85 mg/m², signal in gross tumor samples increased as a function of dose ($R^2 = 0.6$). At slightly higher doses (up to 1.6 mg/m²), no further gain in fluorescence was discernible.

Ratios of fluorescence in gross tumor to normal surrounding tissue (TBR) were calculated for dogs treated with 0.8 mg/m² or higher, when excised non-tumor tissue was available for comparison (17 cases). Regions of tumor and non-tumor tissue were verified by comparison with histology. Examples of cases with high and low/no contrast are shown in Figure 2. Additional ROI analysis is shown in Supplemental Figures S1 and S2. The data show good differentiation in several tumor types, including carcinomas and soft tissue sarcomas (Table 1). There was no clear gain in TBR with increasing dose, which together with the total fluorescence data suggests that an effective dose for imaging peripheral canine tumors at the 1-2 day time point is about 1 mg/m². Given the variability in tumor types and relatively low number of samples in this analysis, continued exploration of dose and TBR is warranted. Sarcomas arising in the jaw (N=2) and vertebral body (N=1) had low uptake and poor to no contrast between tumor and surrounding tissues; it was unclear whether this was due to histologic subtype, anatomic site, or other factors. In one of these cases (patient 17), most of the tissue was a collagenous matrix (stroma) with inflammation and a diffuse infiltrative neoplasm. There were no associations between signal and other study variables, such as breed or body mass.

Intraoperative imaging

A prototype intraoperative imaging system [41, 45] (Figure 3) became available during the study and was utilized for patients 17 through 28 (excluding patient 25 due to technical issues), enabling imaging of tumor beds as well as tumors *in situ* and immediately following excision. Intraoperative and *ex vivo* imaging data are summarized for these patients (Table 2). Peritumoral skin tended to have mildly increased background fluorescence, while uninvolved skin had lower fluorescence. Mucosal tissues also showed background

fluorescence, resulting in lack of specificity and residual non-tumor fluorescence in patient 17. Tumor bed imaging showed little or no background staining in tissues such as trachea, muscle, and fat. The intraoperative imaging showed good subjective concordance with the quantitative *ex vivo* image analysis conducted using the Odyssey scanner (Supplementary Figure S2). The tumors that had overall high intensity and good tumor to normal ratios *ex vivo* also showed high contrast and were easy to detect intraoperatively; this high contrast made it possible to assess residual tumor bed fluorescence prior to closing the surgical site. Contrast between the bulk tumor and surrounding tissues was assessed subjectively by the surgeon, and called positive if clear delineation could be seen. Quantitative imaging of excised tissues on the Odyssey showed that in practice, the surgeon could see the tumor margin if the fluorescence was at least 1.5-fold as intense as surrounding tissues.

The presence or absence of residual fluorescence (foci of tissue with contrast at least as high as gross tumor) was compared with post-operative histologic margin status. In 9 of 11 cases imaged intraoperatively, fluorescence signal enabled delineation of the tumor from adjacent tissues. In 7 of these 9 cases, the presence or absence of residual fluorescence in the tumor bed correlated with histologic margin status, suggesting that intraoperative imaging may provide guidance and additional information to the surgeon at the time of resection. In dog 26, residual tumor was identified at the end of surgery but no further tissue could be safely removed. This is the only dog of the 7 who developed local tumor regrowth to date, despite follow up chemotherapy and pre-surgical local radiation. In the remaining 6 patients, where residual fluorescence was not seen at the end of surgery, all remained tumor free locally until death due to metastasis or unrelated disease, or are still alive. Dog 20 received post-operative radiation therapy, dog 24 received follow up chemotherapy, and dogs 18, 21, 22, and 23 received no adjuvant therapy.

Of the 2 cases where fluorescence did not correlate with histologic margins, dog 21 had a thyroid carcinoma that had invaded the local vasculature within the excised gland. No residual tumor fluorescence was seen but histologically the tumor was seen as emboli into a large caliber vessel at the periphery of the mass. This was by strict definition a microscopic incomplete removal but would have not been visible macroscopically. This dog died of presumed metastatic disease. The second apparently discordant case was dog 19, who had an untreated grade II soft tissue sarcoma on the forelimb. The peritumoral skin was erythematous and ulcerated. Intraoperative imaging of the tumor *in situ* and *ex vivo* showed variable fluorescence within the tumor, some fluorescence in the affected peritumoral skin, and little or no background fluorescence in other areas, including distant skin (Figure 4). Contrast between the tumor and unaffected tissue was almost 20-fold, while contrast between tumor and the peritumoral skin was approximately 5-fold due to the higher background fluorescence in the damaged skin. The tumor was marginally excised, with planned adjuvant radiation therapy, rather than amputation of the limb. Although there was no margin of normal tissue in the excised tumor, and therefore histologic margins are reported as positive, the tumor was a distinct mass and no residual fluorescence was seen in the tumor bed. In this case, the positive margin may have been the true edge or pseudocapsule of the tumor. The patient is doing well and recurrence-free at 24 months post-surgery.

Discussion

The overall goals of this study were to determine a safe and effective imaging dose of BLZ-100, and to investigate the utility, feasibility, and practicality of fluorescence guided surgery in canine patients with solid tumors. We found that BLZ-100 was safe at an effective imaging dose, and demonstrated that tumors could be reliably detected above background using real-time fluorescence imaging during surgery. Imaging results showed good differentiation in several tumor types.

The use of intraoperative imaging during this study enabled investigation of the tumor bed and surrounding tissues that were not excised with the tumor. In cases where tumor to background ratios were determined intraoperatively as well as *ex vivo*, the results were directionally similar but the ratios were different in most cases. This finding could be due to the measurement of fluorescence in different areas of normal tissue in each instance. The instrumentation is also quite different; the Odyssey is a light-tight flatbed scanner, which eliminates interference from room lighting and minimizes scatter. With intraoperative imaging, ambient light and distance from the tissue are variables. The excitation wavelengths and filter configurations of the two instruments are also different, which could contribute to differences in the measured contrast. It is also possible that post-excision tissue processing affected the fluorescence intensity in the samples. These data suggest that *ex vivo* detection provides an estimate of tumor targeting compared to adjacent tissue, but absolute thresholds for intraoperative imaging will have to be determined using the instrumentation and lighting conditions that are used during surgery.

The data obtained with the prototype imaging device were promising enough to spur further development. The commercial version of the prototype, the Solaris imaging instrument, includes a number of technical improvements. Four fluorescence channels, ranging from 470 to 800, are now available to provide support for other dyes in addition to ICG and to enable multiplexing. Multiplexing has been informative in prior studies [46] and there is continued interest in the field, for example to support ratiometric imaging of enzymatic activity or to highlight both tumor and normal structures such as nerves [15]. The four fluorescence channels can be used simultaneously with bright white illumination, which was added to improve visibility within the surgical field. This may eliminate the need for separate surgical lamps, enabling more continuous intraoperative imaging and less spatial interference. Furthermore, while the prototype was operated by keyboard and mouse, the commercial version can be operated via tablet so that a user outside of the sterile field can control the instrument and minimize distraction for the surgeon.

For the full dataset, the highest *ex vivo* fluorescence signals and gross tumor to background ratios were seen in a subset of soft tissue sarcomas, suggesting preferential uptake of the conjugate in these tumor types. Soft tissue sarcomas are a common tumor type in dogs, and are similar between humans and dogs in their presentation and biologic behavior [47]. Prognostic variables in humans include stage of disease, histologic grade, percent necrosis, lymph node involvement and aggressiveness of therapy; all of these have been linked to prognosis in dogs as well [48]. Therapy in both species involves surgery with or without radiation therapy, with completeness of surgical removal often being the most significant

prognostic indicator. These tumors are extremely invasive locally and difficult to control with surgical excision alone. Tumor often extends far beyond what the surgeon perceives to be tumor and even with removal of wide margins, assessed as free of tumor by a pathologist, microscopic disease can still be present in the form of small finger-like projections or satellite lesions separated from the tumor by 1-2 cm [48]. The usual approach to therapy is removal with as wide a margin of normal tissue as possible. Often patients receive follow-up radiation therapy to attempt to eliminate microscopic disease [49, 50]. Larger achieved surgical margins lead to less radiation therapy recommended as a follow up. Less drastic surgery, such as local resection rather than amputation for a sarcoma on a limb, may be possible if tumor limits were delineated by an optical imaging agent, enabling effective local control with less postoperative morbidity.

Limitations in this study include the diverse nature of the tumors enrolled, and the relatively small numbers of cases within each group. In cases for which intraoperative imaging was not available, only a small amount of tissue adjacent to the tumor was available for analysis of contrast. Further studies will be needed in humans with solid tumors to determine whether the fluorescence of these tumors is as reliable as it appears to be in dogs. Not enough dogs were imaged at the time of surgery to draw valid conclusions regarding whether smaller margins could be taken or whether non-fluorescent margins were more likely to be read as clean by pathologists, but the data were encouraging and further veterinary studies are planned.

The study of BLZ-100 in dogs provided key information to support clinical translation of the product. Canine tumors are similar to human tumors in terms of histology, tumor size, local invasion, and background tissue types, whereas mouse tumors are typically much less complex. Due to their large and variable body size, the dogs enrolled in this study enabled a better estimation of effective dose levels in humans, which informed clinical study design and manufacturing efforts. The identification of soft tissue sarcoma as a tumor type with particularly high uptake and contrast has contributed to planning for a human clinical trial in this indication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fluorescence intensity analysis in tumors. (A) Total signal in ROIs from Odyssey scans of gross tumors, grouped by tumor type. ROIs sizes and scan settings were constant across the data set. Linear regression was performed for the whole set, and for the dose groups below and above 0.85 mg/m^2 . Significant correlation between dose and intensity was seen only in the low dose group ($R^2 = 0.6$, $P = 0.02$), suggesting that maximal uptake into tumors is reached at $\sim 0.8 \text{ mg/m}^2$. (B) Odyssey fluorescence scan and (C) H&E stain of tissue sections from patient 12. Comparison with histology was used to verify regions of tumor and non-tumor in tissue samples.

Figure 2.

Imaging and ROI analysis of *ex vivo* tumor and normal tissues. Pseudocolored heat maps of Odyssey images (top), photographs (bottom left,) and H&E (bottom right) are shown for each representative dog. ROIs were chosen within gross tumor (black) and gross normal (yellow) tissue samples. (A) Patient 25 had a sarcoma on the eye lid. TBR is 4.5 compared to immediately adjacent normal fat tissue. (B) Patient 12 had a soft tissue sarcoma in the right front axillary region. TBR is 8.9 compared to normal skin. (C) Patient 23 had a squamous cell carcinoma on the tail. TBR is 13 compared to uninvolved skin. (D) Patient 13 had a subcutaneous hemangiopericytoma, a type of soft tissue sarcoma. TBR is 79 compared to adjacent fat. (E) Patient 17 had a fibrosarcoma in the jaw. TBR is <1.0 compared to a section of lower lip.

Figure 3. Intraoperative NIR imaging system. (A) Prototype instrument used in this study. (B) Commercial version of the instrument, Solaris.

Figure 4.

Intraoperative imaging of a soft tissue sarcoma (patient 19). (A) White light preoperative image of gross tumor showing ulcerated and grossly swollen peritumoral skin. (B) NIR image (40 msec integration time) of tumor *in situ*. (C) Plot of fluorescence intensity along the line drawn through the image in panel B. (D) NIR image of excised tumor, with instrument settings adjusted to maximize contrast in order to highlight the variable appearance of the tumor. This tumor was classified as grade II soft tissue sarcoma with ~50% necrosis.

Table 1

Patient and tumor characteristics, and normalized BLZ-100 dose administered.

ID	Tumor Type	Site	Breed	Sex	Age (yr)	Weight (kg)	Dose (mg/m ²) ^a	TBR ^b
1	Soft tissue sarcoma	Subcutaneous	Labrador mix	M	11.7	24.0	0.44	NA
2	Adenocarcinoma	Lymph node	Poodle mix	M	6.5	6.7	0.28	NA
3	Fibrosarcoma	Subcutaneous	Rhodesian Ridgeback	F	11.7	39.1	0.25	NA
4	Hemangiosarcoma	Jaw	Labrador Retriever	M	10.7	30.9	0.30	NA
5	Mastocytoma	Cutaneous	Labrador Retriever	F	10.0	30.5	0.50	NA
6	Mastocytoma	Cutaneous	Pit Bull Terrier	F	5.3	26.0	0.56	NA
7	Adenocarcinoma	Lung	American Eskimo	F	11.0	14.3	0.83	3.9
8	Squamous cell	Jaw	English Springer Spaniel	M	5.0	25.8	0.56	NA
9	Chondrosarcoma	Nasal	Labrador mix	F	8.4	29.4	1.03	1.7
10	Adenosquamous carcinoma	Mammary	Pit Bull Terrier mix	F	7.0	22.1	1.12	5.6
11	Soft tissue sarcoma	Mammary	Yorkshire Terrier	F	7.0	3.8	1.62	24
12	Soft tissue sarcoma	Subcutaneous	Border Collie mix	F	3.9	27.9	1.06	8.9
13	Hemangiopericytoma	Subcutaneous	Standard Poodle	F	7.0	33.7	0.94	79
14	Squamous cell carcinoma	Cutaneous	Pit Bull Terrier	F	11.8	28.0	1.06	<1
15	Squamous cell carcinoma	Jaw	Springer Spaniel	M	8.3	23.0	1.21	2.3
16	Fibrosarcoma	Jaw	Golden Retriever	F	10.7	39.6	1.26	3.1
17	Fibrosarcoma	Jaw	Chesapeake Bay Retriever	M	5.0	44.4	1.17	<1
18	Mastocytoma	Cutaneous	English Bulldog	F	9.0	29.3	0.82	8
19	Soft tissue sarcoma	Subcutaneous	Labrador Retriever	F	10.3	25.6	1.13	7
20	Follicular carcinoma	Thyroid	Golden Retriever	F	7.0	37.7	0.87	ND
21	Adenocarcinoma	Thyroid	Boxer	F	6.3	25.2	1.14	ND
22	Adenocarcinoma	Mammary	Brittany Spaniel	F	7.0	22.2	1.24	29
23	Squamous cell carcinoma	Cutaneous	Golden Retriever	F	9.1	34.4	0.93	13
24	Adenocarcinoma	Mammary	Labrador mix	F	13.3	21.8	0.94	14
25	Soft tissue sarcoma	Subcutaneous	Golden Retriever	F	13.1	39.0	0.85	4.5
26	Soft tissue sarcoma	Subcutaneous	Chow mix	F	6.1	33.0	0.95	3.8
27	Meningioma	Brain	Border Collie	F	13.2	17.3	1.47	ND
28	Hemangiosarcoma	Vertebral body	Golden Retriever	M	10.8	32	0.97	ND

^a Dose escalation was based on body weight rather than body surface area, but fluorescence data correlated more closely with surface area. Doses are shown normalized to surface area for consistency.

^b TBR was calculated for dogs that received 0.8 mg/m² or higher doses, using gross tissue *ex vivo* Odyssey images. Cases marked ND had insufficient grossly normal tissue for analysis.

Table 2Summary of intraoperative imaging observations^a

Patient	Tumor type	TBR (gross, <i>ex vivo</i>) ^b	TBR (Intraoperative) ^b	Delineation of tumor during surgery	Residual fluorescence in tumor bed	Involved surgical margins	Local recurrence
17	Fibrosarcoma	<1	<1	no	N/A	no	no
18	Mastocytoma	8	1.6	yes	no	no	no
19	Soft tissue sarcoma	7	12	yes	no	yes	no
20	Follicular carcinoma	ND	ND	yes	yes ^c	yes- invasion into vessels and LN	no
21	Adenocarcinoma	ND	ND	yes	no	yes- invasion into vessels	no
22	Adenocarcinoma	29	5.9	yes, multiple lesions	no	no	no
23	Squamous cell carcinoma	13	6.4	yes	no	no	no
24	Adenocarcinoma	14	2.7	yes	no	no	no
26	Soft tissue sarcoma	3.8	2.1	yes, multiple lesions new and previously irradiated	yes	yes	yes
27	Meningioma	ND	2.5	yes	yes	Yes	N/A ^d
28	Hemangiosarcoma	ND	<1	no	N/A	yes	no

^aResults are shown for the subset of cases for which the prototype intraoperative imaging device was available^bTBR was calculated *ex vivo* when grossly normal tissue was available for comparison, and on intraoperative images when both tumor and normal tissue were imaged simultaneously. ND = not determined.^cAn enlarged lymph node was removed, and was fluorescent on *ex vivo* imaging. There was no other residual fluorescence or evidence of local spread.^dSurgical goal was debulking