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Effects of an unlabeled loading dose on tumor-specific uptake of a fluorescently labeled antibody for optical surgical navigation

Lindsay S. Moore¹, Eben L. Rosenthal², Esther de Boer^{1,3}, Andrew C. Prince¹, Neel Patel¹, Joshua M. Richman⁴, Anthony B. Morlandt⁵, William R. Carroll¹, Kurt R. Zinn⁶, and Jason M. Warram^{1,6}

¹Department of Otolaryngology, University of Alabama at Birmingham, Birmingham, AL, United States of America ²Department of Otolaryngology, Stanford University, Stanford, CA, United States of America ³Department of Surgery, University of Groningen, Groningen, the Netherlands ⁴Department of Surgery, University of Alabama at Birmingham, Birmingham, AL, United States of America ⁵Department of Oral & Maxillofacial Surgery, University of Alabama Birmingham, Birmingham, AL, United States of America ⁶Department of Radiology, University of Alabama at Birmingham, Birmingham, AL, United States of America

Abstract

Purpose—Intraoperative optical imaging to guide surgeons during oncologic resections offers a unique and promising solution to the ambiguity of cancer margins to tactile and visual assessment that results in devastatingly high rates of positive margins. Sequestering of labeled antibodies by normal tissues with high expression of the antibody target, or “antigen sinks”, diminishes the efficacy of these probes to provide contrast between the tumor and background tissues by decreasing the amount of circulating probe available for uptake by the tumor and by increasing the fluorescence of non-tumor tissues. We hypothesized that administering a dose of unlabeled antibody prior to infusion of the near infrared (NIR) fluorescently-labeled antibody would improve tumor-specific uptake and contrast of the fluorescently-labeled probe by occupying extra-tumoral binding sites, thereby increasing the amount of labeled-probe available for uptake by the tumor.

Procedures—In this study, we explore this concept by testing two different “pre-load” doses of unlabeled cetuximab (the standard 10mg test dose, and a larger, experimental 100mg test dose) in six patients receiving cetuximab conjugated to the fluorescent dye IRDye800CW (cetuximab-IRDye800CW) in a clinical trial, and compared the amount of fluorescent antibody in tumor and background tissues, as well as the tumor-specific contrast of each.

Results—The patients receiving the larger preload (100 mg) of unlabeled cetuximab demonstrated significantly higher concentrations (9.5 vs. 0.1 μ g) and a longer half-life (30.3 vs. 20.6 days) of the labeled cetuximab in plasma, as well as significantly greater tumor fluorescence (32.3 vs. 9.3 relative fluorescence units) and tumor to background ratios (TBRs) (5.5 vs. 1.7).

Corresponding Author: Jason M. Warram, PhD, Assistant Professor, Departments of Otolaryngology, Neurosurgery, and Radiology, The University of Alabama at Birmingham, 1670 University Blvd. Birmingham, AL 35294, 205.996.5000 (p) 205.934.3993 (f) mojack@uab.edu(e).

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Conclusions—Administering a preload of unlabeled antibody prior to infusion of the fluorescently labeled drug may be a simple and effective way to improve the performance of antibody-based probes to guide surgical resection of solid malignancies.

Keywords

Fluorescence guided surgery; loading dose; cetuximab; Cetuximab-IRDye800; head and neck cancer; optical surgical navigation; unlabeled preload

INTRODUCTION

The margins of solid malignancies are ambiguous and difficult for even the most experienced surgeons to identify by tactile and visual assessment alone during surgical resection. Incomplete tumor resections with positive margins are common, approaching 50% in some cancer types, and are correlated with locoregional recurrence and poor patient outcomes [1–3]. Fluorescence-guided surgery has already been shown to increase the rate of complete resections and improve oncologic and functional outcomes [4–5], and a wealth of pre-clinical data and clinical trials predict the impending widespread implementation of this technology into the operating room [6–18]. While a number of fluorescent constructs are being explored, fluorescently-labeled antibodies targeting cancer-specific markers are perhaps the most studied, with probes targeting human epidermal growth factor receptor 1 (EGFR) [19] and 2 (HER2) [20–21], vascular endothelial growth factor (VEGF) [19–20], carcinoembryonic antigen (CEA) [22], carbohydrate (cancer) antigen 19-9 (CA19-9) [23], and receptor tyrosine kinase (KIT) [24] and others utilized in a variety of cancer types [6]. Importantly, there are currently multiple clinical trials ongoing using fluorescently labeled antibodies to image cancer (NCT02113202, NCT01972373, NCT02129933, NCT01508572, NCT01987375, NCT02736578, NCT02415881)[6].

Antibodies are desirable vectors to carry fluorescent molecules to cancerous tissue for a number of reasons. The toxicity of systemically administered antibodies is generally low and adverse side effects are rarely severe [25–27]. Importantly, antibodies exhibit active and specific targeting of cancer [25, 28], as compared to the passive, non-specific mechanism of enhanced permeability and retention (EPR) utilized by other fluorescent probes [29–30]. The long elimination half-lives of antibodies result in increased time for tumor exposure and uptake [26, 31], in contrast to smaller fluorescent probes that are often cleared too quickly and with suboptimal target binding [32]. The structure of antibodies is highly amenable to chemical manipulation, and the large number of active binding sites present on each provides an optimal scaffold for conjugating multiple fluorescent molecules [33–34]. Given their increasing use as therapeutics, many antibodies have pre-existing FDA approval for human use and well-established patient safety profiles [25, 27–28, 31], which can ease and expedite their clinical translation as imaging agents [6]. Finally, the over expression of antibody targets is often found in more than one tumor type, affording the opportunity for a single fluorescently-labeled antibody for multiple cancer types.

However, experience with the pharmacokinetics and pharmacodynamics of antibodies offers insight into the potential complications of using fluorescently-labeled antibodies to provide

tumor-specific contrast during surgery. While tumors may overexpress antibody targets, these receptors are also endogenously expressed, sometimes ubiquitously, in many non-cancerous tissues, leading to off-target binding [25, 31, 35]. Cetuximab demonstrates high levels of binding to especially EGFR-rich tissues, including the skin and liver [27, 31, 35]. This target-mediated elimination, involving target receptor binding, internalization, and degradation, is the primary elimination route for many antibodies, including cetuximab, and is largely responsible for the nonlinear elimination kinetics they exhibit [25, 27–28, 31, 35–37]. Additionally, the accumulation of circulating labeled antibodies by normal tissues occurs more rapidly than in tumor tissue, which has slower uptake kinetics due to inherently immature vascularization and elevated interstitial pressures [25, 28, 35]. Antibodies are also metabolized, although in less significant quantities, by cells of the reticuloendothelial system (RES) and by non-specific phagocytosis [25, 31, 36]. Sequestering of labeled antibodies by these off-target sites may negatively affect the tumor specific contrast by decreasing the amount of circulating probe available for tumor uptake and by increasing the fluorescence of non-tumor background tissues.

Although rare, the possibility of serious hypersensitivity and infusion reactions is another potential disadvantage to using antibodies as fluorescence vectors [26–27, 37]. For cetuximab this reaction rate is ranges from 3%–20% depending on the geographic location [38]. In order to screen for individuals who may develop a serious adverse reaction to cetuximab, a small “test dose” is routinely administered prior to infusion of the full treatment dose (250 mg/m²) in patients undergoing antibody-based cancer therapy [31, 37]. Based on this premise, patients recruited to be included in the study were administered a test dose of unlabeled cetuximab (10 mg or 100 mg) prior to infusion of cetuximab conjugated to the near-infrared (NIR) fluorescent dye IRDye800CW (cetuximab-IRDye800CW) so that any immune responses to the cetuximab could be identified prior to the study drug infusion. Two patients exhibited reactions to the test dose, and were therefore unable to participate in the study [8]. While hypersensitivity reactions were the driving force for the test dose administration, we hypothesized that the unlabeled antibody dose given first would also improve tumor-specific uptake and contrast in the tumor, by saturating the EGFR in normal tissues with the unlabeled antibody. In this study, we explore this concept by testing two different “preload” doses of unlabeled cetuximab in six patients that subsequently received cetuximab-IRDye800CW during a clinical trial evaluating the safety and specificity of cetuximab-IRDye800CW in patients with HNSCC [8]. We then compared the amount of circulating fluorescent antibody, tumor and normal tissue fluorescence, and the tumor-specific contrast of each preload dose cohort.

METHODS

Study design

Tissue imaging and blood plasma were collected by the ablative surgeon from 6 consented patients enrolled in a clinical trial (Clinicaltrials.gov Identifier: NCT01987375) evaluating the safety and tumor-specificity of systemically injected cetuximab-IRDye800CW (25 mg/m²) for surgical navigation in patients with HNSCC, as previously reported [8]. For this study, one hour prior to cetuximab-IRDye800CW infusion, patients (n=3) received either 10

mg unlabeled cetuximab, per standard of care, or a 100 mg unlabeled cetuximab. Per trial design, patients then received one hour infusion of cetuximab-IRDye800CW 3–4 days prior to the scheduled surgical procedure. Informed consent was obtained from all individual participants included in the study [8]. All patient data were anonymized and all experiments using the specimens were conducted in accordance with the rules and regulations approved by the University of Alabama Institutional Review Board.

Plasma analysis

Pharmacokinetic assessments were performed prior to infusion, 2 hours, 24 hours, day 3, day 4, day 15, and day 30 post cetuximab-IRDye800CW infusion as previously described [39]. Aliquots of plasma samples (2.5µL) were mixed with 4X sample buffer and resolved by NuPAGE 4–12% Bis-Tris gel (Invitrogen Corporation; Carlsbad, CA). A known amount of cetuximab-IRDye800CW was assessed by gel electrophoresis and imaged at 800nm (Pearl Impulse) to verify cetuximab-IRDye800CW at the 150kDa protein marker. For each cohort, total mg of cetuximab-IRDye800CW was calculated at each time-point after quantification using Image Studio software (LI-COR Biosciences). Mean fluorescence intensity from size-matched ROI's was recorded and the mg of cetuximab-IRDye800CW contained in each band was calculated based on mean counts from a set of cetuximab-IRDye800CW standards. Total plasma mg of cetuximab-IRDye800CW at each time point was determined based on patient dose and total body blood weight. Total plasma values were averaged across patients for each cohort at every time point, and a scatter plot was generated to determine plasma clearance of cetuximab-IRDye800.

Optical imaging

The LUNA imaging system (Novadaq, Toronto, Ontario, Canada) was used to acquire open-field, near-infrared fluorescence images of the primary tumor site (oral cavity or cutaneous), ipsilateral neck, forearm, and oral mucosa in the pre-operative clinic 2 hours post-injection of cetuximab-IRDye800CW (day 0) followed by daily imaging and prior to resection on the day of surgery (day 3 or 4 post-infusion) [8]. Image settings were constant throughout acquisition with the camera at a constant distance of 30cm.

Fluorescence Analysis

Fluorescence was quantified using integrated instrument software, SpyQ version 1.2 (Novadaq, Toronto, Ontario, Canada). Relative fluorescence units (RFU) were measured for tumor and background (approximately 3 to 4 centimeters from the edge of gross tumor) and averaged among six individual frames per imaging timepoint. Tumor-to-background ratio (TBR) was calculated by dividing tumor RFU by respective background RFU as previously described [40].

RESULTS

In order to elucidate the effects of unlabeled cetuximab on tissue uptake of the fluorescent probe cetuximab-IRDye800CW, six patients with HNSCC enrolled in a clinical trial evaluating the safety and specificity of the probe were given either a 10mg or 100mg dose of unlabeled cetuximab prior to administration of the study drug. Fluorescence-imaging with

an open-field NIR imaging device (LUNA) was used to evaluate the fluorescence of tumor and background tissue at several time points before and after drug administration. Samples of patient plasma were also collected and used to determine the amount of cetuximab-IRDye800CW in each sample at each time point. Table 1 displays key characteristics of patients in each of the two pre-treatment dose cohorts. Both of the cohorts were comprised of two male patients and one female patient. The average age was 62 for the 100 mg cohort and 57 for the 10 mg cohort. There were two oral cavity tumors and one cutaneous tumor in each cohort. All patients received 25 mg/m² of cetuximab-IRDye800CW, therefore, there was no significant difference ($P=0.8$) between the dose of cetuximab-IRDye800CW in cohort 1 (49.6 mg) and cohort 2 (48 mg).

Figure 1 depicts the average amount of cetuximab-IRDye800CW detected in the blood plasma of patients in each unlabeled dose cohort (10mg or 100mg) at each collection time point over 15 days. There was no significant difference between the two cohorts on day 0 (100 mg=51.18 μ g $\pm$ 6.7, 10 mg=41.23 μ g $\pm$ 14.8, $P=0.17$), which represented the peak amount of labeled drug detected in the plasma for both cohorts. However, there was significantly ($P<0.05$) more cetuximab-IRDye800CW in the plasma of patients receiving the 100 mg unlabeled preload compared to those receiving the 10 mg unlabeled preload dose 1,3, and 4 days post-infusion. On day 1 post-infusion, the average amount of cetuximab-IRDye800CW in patients receiving the 100 mg preload was 34.5 μ g ($\pm$ 10.7), and only 18.3 μ g ($\pm$ 6.1) in the 10 mg preload cohort ($P=0.04$). On day 3 post-infusion, 14.3 μ g ($\pm$ 1.5) cetuximab-IRDye800CW was detected in the plasma of patients given the 100 mg loading dose and nearly one third of that amount in the plasma of patients receiving the 10 mg loading dose (5.7 μ g $\pm$ 5.3, $P=0.03$). The greatest difference in the amount of cetuximab-IRDye800CW detected in the plasma of patients in the two cohorts occurred on day 4 post-infusion. Those patients in the 100 mg preload cohort had an average of 9.5 μ g ($\pm$ 4.2) cetuximab-IRDye800CW in the plasma, while the labeled drug had dropped to nearly undetectable amounts in those patients who received the 10 mg loading dose (0.1 μ g $\pm$ 0.12, $P=0.008$). No patient from either cohort had detectable levels of the labeled cetuximab in the plasma on days 15 or 30 post-infusion.

Figure 1 also illustrates that the rate of clearance of the cetuximabIRDye800CW from the plasma was less in patients who received the 100 mg preload of unlabeled cetuximab compared to those who received the 10 mg preload. The average half-life of cetuximab-IRDye800 for the 100 mg preload cohort was 30.33 hours ($\pm$ 7.3), which was significantly longer than that of the 10 mg preload cohort (20.6 hours $\pm$ 4.0, $P=0.05$).

Figure 2 demonstrates the average fluorescence of the forearm skin, oral mucosa, and tumor tissue of the patients in the 100 mg preload group compared to the 10 mg preload group at the day of infusion of cetuximab-IRDye800CW (day 0), day 1 post-infusion, and day 3 or 4 post-infusion (day of surgery). There was no significant difference between the average tissue fluorescence of the oral mucosa in patients receiving the 10 mg preload dose and those given 100 mg of unlabeled cetuximab at any of the imaging time points (Fig 2A). The mean fluorescence of the skin of patients in the 10 mg preload dose cohort was significantly greater than that of patients in the 100 mg cohort on day 1 post-infusion ($P=0.007$). However, there was no significant difference in mean fluorescence of patient skin between

the two cohorts on day 0 or on the day of surgery (Fig 2B). Figure 2C demonstrates that the average fluorescence of tumor tissue in the 100 mg preload cohort ($32.3 \text{ RFU} \pm 18.8$) was significantly greater than the tumor fluorescence of the 10 mg preload cohort (9.3 ± 4.9) on the day of surgery ($P=0.05$).

Finally, Figure 2D depicts the trending increase in the tumor to background ratio (TBR) of roughly 1 unit per time point for the 100 mg preload cohort over time compared to the stable contrast ratio of the 10 mg preload cohort (1.7, 1.8, and 1.7 on day 0, 1, and 3/4, respectively). The mean TBR was more than two-fold greater for the 100 mg preload dose cohort (5.5 ± 2.6) than for the 10 mg cohort (1.7 ± 0.7) on the day of surgery ($P=0.04$).

DISCUSSION

Fluorescence-guided surgery is a promising technique to guide the surgical resection of solid malignancies and antibodies have been shown to be an effective imaging agent in humans [6, 8]. However, sequestration of these antibodies by non-tumor tissues endogenously expressing the target receptor may decrease antibody bioavailability and tumor-specific binding. This concept is commonly utilized in the field of radioimmunotherapy, in which a “cold” preload dose of unlabeled antibody is used to improve the bioavailability and targeting of subsequently administered radiolabeled antibodies intended to eradicate a malignant target [41–42]. We evaluated the effects of unlabeled cetuximab preloading on the pharmacokinetics and tumor-targeting of systemically injected cetuximab-IRDye800CW in human patients with HNSCC.

Patients who received the larger unlabeled preload dose (100 mg) demonstrated greater peak concentrations and a longer half-life of the cetuximab-IRDye800CW in blood plasma. Importantly, almost no circulating labeled drug was present in the plasma in the 10 mg preload cohort at the time of surgery, while the plasma of patients in the 100 mg preload cohort contained nearly 100 times more labeled drug at that time. It is likely that this increase in the amount of circulating cetuximab-IRDye800CW and its longer biological half-life in the 100 mg cohort was the result of increased off-target receptor occupancy and tissue sequestration by the larger dose of unlabeled antibody preload, substantially increasing the uptake of the cetuximab-IRDye800CW in tumor. One study using radiolabeled antibodies observed similar results, noting a significant increase in the concentration and resident time in the blood stream of the radiolabeled antibody when preload dose of unlabeled antibody was used [42].

It is important to note that the highest preload dose used in this study (100 mg) is only a fraction of the therapeutic dose of cetuximab (250 mg/m^2) [31, 37]. No increase in adverse events occurred in the group receiving the larger preload dose, suggesting a preload of at least 100 mg can be administered without increased toxicity. It is also important to note that, even when a larger preload was used, there was no detectable cetuximab-IRDye800CW in patients in either cohort at days 15 and 30, meaning that this increase in half-life does not risk patient safety by prolonging drug circulation beyond expected time frames in immunotherapy.

Furthermore, there was no significant difference in patient age, weight, or cetuximab-IRDye800CW dose between the two cohorts, and the groups were matched for gender and primary tumor location, suggesting that the significant increase in the TBRs of patients who received the 100 mg dose is not an artifact of these confounding variables. Additionally, the findings in this study are not due to differences in EGFR expression of the tumors, as a separate analysis of tumor tissue of the patients in this clinical trial found no significant difference in EGFR expression between cohorts [43]. Thus, this significant difference in the pharmacokinetics and TBRs between the two preload dose cohorts is most likely attributable to the difference in the unlabeled preload dose.

In conclusion, this study conducted in humans containing SCC of the head and neck found that administration of a 100 mg preload dose of unlabeled cetuximab prior to systemically injected cetuximab-IRDye800 yielded slower plasma clearance and increased tumor-specific uptake of the fluorescently-labeled drug, as well as improved fluorescence contrast when compared to the standard 10 mg preload dose. These findings were consistent with a number of radioimmunotherapy studies that found increased tumor-specific uptake of radiolabeled antibodies following a “cold” preload dose [41–42, 44].

To the best of our knowledge, this is the first study comparing the effects of different preload doses of an unlabeled antibody on the performance of the fluorescently-labeled antibody as an optical contrast agent to guide oncologic surgical resection. This study was limited by the small number of patients, a constraint inherent to the standard “3+3” dose-escalation study design of the overarching clinical trial. In order to draw definitive conclusions about the optimal preload of unlabeled antibodies in antibody-based fluorescence guided surgery, larger studies testing a range of preload doses should be conducted, as has been done with radioimmunotherapy [41, 45]. Also, given the variability among different antibodies and their target receptors, conclusions should be limited to the specific antibody investigated. However, this study offers valuable insight into the potential for a preload of unlabeled antibody to improve the bioavailability and performance of antibody-based fluorescent probes to navigate the surgical resection of solid malignancies.

CONCLUSION

Fluorescence-guided surgery is a promising technique but in order to maximize the efficiency of fluorescent probes efforts must be made to improve tumor-specific uptake of the probe. This study demonstrated enhanced pharmacokinetics, increased tumor-specific uptake, and improved optical contrast of fluorescently-labeled cetuximab in patients with HNSCC when a preload of 100 mg unlabeled antibody was administered, compared with 10 mg. While further studies and dose-range testing must be done to validate the efficacy of this technique, this strategy may provide a simple method for improving the performance of antibody-based fluorescence probes to guide oncologic resections without adding a substantial burden to the approval process.

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Figure 1. Plasma pharmacokinetics of Cetuximab-IRDye800CW

Average amount of Cetuximab-IRDye800 detected in the blood plasma of patients in each unlabeled pre-treatment dose cohort (10mg or 100mg) at each collection time point over 15 days.

Figure 2. Tissue fluorescence

Average fluorescence (relative fluorescence units) of patient (a) skin, (b) oral mucosa, (c) tumor tissue and the tumor to background ratios (d) for each unlabeled pre-treatment dose cohort (10mg or 100mg) at Day 0,1, and 3/4 (day of surgery).

Age, sex, primary tumor site, and dose of cetuximab-IRDye800CW for each patient in the two unlabeled cetuximab preload dose cohorts (100mg and 10mg).

Table 1

Patient Characteristics

Unlabeled Cetuximab Dose (mg)	Patient Number	Age	Sex	Cancer Site	Cetuximab-IRDye800CW Dose (mg)
Cohort 1: 100 mg	1	77	M	Oral Cavity	44.5
	2	40	M	Oral Cavity	59.0
	3	69	F	Cutaneous	45.5
Cohort 2: 10 mg	4	69	F	Oral Cavity	52.0
	5	38	M	Oral Cavity	45.0
	6	64	M	Cutaneous	47.0