Near-infrared imaging of brain tumors using the Tumor Paint BLZ-100 to achieve near-complete resection of brain tumors

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Object. The intraoperative clear delineation between brain tumor and normal tissue in real time is required to ensure near-complete resection without damaging the nearby eloquent brain. Tumor Paint BLZ-100, a tumor ligand chlorotoxin (CTX) conjugated to indocyanine green (ICG), has shown potential to be a targeted contrast agent. There are many infrared imaging systems in use, but they are not optimized to the low concentration and amount of ICG. The authors present a novel proof-of-concept near-infrared (NIR) imaging system using a standard charge-coupled device (CCD) camera for visualizing low levels of ICG attached to the tumors. This system is small, inexpensive, and sensitive. The imaging system uses a narrow-band laser at 785 nm and a notch filter in front of the sensor at the band. The camera is a 2-CCD camera, which uses identical CCDs for both visible and NIR light.

Methods. The NIR system is tested with serial dilution of BLZ-100 from 1 μ M to 50 pM in 5% Intralipid solution while the excitation energy is varied from 5 to 40 mW/cm². The analog gain of the CCD was changed from 0, 6, and 12 dB to determine the signal-to-noise ratio. In addition to the Intralipid solution, BLZ-100 was injected 48 hours before euthanizing the mice that were implanted with the human glioma cell line. The brain was removed and imaged using the NIR imaging system.

Results. The authors' results show that the NIR imaging system using a standard CCD is able to visualize the ICG down to 50 nM of concentration with a high signal-to-noise ratio. The preliminary experiment on human glioma implanted in mouse brains demonstrated that BLZ-100 has a high affinity for glioma compared with normal brain tissue. Additionally, the results show that NIR excitation is able to penetrate deeply and has a potential to visualize metastatic lesions that are separate from the main tumor.

Conclusions. The authors have seen that BLZ-100 has a very high affinity toward human gliomas. They also describe a small, cost-effective, and sensitive NIR system for visualizing brain tumors tagged using BLZ-100. The authors hope that the use of BLZ-100 along with NIR imaging will be useful to delineate the brain tumors in real time and assist surgeons in near-complete tumor removal to increase survival and reduce neurological deficits.

(<http://thejns.org/doi/abs/10.3171/2013.11.FOCUS13497>)

KEY WORDS • fluorescence • imaging • glioma • ligand • indocyanine green • Tumor Paint

GLIOMAS are the deadliest and commonest tumors of the CNS. The current 18-month survival rate of glioblastoma patients treated with complete resection ranges from 15% to 34%, making glioma one of the most lethal tumors.⁶ Additionally, these tumors' deleterious effects are visible in the patient's quality of life, their family, and the health care system, with a mean survival of 14 months. Gliomas have a reported incidence of 10 per 100,000 per year and result in 13,000 deaths per year, accounting for more years lost than any other tumor.¹¹ Although multiple options such as chemotherapy

and radiation therapy are available for the treatment of gliomas, due to the inability of chemotherapeutic agents to cross the blood-brain barrier and the ineffectiveness as well as adverse effects of radiation therapy, resection remains the primary treatment.^{10,13,27} The aggressiveness of tumor surgery and extent of resection in prolonging patient survival is controversial,^{11,12,18} however, there is evidence that minimizing residual tumor will decrease the likelihood of recurrence.^{3,30} There are multiple publications over the past couple of decades that have demonstrated that surgical extent is the single most important determinant of outcome and the most important predictor of longer survival.^{4,7,28} Evidence also suggests that more complete resection of gliomas correlates with a higher progression-free interval and may prolong life expect-

Abbreviations used in this paper: CCD = charge-coupled device; CTX = chlorotoxin; HD = high-definition; ICG = indocyanine green; NIR = near-infrared.

tancy.^{11,12,18} One of the primary challenges of surgical removal of brain tumors is to identify and visualize the tumor tissue separate from the normal cortex. Balancing aggressive resection against the necessity to limit resection of surrounding normal brain tissue is the core goal of brain tumor surgery. Intraoperative fluorescence-guided resection can facilitate this goal by defining the location of the tumor and delineating the brain-tumor border.²⁶ Thus, newer technologies are needed that can accurately distinguish between the tumor and normal brain and guide tumor resection in real time.

Tumor-Specific Ligand

Targeting molecules such as antibodies and peptides are being investigated as tumor-specific ligands. One such ligand is chlorotoxin (CTX), a 36-amino acid peptide initially isolated from the venom of the scorpion *Leiurus quinquestriatus*. The peptide has demonstrated specificity as a tumor-targeting agent in a variety of formats, including radiolabeled, conjugated to fluorescent tags, or incorporated into nanoparticles.^{1,29} All clinical trials to date conducted with CTX indicate that it has negligible toxicity in humans. These unique properties make CTX attractive as a targeted imaging agent for cancer. In transgenic and xenograft mouse models of glioma, medulloblastoma, prostate cancer, sarcoma, and colorectal cancer, a CTX-Cy5.5 conjugate ("Tumor Paint") was shown to bind to both primary tumor and metastatic lesions.³⁷ Subsequent work with CTX conjugated to a variety of near-infrared (NIR) fluorophores confirmed and extended these results, supporting the peptide's specificity and breadth of tumor recognition.²⁰ BLZ-100 is a CTX-indocyanine green (ICG) conjugate that is being advanced toward human clinical trials.

In the past few years there has been an increased interest in NIR light fluorescence imaging.³² Near-infrared contrast agents such as ICG offer well-recognized advantages over ultraviolet or visible light imaging. These include better tissue penetration of incident light, less scatter of emitted photons, and low tissue autofluorescence.³² There are multiple imaging systems that have been developed by both academic and industrial groups.^{5,8,9,17,19,21,22,24,25,31,33,38,39} There are separate systems for open surgeries, laparoscopic surgeries, and robot-assisted surgeries that vary based on specific challenges. Some of the important criteria for developing a new imaging system are based on concentration of the fluorophores, type of tissue due to varying tissue penetration depths, ergonomics, and cost. Additionally, NIR imaging systems have to optimize the excitation light fluence, and there is a choice between coherent and incoherent light sources. Incoherent light sources are easier to work with, require fewer precautions for skin and eye exposure, and face fewer regulatory hurdles, but require high energy to produce adequate fluorescence. Coherent sources (lasers) are usually restricted due to skin and eye exposure to 10–25 mW/cm² to negate the need for protective glasses,³² but if the photobleaching effect on the fluorophore is absent at higher energies and the use of protective glasses is not an issue during the surgery, it may be possible to use higher energy. Considering these factors, we would like to report development of a "proof-of-concept" intraopera-

tive NIR imaging system to detect a novel tumor ligand attached to an NIR dye (ICG) for use in brain tumor surgeries.

Methods

Fluorescence Imaging System

To address the need for a clinically relevant tool for NIR fluorescence-guided resection of tumors, we developed a camera system that simultaneously acquires both white light and NIR images and combines these images via superimposition on a high-definition (HD) video monitor. The proof-of-concept system consists of a dual HD charge-coupled device (CCD) camera that splits incident light into 2 pathways, one for white light and the other for NIR light (AD-130GE, 1/3", 1296 × 966, 31 fps, GigE; JAI, Inc.) (Fig. 1).

A fixed focal length lens (16-mm VIS-NIR Compact Fixed Focal Length Lens, Edmund Optics) was attached using a C-mount. A thin filter holder (NT56–353, C-Mount Thin Lens Mount, 25/25.4-mm diameter, Edmund Optics) was attached in front of the lens, which housed a 785-nm notch filter to filter out the excitation light from the return image (785-nm StopLine single-notch filter, NF03–785E-25, Semrock). Near-infrared excitation is provided via a 785-nm laser diode (LD785-SH300, Thorlabs), while white light is provided through a commercially available xenon light source (Storz). The camera acquires both white light and NIR fluorescence images. These are captured via a GigE interface to a computer. The NIR images are given a transparency mask, given a false color, and added to the white light image. The resultant HD-quality images superimposed with fluorescent maps of BLZ-100 distribution can be used to direct intraoperative detection and resection of tumor. The camera system can detect BLZ-100 in nanomolar concentrations. Since the images are collected digitally, the detection sensitivity can be adjusted utilizing analog and digital gain and threshold to maximize detection in the NIR, as well as providing artificial color maps.

BLZ-100

The CTX peptide was synthesized using standard solid-phase methods. After refolding, the peptide was conjugated to an active ICG derivative using standard NHS ester chemistry. The conjugate underwent high performance liquid chromatography purification.

Intralipid Studies

To test the sensitivity of the instrument to detect BLZ-100, a range of BLZ-100 concentrations were prepared in 5% Intralipid emulsion (Sigma Aldrich). The BLZ-100 concentrations tested were 1 μ M, 500 nM, 50 nM, and 50 pM in 5% Intralipid emulsion, and 20% Intralipid emulsion was tested with no BLZ-100 as a control. The laser fluence was varied to determine the optimal fluence rate for each BLZ-100 concentration. The following fluence rates were tested: 5, 10, 15, 20, 30, and 40 mW/cm². The analog gain on the CCD was set at 0 dB, 6 dB, and 12 dB, and the fluorescence intensity was noted.

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Fig. 1. Schematic showing the prototype NIR imaging system.

Intracranial Tumors in Animals

BLZ-100 was tested in mice implanted with human glioblastoma cells along with ICG as control. The mice were treated according to the approved Cedars-Sinai Medical Center Institutional Animal Care and Use Committee protocols. Athymic mice (NCR-nu/nu homozygous) were obtained from the National Cancer Institute. Human glioblastoma cells (LN229) were stereotactically implanted at 5×10^5 cells/mouse into the right basal ganglia field of mice under ketamine and dexmedetomidine intraperitoneal anesthesia, as reported previously.¹⁴ The animals were closely monitored and used for the experiment about 6 weeks postinoculation, when the tumors had reached maximum size.

In 1 mouse that was under inhalation isoflurane anesthesia, vascular contrast dye (20 nmol ICG) was injected intravenously once via the tail vein using a 30-gauge needle on a 1-ml syringe at a rate of 50 μ l over 3 seconds. Another mouse was injected in a similar manner with 20 nmol of BLZ-100. One set of mice received phosphate-buffered saline as control. After drug administration, the mice were observed for several hours for any physical symptoms or behavioral changes. Forty-eight hours after injection, animals were anesthetized via intraperitoneal injection with a combination of ketamine and dexmedetomidine and were euthanized via cervical dislocation.

The mice were then placed securely in the stereotactic apparatus, and a 10-mm midline incision was made in the scalp, as for the tumor cell inoculation. A craniectomy was then performed over the tumor site. Once the skull was removed, direct in situ tumor imaging was

performed, after which the brain and other organs were removed for individual imaging and processing. Direct imaging of the tumor site was done after euthanasia.

Results

Intralipid Study

To quantitate the sensitivity of the CIRCAM for BLZ-100, a series of dilutions were tested in Intralipid emulsion, which mimics the light scattering and absorption properties of tissue. At 1 μ M BLZ-100, the CCD was able to record fluorescence at all the fluence rates and all the CCD gains. The imaging system was able to record fluorescence at the lowest excitation fluence of 5 mW/cm² and with no analog gain on the CCD (Fig. 2).

The fluorescence increased when the concentration of BLZ-100 was decreased to 500 nM (0.5 μ M) as observed in Figs. 3 and 4 (6 dB and 12 dB gain). This is believed to be due to reduction in fluorescence quenching.

When BLZ-100 was diluted to 50 nM of concentration, the fluorescence was difficult to discern at 0-dB gain on CCD, but when the analog gain of the CCD was increased to 6 dB, fluorescence could be seen at 20 mW/cm² of laser fluence. Although the samples demonstrated visible fluorescence when CCD gain was set at 12 dB at all the excitation energies, there was a significant increase in the CCD noise (Fig. 4). At 50 pM, fluorescence from BLZ-100 was barely detectable at 40 mW/cm² with 12 dB of gain. Finally, 20% Intralipid solution was tested, which showed no fluorescence at any excitation energy and CCD gain (Fig. 4).

Fig. 2. Fluorescence from BLZ-100 mixed in 5% Intralipid emulsion was recorded at varying laser fluence with 0-dB analog gain. As seen in the image, there is no discernible fluorescence at a low concentration of 0.5 μM . The change in the fluorescence area was due to Gaussian profile of the laser diode emission.

Fig. 3. Fluorescence from BLZ-100 mixed in 5% Intralipid emulsion was recorded at varying laser fluence with 6-dB analog gain. As seen in the results with the analog gain, it is possible to visualize BLZ-100 down to 50 nM concentration at fluence of 20 mW/cm².

Fig. 4. Fluorescence from BLZ-100 mixed in 5% Intralipid emulsion was recorded at varying laser fluence with 12-dB analog gain. As seen in the image, ICG fluorescence was visible down to 50 pM with a high signal-to-noise ratio when excited using 40 mW/cm².

In Vivo Study (Brain)

To demonstrate effective discrimination between BLZ-100–labeled tumors and surrounding normal brain tissue, mice bearing orthotopic glioma tumors were treated with BLZ-100 and were imaged 48 hours later. The mice injected with BLZ-100 showed a significant fluorescence from the glioma (Figs. 5 and 6), where there was no fluorescence observed from the mice injected with plain ICG. BLZ-100 accumulated in the glioma. Additionally, fluorescence from BLZ-100 was able to show a deeper metastasis (Fig. 5). The organs harvested from the mice were also recorded for fluorescence (Fig. 7). There was no visible fluorescence from the stomach, heart, lung, and skin. The kidney and liver showed detectable amounts of BLZ-100.

Discussion

Near-Infrared Imaging and Tumor Ligands

Compared with intraoperative x-ray fluoroscopy, ultrasonography, CT, and MRI, targeted NIR imaging affords the combination of tumor specificity, low cost, simplicity, and safety, without exposing patients and personnel to ion-

izing radiation.³² The NIR technique involves the use of an imaging system or device along with a contrast fluorophore.^{2,32} Multiple optical imaging systems are currently out on the market, and pertinent contrast agents include methylene blue and ICG.^{2,32} The technology has seen clini-

Fig. 5. Left: White light image overlaid with an NIR image of a brain slice with implanted glioma. **Right:** Image from the NIR channel, CCD gain was set at 0 dB, and excitation fluence was 40 mW/cm². Notice the satellite metastatic lesion separate from the main tumor body. Due to high penetration depth of the excitation light, NIR imaging is able to visualize deep tumors, not seen by visual inspection.

FIG. 6. **Left:** White light image overlaid with an NIR image of sections through the brain sample implanted with human glioma cells. **Right:** A separate NIR channel.

cal applications in multiple fields including laparoscopy, hepatology, coronary artery surgery, vascular surgery, and surgical oncology.^{2,32} In particular, there has been a lot of interest in NIR imaging using ICG, with reported uses in detection of breast cancer, melanoma, otolaryngological malignancies, and liver metastasis.^{15,16,22,23,33–36} Indocyanine green (ICG) is a water-soluble molecule that has been used in angiographic, cardiac, hepatic, and oncological applications.^{3,26} Its minimal side effects and positive safety profile have made it an increasingly used compound in multiple disciplines. In neurosurgery, it has been demonstrated to outline tumor boundaries and differentiate low-grade from high-grade tumor cells.³ It has also been routinely used to aid aneurysm and arteriovenous malformation surgery through ICG video angiography.³

Recently, NIR technology has been used alongside fluorescent tumor ligands. These cancer-targeted molecules can be used to specifically isolate tumors for resection, and combining them with fluorescent molecules and NIR technology can make them even more powerful tools. The currently available NIR imaging systems are optimized for high-volume and high-concentration ICG for visualizing vasculature or sentinel lymph nodes. Additionally, the imaging systems are bulky and many are as big as the operating microscopes. An important criterion for us in designing the system for brain tumor imaging was to have a small profile so as to allow the surgeon to use the operating microscope separate from the NIR imaging system. Most of the traditional NIR systems available use 2 separate sensors for visible and NIR channels. The NIR light is redirected separate from the visible light using a beam splitter. Although this framework allows for the use of a separate high-sensitivity infrared optimized camera, it also adds to the weight and size of the system. To avoid this problem we have designed a new system that uses the same sensor for both visible and infrared channels. However, to test the hypothesis that a single sensor with the NIR filter removed can have enough sensitivity to record fluorescence from the brain tumor, we used the JAI AD-130GE camera, which uses identical CCDs for both infrared and visible channels. From our initial results we can report that BLZ-100 fluorescence can be recorded by a CCD, which is neither cooled nor intensified.

We also chose to use laser excitation instead of an incoherent light source such as xenon lamps. This allowed us to use a very narrow notch filter instead of a broad long-pass filter to remove the excitation light and collect

FIG. 7. White light images overlaid with NIR images of organs showing fluorescence from the residual BLZ-100. There is very low or no discernible fluorescence from stomach (**A**), skin (**C**), heart (**D**), and aorta (**E**), while the kidney (**B**) and liver (**F**) show significant fluorescence as ICG is excreted through the kidney.

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maximum fluorescence. Laser light is also more efficient in generating fluorescence from the ICG than incoherent light. Thus, by optimizing both the excitation as well as fluorescence light paths, we were able to achieve high sensitivity with very low noise.

Conclusions

We have demonstrated as a proof-of-concept that it is possible to use a standard CCD for recording fluorescence from brain tumor using BLZ-100, a tumor ligand conjugated to ICG. This will enable us to reduce the size and cost of the imaging system. The use of a narrow-band laser at the peak absorption of ICG allows us to use low excitation fluence, and use of a narrow notch filter in front of the sensor will allow us to gather maximum fluorescence.

Acknowledgment

We thank Dr. David Kittle for his help with the arrangement and formatting of the images.

Disclosure

The authors thank Stacy Hansen and Disha Sahetya from Blaze Bioscience, Inc., Seattle, for providing the BLZ-100 samples. The study was internally funded by the Department of Neurosurgery at Cedars-Sinai Medical Center. Dr. Mamelak has ownership in Teal Light Surgical. Dr. Parrish-Novak is an employee of Blaze Bioscience, Inc.

Author contributions to the study and manuscript preparation include the following. Conception and design: Butte, Mamelak, Parrish-Novak. Acquisition of data: Butte, Gangalum, Chesnokova. Analysis and interpretation of data: Butte. Drafting the article: Butte, Drazin, Shweikeh, Gangalum. Reviewed submitted version of manuscript: Mamelak, Parrish-Novak. Administrative/technical/material support: Mamelak, Parrish-Novak, Gangalum, Chesnokova, Ljubimova, Black. Study supervision: Mamelak, Parrish-Novak.

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Manuscript submitted October 18, 2013.

Accepted November 26, 2013.

Please include this information when citing this paper: DOI: 10.3171/2013.11.FOCUS13497.

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