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Measuring microdose ABY-029 fluorescence signal in a primary human soft-tissue sarcoma resection

Kimberley S. Samkoe^{*,a,b,c}, Hira Shahzad Sardar^b, Jason Gunn^b, Joachim Feldwisch^d, Konstantinos Linos^{a,e}, Eric Henderson^{a,f}, Brian Pogue^c, Keith Paulsen^c

^aGeisel School of Medicine, Dartmouth College, Hanover, NH 03755;

^bDepartment of Surgery, Dartmouth-Hitchcock, Lebanon, NH, 03756;

^cThayer School of Engineering, Dartmouth College, Hanover, NH, 03755;

^dAffibody AB, Solna, Sweden, 171 69;

^eDepartment of Pathology, Dartmouth-Hitchcock, Lebanon, NH, 03756;

^fDepartment of Orthopaedics, Dartmouth-Hitchcock, Lebanon, NH, 03756

Abstract

Microdose administration of ABY-029, an anti-epidermal growth factor receptor Affibody molecule conjugated to IRDye 800CW, is being studied in a Phase 0 trial for resection of soft-tissue sarcomas. The excised tissue of a single patient in the microdose administration group was imaged with both a wide-field fluorescence surgical system and a flat-bed scanning fluorescence imaging system. Here the resultant fluorescence from a breadloaf section of the primary tumor specimen and six region-specific tissue samples collected from that breadloaf are compared using these two imaging systems – a flatbed, black-box, fluorescence scanning system, the Odyssey CLx, and an open-air, wide-field, pre-clinical surgical imaging system, the Solaris. Fluorescence signal is compared using a variety of methods including: mean, standard deviation, variance, tumor-to-background ratio, biological-variance ratio, and contrast-to-noise ratio. The images produced from the Odyssey scanner have higher signal variance but more accurately represent the EGFR expression in small tissue sections. The Solaris system has higher depth sensitivity and volume averaging, and as such has lower signal variation and higher contrast-to-noise ratio.

Keywords

soft-tissue sarcoma; epidermal growth factor receptor; ABY-029; microdose; fluorescence guided surgery; molecular targeted fluorescence

1. INTRODUCTION

Fluorescence-guided surgery (FGS) is a developing imaging approach that aims to improve the safety and efficacy of surgery by maximizing the tumor tissue excised from patient tissue, and minimizing damage to essential physiological structures. Molecular targeted FGS

*Corresponding author: Kimberley Samkoe, PhD; kimberley.s.samkoe@hitchcock.org; office: 603-650-7618.

utilizes targeted fluorescent reporters that bind to specific proteins within tissues of interest, with higher binding capabilities tumors as compared to normal tissues due to overexpression of key proteins. Recently, we have been motivated to investigate FGS using the US Food and Drug Administration (FDA) exploratory Investigative New Drug (eIND) pathway that emphasizes microdose studies (Phase 0) prior to pursuing more expensive Phase 1 trials [1]. The eIND pathway is additionally appealing because production, safety testing, and Phase 0 trials of fluorescent protein molecules is feasible with a single National Institute of Health (NIH) grant [2].

Previous studies have pioneered the clinical implementation of FGS using therapeutic epidermal growth factor receptor (EGFR)-targeting antibodies labeled with near-infrared dyes, such as IRDye® 800CW (LI-COR Biosciences, Inc.). The trailblazing Phase I safety and dose escalation study of cetuximab-IRDye800CW for human FGS demonstrated tumor-to-background ratios (TBR) in the range of 4–5X with sub-therapeutic doses, 2–5 days post-administration [3]. However, microdose administration (1% therapeutic dose) of these antibody agents did not yield significant TBRs *in vivo*, and adverse events typical of anti-EGFR therapy were experienced by patients at the higher doses required for successful FGS [3]. Since then, several Phase I and II clinical trials have been reported (or are ongoing) using these fluorescent antibodies for resection of head and neck (NTC01987375) [3–5], glioma (NTC02855086), and pancreatic (NTC03384238) cancers. However, long administration-to-surgery times are still required to obtain suitable contrast due to the long plasma half-life of antibodies. Thus, application of an EGFR-targeted agent that could be administered at microdose levels, provide suitable contrast between tumor and normal tissues, and reduced infusion-to-excision time is desired.

Several pre-clinical studies have successfully investigated FGS for soft-tissue sarcoma resection [6–12]. A single pilot study of two human patients performed by Holt *et al.* (2015) [6] utilized the indocyanine green (ICG) ‘second window’, where high dose (5 mg/kg) ICG is injected 24-hours prior to surgery. In this case, TBRs as high as 10.3 were observed but contrast was based solely on the enhanced permeability and retention (EPR) effect, and not specific molecular targeting. EGFR overexpression is common with estimates of 60% in all categories and as high as ~70–90% in subsets of the disease, such as leiomyosarcomas and myxofibrosarcomas [13]. Additionally, soft-tissue sarcomas require complete surgical removal for cure [14], yet ~23% of surgeries result in residual positive margins [15]. It is feasible that patients with soft-tissue sarcomas that are EGFR positive may benefit from molecular-targeted FGS using an EGFR-targeted imaging agent, such as ABY-029.

Here, we describe a single patient that received microdose (30 nanomoles, 237 µg) administration of ABY-029 prior to entering surgery. ABY-029 is presumed to have minimal toxicity due to its rapid clearance, high No Observed Adverse Effect Level (NOAEL; 1000× human microdose or 24.5 mg/kg in rats [16]), and low risk of immunogenicity. After the tumor was excised, the primary tumor specimen was evaluated for ABY-029 fluorescence using a pre-clinical surgical imaging system, to recapitulate *in situ* surgical results, and a flat-bed fluorescence scanning system that is often used to image excised tissue. We present initial results this patient, that suggests microdose administration of ABY-029 is sufficient to

distinguish tumor from normal tissue and the EGFR expression closely matches fluorescence signal.

2. MATERIALS AND METHODS

2.1 ABY-029

ABY-029 was manufactured under Good Manufacturing Practices (GMP) conditions at the University of Alabama Birmingham Vector Production Facility as previously described [16]. ABY-029 was by packaged in single microdose aliquots – defined by the FDA as 30 nanomoles (237 μg) in an amber vial dissolved in 5 mL of sterile saline. ABY029 was used under an eIND 122681 for allowance of experimental human studies.

2.2 Patient Enrollment

The soft-tissue sarcoma, ABY-029 Phase 0 study (NTC03154411) protocol was approved by the Dartmouth College Center for Protection of Human Subjects (CPHS) Institutional Review Board (IRB). The subject participated under informed consent by signing a HIPAA-compliant form to document this understanding. The patient was enrolled by meeting the following criteria: tumor judged to be suitable for open surgical resection based on preoperative imaging studies, subject 18 years of age, and valid consent signed by subject. Patients were excluded from the study if they were on any anti-EGFR therapies, either investigational or FDA approved, or were female and pregnant, or breastfeeding. The patient had a preoperative histological diagnosis of primary soft-tissue sarcoma. Standard H&E and epidermal growth factor receptor (EGFR) immunohistochemistry (IHC) were performed on diagnostic tumor biopsy samples. EGFR IHC was scored by a clinical pathologist (author KL) based on a previously determined scale of 0–3+ [17], where 0 = no staining; 1+ = partial membranous staining around cells; 2+ = weak membranous staining completely around cells; and 3+ = strong membranous staining completely around cells. A zero (0) score was considered EGFR negative, and scores from 1+ to 3+ were classified as EGFR positive (EGFR+).

2.3 Soft-tissue sarcoma study data

On the day of surgery, the patient was administered a microdose of ABY-029 (30 nanomoles, 237 μg) by 5 mL intravenous bolus, 1–3 hours prior to surgery. Sarcomas were removed as a single specimen by wide local excision and transferred to clinical pathology where the tissue was inked and breadloafed into ~ 2.5 cm sections. One representative slice was selected and scanned for fluorescence in the 800 nm channel on an Odyssey CLx (LI-COR Biosciences, Inc., Lincoln, NE) at low resolution (337- μm) and then imaged using the 750 nm channel (735nm excitation, 770–809 nm emission collection) of the Solaris (Perkin Elmer, XXX, MA). Six ~1-cm² regions—three high-fluorescence and three low fluorescence locations – were selected from the slice and rescanned at higher resolution (42- μm) on the Odyssey and again on the Solaris. All fluorescent images were collected with calibration paper in the field-of-view. These small tissue sections were blocked, fixed and stained for pathology.

2.4 Pathology

Routine H&E staining and EGFR IHC was performed on the six ROI tissue specimens by Pathology Translation Research Services at Dartmouth-Hitchcock. EGFR IHC staining was completed as described previously [17]. Sections of placenta were used as positive controls, and surgical tissue stained only with the secondary antibody was used as a negative control. EGFR IHC stained tissue was assigned a score from 0–3+ by clinical pathologist (author KL).

2.5 Ex vivo fluorescent image analysis

ABY-029 fluorescent images from the Odyssey and Solaris were individually background subtracted and then normalized to the maximum signal generated from Odyssey CLx test paper, provided by the manufacturer, prior to fluorescence intensity determination.

2.5.1 Breadloaf Section of Primary Sample—For the primary sarcoma breadloaf specimen, the mean, standard deviation (st dev) and variance were calculated for the whole specimen, tumor region, normal tissue (fat and muscle), fat, and muscle. The tumor-to-background ratio (TBR), biological-variance ratio (BVR), and contrast to noise ratio (CNR) were calculated by using equations 1–3, respectively:

$$TBR = \frac{I(FL)_{TUM}}{I(FL)_{NORM}} \quad (1)$$

$$BVR = \frac{I(FL)_{TUM}}{SD(FL)_{NORM}} \quad (2)$$

$$CNR = \frac{I(FL)_{TUM} - I(FL)_{NORM}}{\sqrt{(SD(FL)_{TUM})^2 + (SD(FL)_{NORM})^2}} \quad (3)$$

where $I(FL)$ represents the mean fluorescent intensity, and $SD(FL)$ is the standard deviation of the fluorescent intensities in the tumor (TUM) or normal tissues (NORM).

2.5.2 Region-of-Interest Sections of Primary Breadloaf Sample—For each of the tumor ROI sections, the average fluorescence intensities from the Odyssey and Solaris were determined and plotted against the average intensity of the corresponding IHC stain, assessed with the H DAB Color Deconvolution add-on in FIJI (FIJI is Just Image J, LOCI, Madison, WI) [18, 19]. Fluorescence intensity was plotted against IHC stain intensity and fit with a linear regression using Pearson's correlation.

3. RESULTS AND DISCUSSION

The enrolled patient had a diagnosis of undifferentiated pleomorphic sarcoma, with a 2/3+ EGFR IHC score on the preliminary diagnostic biopsy. The sarcoma was extracted 4.12 hours after administration of ABY-029, and the specimen was sent directly to pathology for processing. The breadloaf section of the primary specimen chosen for imaging is shown in Figure 1, with regions of tumor, fat, and muscle indicated. Note that for analysis, ‘whole specimen’ is tumor, fat, and muscle together, while ‘normal tissue’ is a considered a combination of fat and muscle. ABY-029 fluorescence resulting from microdose administration is clearly observed in both the Odyssey CLx and Solaris imaging systems (Figure 2).

Table 1 reports the typically calculated parameters of fluorescence intensity in a variety of regions-of-interest, including the mean signal, standard deviation, and the variance. The mean calibrated fluorescence intensity and the standard deviation of the intensity of the Whole Specimen are on the same order of magnitude for the Solaris and Odyssey systems. However, when looking at the Tumor region alone, while the mean fluorescence intensities between to two systems are on the same order of magnitude, in the Odyssey images the standard deviation is an order of magnitude higher and the variance is two orders of magnitude higher than in the Solaris images. The normal tissues, and fat and muscle considered alone, again only differ considerably in the variance. It is interesting to note that the tumor and normal tissues have similar variance when imaged in the Solaris system, but the tumor tissue displays much higher variance (~2 orders of magnitude) as compared to the normal tissues when imaged with the Odyssey scanner.

The most often reported value in clinical and pre-clinical fluorescence-guided surgery is the tumor-to-background, or the tumor-to-normal tissue, ratio (TBR or TNR). However, there is current debate whether TBR is the best value to report during fluorescence guided surgery, because it does not take into consideration the variance of the signal, has been shown not to increase with increasing fluorophore dose, and is dependent on the dynamic range of the instruments [20]. In Table 2, the TBR values of tumor-to-normal tissue are summarized in addition to the biological-variability ratio (BVR) and the contrast-to-noise ratio (CNR). The Odyssey scanning system provides a higher TBR than the Solaris between the tumor and normal surrounding tissues. BVR is the biological equivalent to the signal-to-noise ratio (SNR) that is often calculated for imaging systems using instrument noise as the background. Here, the Odyssey fluorescence signal in the tumor is 5.7 times higher than the ‘noise’ or the signal in the normal tissue. The Solaris only displays a BVR of 3.6, indicating that the Odyssey is better at detecting differences in the tissue types. The CNR is a measure that determines overall image quality. Here, we show that the Odyssey has a lower CNR than the Solaris for the same tissue sample (0.8 and 1.1, respectively) indicating that even though the mean fluorescence signal is high in the tumor, the overall noise in the image is also high making it more difficult to distinguish true signal [20, 21].

Six specific regions of interest (Figure 4) were isolated from the primary specimen breadloaf and imaged separately on the Solaris and the Odyssey at high resolution. The regions were selected so that 3 contained high-fluorescing regions and 3 contained moderate-to-low

fluorescence regions in order to maximize the variations in EGFR expression. Necrotic regions were avoided as they were very low in fluorescence intensity and anticipated to be devoid of EGFR based on pre-clinical experience (data not shown). After imaging, these sections were formalin fix and stained for H&E and EGFR IHC. All the ROI sections were assigned an EGFR score of 3+, with the exception of ROI 5 that was assigned a score of 2+. It is clearly visible in Figure 4 that the ABY-029 fluorescence is spatially similar to the EGFR IHC staining patterns, especially in the case of the Odyssey. The Solaris images in general follow similar spatial patterning to the EGFR IHC but are more blurred in nature. The fluorescence intensity from each tumor section was analyzed for both the Odyssey and the Solaris systems and plotted against the EGFR IHC stain intensity, isolated from the brown coloring in the images (Figure 5). Interestingly, the fluorescence intensity of ABY-029 is moderately, positively correlated to the EGFR IHC stain intensity ($r = 0.48$) using Pearson's correlation. However, the fluorescence intensity measured from the Solaris images is moderately, negatively correlated to the EGFR IHC stain intensity ($r = -0.34$). These results should be taken lightly as they only represent one patient sample but it does indicate that the Odyssey fluorescence intensity most closely matches the EGFR expression. The correlation could be further improved by weighting the intensities by area fraction of EGFR expression, or only analyzing regions that are EGFR positive.

The patient tissue presented here represents the only sample in the microdose group that was imaged using both the Odyssey and the Solaris systems. In the higher dose groups, not presented here, all patient tissue was imaged with both instruments, and therefore more significant results will be presented and analyzed at a later date. However, from this preliminary data we can conclude that the fluorescence signal from the Odyssey system best represents the molecular EGFR expression, at least on the surface of the specimen, but is more susceptible to fluorescence intensity variations. This is likely due to the Odyssey scanner having less depth sensitivity than the Solaris scanner [22, 23]. The surface-weighted nature of the signal collection means that there is less volume-averaging of the fluorescence signal and therefore the Odyssey best represents the molecular EGFR expression on the surface of the tissue section. The Solaris, which is capable of imaging to much greater depth [22, 23], is likely to have higher volume averaging and thus will be susceptible to signal intensity throughout the specimen, rather than just the surface. This can be noted in regions like the center of the tumor, which is likely necrotic. In the Odyssey image, this region is relatively devoid of fluorescence, but is not as dark relative to the rest of the tissue in the Solaris image. Although, there is a possibility that these differences are due to the emission collection parameters of the two systems - the Solaris collects emission from 770–809 nm whereas the Odyssey collects fluorescence above 800 nm – the same trend is not observed in other low intensity regions. Therefore, it is most likely that this discrepancy between the two systems can be attributable to volume averaging. In the future, performing serial step-sections through the sample could explain these discrepancies.

Additionally, it is important to note that all of the values reported here today will change temporally as the length of time between administration to imaging is lengthened. At shorter time periods, it is anticipated that there would be a smaller difference between the tumor and normal tissues, but overall fluorescence intensity would be higher, and variance would likely be lower. At longer time periods, as the unbound fluorophore is cleared from normal tissues

and the tumor, it is anticipated that the overall signal will decrease, the difference between the tumor and normal tissue will increase and the spatial variation in signal will increase. Therefore, it will be increasingly important as fluorescence guided surgery moves towards clinical approval, that the time points in which the measurements are collected by considered and methods to reduce inter-sample temporal variations minimized.

4. CONCLUSIONS

ABY-029 is readily detectable in *ex vivo* soft-tissue sarcomas using both a fluorescence scanning system and a wide-field imaging system that is intended to recapitulate a clinical system. For the larger breadloaf sample, fluorescence intensities are similar for the same tissues imaged with both systems but signal variation is substantially higher in the Odyssey scanning system, most likely due to the surface weighting nature of the instrument. For the smaller ROI tissue sections collected from the primary breadloaf sample, there are discrepancies in the reported fluorescence values. This is likely due to differences in the lower detection limits of the imaging systems and the effects of volume averaging. However, as demonstrated here using the Odyssey scanner, ABY-029 fluorescence is positively correlated with EGFR expression. Additional patient samples will be analyzed to further confirm this conclusion.

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Figure 1.

Breadloaf of a resected primary undifferentiated, pleomorphic sarcoma (*left*). Regions of tumor (T), muscle (M), and fat (F) are denoted (*right*). Note that “Whole Specimen” indicates tumor + muscle + fat, and “Normal Tissue” will be considered to be muscle + fat regions of interest together.

Figure 2.

Comparison of imaging systems. The Odyssey is a black-box, fluorescence, point-scanning system with high resolution and low depth sensitivity and produces images (*left*) that are highly detailed and highly variable in signal intensity. The Solaris (*right*) is an open-air, wide-field fluorescence system with lower resolution and high depth sensitivity. The Solaris produces images (*right*) that are less detailed with less signal variation.

Figure 3.

Region-of-interest (ROI) selection for correlation to EGFR expression. ROIs were selected from the Odyssey fluorescence image (*left*) and then translated to the primary specimen breadloaf (*right*) using colored O-rings. Three regions with high fluorescence intensity (*pink*, ROIs 1–3) and three areas of low-to-moderate fluorescence intensity (*yellow*, ROIs 4–6) were selected to represent a range of EGFR expression. Necrotic regions (Tumor Core, Figure 2), with very little to no fluorescence signal were avoided.

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Figure 5.

Correlation between fluorescence intensity measured by the Odyssey (*solid circles*) and the Solaris (*open circles*). The best linear fit is indicated for both the Odyssey (*solid line*) and the Solaris (*dashed line*) and the corresponding Pearson correlation is reported for both.

Table 1.

Comparison of fluorescence intensity mean, standard deviation (St Dev), and variance in different regions-of-interest (ROIs) of the primary breadloaf sarcoma specimen

Regions-of-Interest	SOLARIS			ODYSSEY		
	Mean	St Dev *	Variance	Mean	St Dev	Variance
Whole Specimen	1.1E+02	1.3E+02	1.7E+03	4.5E+02	4.4E+02	2.0E+05
Tumor	1.1E+02	4.3E+01	1.8E+03	5.5E+02	4.8E+02	2.3E+05
Fat	6.4E+01	2.0E+01	4.1E+02	1.6E+02	9.7E+01	9.5E+03
Muscle	9.0E+01	2.5E+01	6.2E+02	1.7E+02	4.7E+01	2.2E+03
Normal (Fat + Muscle)	5.8E+01	3.2E+01	1.0E+03	1.6E+02	9.8E+01	9.6E+03

*
St Dev = standard deviation

Table 2.

Comparison of methods to compare signal between tissue regions-of-interest (ROIs)

	Tumor to Normal Tissue		
	TBR	BVR	CNR
Solaris	2.0	3.6	1.1
Odyssey	3.4	5.7	0.8

Abbreviations: TBR = Tumor-to-background, BVR = Biological-variance ratio, CNR = Contrast-to-noise ratio