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In Vivo Bio-imaging Using Chlorotoxin-based Conjugates

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Abstract

Surgical resection remains the primary component of cancer therapy. The precision required to successfully separate cancer tissue from normal tissue relies heavily on the surgeon's ability to delineate the tumor margins. Despite recent advances in surgical guidance and monitoring systems, intra-operative identification of these margins remains imprecise and directly influences patient prognosis. If the surgeon had improved tools to distinguish these margins, tumor progression and unacceptable morbidity could be avoided. In this article, we review the history of chlorotoxin and its tumor specificity and discuss the research currently being generated to target optical imaging agents to cancer tissue.

Keywords

Chlorotoxin; Near-infrared Dye; Optical Imaging; Cancer; Tumor Paint

INTRODUCTION

Cancer is the second leading cause of death in the United States with over 1.5 million people diagnosed each year [1]. Surgery is often the first and most important step in the long process of cancer treatment for patients. The extent of tumor resection is frequently the primary indicator of whether a patient will suffer a relapse. Since most tumors that recur do so at the original site of the lesion it is imperative that as much of the tumor is removed as possible. However, it is often difficult for a surgeon to distinguish normal from neoplastic tissue, and despite the surgeons expertise tumor tissue is sometimes left behind. Current technologies, such as magnetic resonance imaging (MRI), do not enable surgeons to directly visualize tumor margins in a real time intra-operative setting. Research in developing tumor specific intra-operative imaging agents that enable surgeons to completely resect neoplastic tissue may have a significant impact on patient survival.

Nowhere in the body is removing tumor tissue while sparing normal tissue more important than in the brain. In the case of medulloblastoma, the most common malignant pediatric brain cancer, 80% of patients have a five-year survival rate when a near complete resection is achieved. This compares to a 50%-65% five-year survival rate if more than 1.5 cm of residual tissue remains post-surgery [2]. In the brain however, surgical precision is required because removing even a small amount of normal brain tissue could leave a patient unable to speak or walk. It is therefore imperative that neurosurgeons have the capability to

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Conflict of interest: The authors hold interest in Blaze Bioscience, which intends to commercialize Tumor Paint

distinguish tumor tissue from normal healthy brain structures. In an effort to provide surgeons with real-time biophotonic information to clearly define tumor margins and small foci of cancer cells, we recently developed a chlorotoxin-based molecular imaging probe as an effective targeting agent for many types of cancer. The bioconjugate, known as “Tumor Paint” specifically binds to tumor cells without binding normal tissue [3]. In addition, Tumor Paint retains the ability of native chlorotoxin to cross the blood brain barrier, a necessary requirement for imaging brain tumors. Tumor Paint fills the gap in current technology by allowing surgeons to visualize tumor tissue in a real-time intra-operative setting.

In addition to demarcating tumor margins in the brain, surgeons also face challenges when trying to determine the extent of metastatic disease and lymph node infiltration in neoplasms of the prostate and colon. Tumor paint technology may provide surgical oncologists a valuable tool for real-time tumor detection leading to an increase in positive outcomes for patients.

Chlorotoxin

The crude venom from the scorpion *Leiurus quinquestriatus* was previously shown to inhibit reconstituted small-conductance chloride ion channels from rat epithelia and rat brain [4]. The active compound of this venom was purified and characterized as a small, basic, 36 amino acid peptide with considerable sequence homology to a class of previously described peptides of the four-disulfide core protein family, including the short insectotoxins, neurophysins, and agglutinins [5, 6]. Structural analysis by solution NMR indicates that the peptide consists of an alpha-helix linked by three disulfide bridges to a small, three-stranded, antiparallel beta-sheet. The fourth disulfide bridge links the N-terminal cysteine to the rest of the molecule (Figure 1) [7]. The active component of the venom responsible for the inhibitory effect on chloride channels was named chlorotoxin [5].

A series of studies following the initial finding on the effects of chlorotoxin on chloride ion channels suggested that a glioma-specific chloride channel facilitates shape changes during glioma cell migration and invasion [8, 9] and that these channels are specifically targeted by chlorotoxin. However, these results have been refuted [10]. First, the current was activated under non-physiological conditions and attempts to reproduce these experiments using the same cells under identical conditions have failed to confirm the original findings [10]. In addition, since verified chloride ion blockers failed to block the ion current, the investigators concluded that chlorotoxin blocks neither volume-regulated nor calcium-activated chloride channels and that they are therefore not the natural target of chlorotoxin [10].

Previous studies demonstrate chlorotoxin specifically targets gliomas in which human glioma cells were xenografted into the cerebrum of SCID mice [11]. More recently, immunohistochemical screening of over 250 human biopsy samples showed selective binding of chlorotoxin to all grades of malignant gliomas and tumors of neuroectodermal origin, including medulloblastomas, neuroblastomas, melanomas, PNETs, and small cell lung carcinoma [12]. Under the same staining conditions corresponding normal tissues were consistently negative for chlorotoxin.

Due to chlorotoxin's ability to specifically bind to malignant glioma, it is currently being evaluated in human clinical trials as a targeted radiotherapy agent and an imaging peptide for determining the extent of glioma invasion [13, 14]. In phase I clinical trials, ¹³¹I-TM-601, an ¹³¹I-labeled synthetic version of chlorotoxin, specifically targeted tumor tissue following an intracavitary injection [14]. ¹³¹I-TM-601 was safely administered to patients with recurrent high-grade glioma with no grade 3 or 4 toxicities [14]. These results indicate that ¹³¹I-TM-601 can be safely administered to humans and further phase II clinical trials

are warranted to evaluate its efficacy as a radiotherapy agent. ^{131}I -TM-601 was also shown to be an effective imaging agent for evaluating tumor extent in phase I/II clinical trials and can be further improved with isotopes that have better imaging capabilities [13]. These studies indicate that chlorotoxin is safe to administer in patients with brain tumors and may be an important imaging tool for determining the extent of neoplastic growth.

Since chlorotoxin shows a high specificity toward gliomas and tumors of neuroectodermal origin [12], studies were initiated using a recombinant His₆-chlorotoxin as a probe to isolate and characterize the true physiological target from glioma cells [15]. Contrary to the original idea that a specific chloride channel is the direct target of chlorotoxin, matrix metalloproteinase-2 (MMP2) was co-purified by affinity chromatography as part of a macromolecular complex containing MMP2, membrane type-I MMP (MT1-MMP), $\alpha_v\beta_3$ integrin, and tissue inhibitor of metalloprotein-2 (TIMP-2). Further experiments demonstrated that chlorotoxin inhibits MMP2 enzymatic activity and causes reduction of cell surface expression of MMP2 and that chlorotoxin does not bind to MMP-1, MMP-3, or MMP-9 [15]. MMP2 is part of a lipid raft anchored protein complex that includes MT1-MMP and TIMP-2 [16, 17], and MMP2 is involved in the enzymatic degradation of the extracellular matrix [18, 19]. It belongs to the family of zinc dependent endopeptidases and is expressed in numerous tumor types [20, 21] contributing to the invasive potential of tumors [22]. It has been suggested that the binding of chlorotoxin to the cell surface leads to internalization of the entire lipid raft anchored complex containing MMP2, MT1-MMP, TIMP-2, chloride channels, and other proteins, thereby eliminating the functional chloride ion channel [15, 23]. Preliminary evidence using filipin, a cholesterol binding agent known to disrupt the caveolae endocytosis pathway, demonstrated decreased chlorotoxin-induced MMP2 internalization in samples treated with filipin [15, 17]. Combined, these data made MMP2 the leading chlorotoxin target candidate, but direct evidence of interaction between the proteins is lacking, as is evaluation of the other candidate targets that were co-isolated with MMP2 by chlorotoxin affinity chromatography. It was recently reported that annexin A2 also acts as a target for TM601, a chemically synthesized, biotinylated version of chlorotoxin [24]. However, based on their studies, the authors also stated that membrane type metalloprotease-1 bound TM601, and additional proteins in macromolecular complexes may contribute to its binding. The molecular basis of chlorotoxin's specificity towards cancer is still unclear since annexin A2 is expressed intracellularly in all cells. Further studies are clearly needed to conclusively identify the molecular target of chlorotoxin.

Near-infrared Fluorophores

Fluorophores that emit in the near-infrared (NIR) range (600–900 nm) have become popular as *in vivo* imaging agents. Since absorption of light by hemoglobin and lipids occurs below 600 nm and absorption of light by water occurs above 900 nm, light scattering and tissue absorption is decreased while tissue penetration is increased when imaging is performed within the NIR range. In addition, tissue autofluorescence is high when wavelengths in the visible spectrum are used causing an increase in noise or background resulting in a reduction in the ability to detect specific signal from the imaging agent (Figure 2). These principals of NIR fluorescence in biological systems are extensively discussed in reviews by Ralph Weissleder and John Frangioni [25, 26].

Technology that takes advantage of the naturally occurring autofluorescence inherent in endogenous molecules is being developed as a method of resolving tumor margins and detecting invasive disease in glioma tumors [27–29]. Time-resolved laser induced fluorescence spectroscopy is a technique that measures the unique fluorescence lifetime of endogenous biomolecules thus providing a specific profile for each cell type within a tissue. This information can be converted into color images that could be used by a surgeon to discriminate normal from neoplastic cells. In addition to delineating tumor margins, this

technology may be used as a rapid intra-operative diagnostic tool for differentiating low-grade or high-grade glioma [30]. Autofluorescent detection has also been used in colonoscopy procedures to improve detection of neoplasms of the colon. In patients however, autofluorescent imaging technology failed to improve the detection of neoplastic lesions due to low levels of specificity [31]. While this technology is appealing because it does not require the use of imaging probes it remains to be determined if it is sensitive and specific enough to be used in a clinical setting.

Cyanine based dyes are a broad family of fluorescent molecules. These dyes are chemically synthesized fluorescent organic molecules containing an odd number of methine groups with alternating single and double bonds (polymethines). The polymethine group connects two nitrogens within two separate aromatic moieties. Various reactive groups such as *N*-hydroxysuccinamide, and maleimide can be attached to the side chain of the nitrogens allowing different chemistries to be utilized to react with primary amines and sulfhydryl groups respectively on larger macromolecules. The chemical nature of the cyanine dyes and the length of the polymethine moiety can be controlled to allow fluorescence of the molecule within a broad range (200–900 nm) of the electromagnetic spectrum covering the ultraviolet (UV) to infrared (IR) range.

Cyanine fluorophores are bright fluorescent dyes with high molar extinction coefficients that generate good quantum yields. Newer generations of these molecules have been chemically modified to be soluble in aqueous solutions, stable in DMSO and stable within a pH range of 3–10. They also show increased photo-stability compared to earlier generations of cyanine dyes.

Currently, only a few fluorophores are available and approved by the FDA for use in the clinic. Indocyanine green (ICG), [32] fluorescein-labeled serum albumin, [33] and 5-aminolevulinic acid-induced porphyrins [34, 35] have all been used intra-operatively to enhance tumors and delineate tumor margins during fluorescent guided resections of glioblastoma multiforme. However, of these only ICG fluoresces in the near-infrared range and is suitable for deep tissue penetration. Cyanine based fluorophores that emit in the NIR spectrum are also being developed and tested for *in vivo* applications. One such dye, IRDye 800CW, has been developed and shown to be efficacious when used for *in vivo* imaging applications [36, 37]. IRDye 800CW has recently been shown to exhibit no toxic effects in an extensive toxicity study in rats [38]. Figure 3 shows the structure of Cy5.5 (Figure 3A) and IRDye 800CW (Figure 3B), two of the commercially available NIR dyes sold by Li-Cor Biosciences and GE Health Sciences respectively. A summary of selected NIR dyes is shown in Figure 3C.

Chlorotoxin-based Imaging Agents

Our lab reported the development of “Tumor Paint”, a chlorotoxin: Cy5.5 (Ctx: Cy5.5) bioconjugate. Its efficacy as an *in vivo* imaging agent was demonstrated in mouse models of glioma, medulloblastoma, prostate cancer, intestinal cancer, and sarcoma [3]. We addressed the sensitivity of this agent for detecting cancer foci and metastasis noninvasively and under simulated surgical operating conditions. The technique combines an intuitive visual guide for the surgeon with the potential for significant improvement in accuracy and safety.

Preclinical development of the Ctx: Cy5.5 bioconjugate determined that the compound has pharmacokinetic and biodistribution properties that make it suitable for use in surgical oncology practice. In these studies, Ctx: Cy5.5 bound to tumor tissue as early as one day after injection and could be detected in tumors as long as 14 days after injection [3]. The prolonged retention of Ctx: Cy5.5 in tumor tissue in mice is consistent with that observed for ¹³¹I-TM-601 in human clinical trials in patients with high grade glioma [13]. In these

phase I/II clinical trials, free circulating ^{131}I -TM-601 was cleared 48 hours after injection with tumor specific signal remaining for up to eight days after injection [13]. The extended retention time of Ctx:Cy5.5 in preclinical studies is promising, however, it is unknown how the conjugate will behave in human surgical settings. Factors that could lead to a reduction in tumor cell specificity such as leakage of the probe out of the cells into surrounding normal tissue during surgery and bioconjugate integrity over long periods of time are to be determined.

Biodistribution studies of chlorotoxin imaging agents indicate that chlorotoxin is cleared mainly by the kidney with some accumulation in the liver [3]. These studies indicate that intra-operative imaging in tissues other than kidney and liver, particularly in brain, prostate, and intestines where background signal is very low, will be beneficial in human clinical practice.

Medulloblastoma is the most common malignant brain tumor in children. Children diagnosed with medulloblastoma undergo extensive and complicated tumor resection surgery. In some cases, it is difficult for a neurosurgeon to demarcate normal from diseased tissue. The trade off of complete surgical resection without causing damage to normal brain has drastic consequences for the patient. Studies have shown that post-operative residual disease of more than 1.5 cm results in a 50%–65% five-year survival rate; this compares to an 80% survival rate over the same period for standard risk patients where a complete resection of tumor tissue was achieved [2]. Advances in intra-operative technologies that improve the distinction between normal brain and tumor tissue is highly desired.

Ctx:Cy5.5 has been extensively tested in mouse models of medulloblastoma by us [3]. Ctx:Cy5.5 clearly demarcated tumor margins in the genetically engineered ND2:SmoA1 mouse medulloblastoma model. The bioconjugate effectively labeled tumor cells in mice with bulky advanced tumors as well as subclinical neoplasms where only small foci of cancer cells were present (Figure 4). These studies demonstrate the tumor specificity of Ctx:Cy5.5 and its ability to clearly delineate tumor cells from normal brain tissue [3].

Ctx:Cy5.5 also targets prostate cancer [3]. The bioconjugate targeted and highlighted tumors in the prostate epithelium in the TRAMP mouse model of prostate cancer, where the SV40T gene is expressed in the prostate epithelium [39]. In addition, small metastatic foci of cells in lymph nodes were tagged and detected with this molecule (Figure 5).

Current statistics indicate that in the United States an estimated 217,730 men will develop prostate cancer each year [1]. Prostate cancer is the second most common cause of cancer death in men accounting for 29,093 deaths per year [1]. One important prognostic factor in prostate cancer survival is the detection and removal of cancer positive lymph nodes, which decreases a patient's risk of tumor recurrence. Current imaging technology does not accurately detect lymph node metastasis, therefore surgical dissection is the current state of the art in lymph node cancer detection [40]. Standard pelvic lymph node dissection (PLND) removes many but not all of the lymph nodes that drain from the prostate. It has been debated if a more radical and extend lymphadenectomy would increase the chances of removing cancer positive lymph nodes therefore decreasing the likelihood of recurrence [41]. Ctx:Cy5.5 has the ability to help surgeons detect and resect all cancer positive lymph nodes while sparing the patient from drastic and extended lymphadenectomy. Selected removal of cancer positive lymph nodes would decrease the occurrence of medical complications associated with both standard and extended PLND while ensuring the patient has the greatest survival benefit.

In the United States cancers of the colon and rectum are the third leading cause of cancer related deaths with an estimated 72,090 new cases expected in 2010 [1]. Colonoscopy

remains the most important colorectal cancer screening method for detecting early pre-symptomatic tumors with over 14 million procedures performed in the United States per year [42]. Current technology detects small precancerous polyps, which are surgically removed. However, many non-cancerous polyps are often unnecessarily dissected increasing the risk of adverse complications for the patient [43]. Conversely, data suggests that 26% of adenomatous polyps [44] and some distinct polyp subclasses, such as flat polyps, are often missed during colonoscopy [43]. In one study, flat polyps were discovered in 9.35% of patients and were associated with an increased incidence of carcinoma cells than the more common polypoid lesions [45]. Ctx: Cy5.5 delineated intestinal cancers from normal tissue in the *Apc^{1638N}* mouse model of familial adenomatous polyposis (FAP) [3, 46] demonstrating that chlorotoxin based imaging agents bind to cancerous lesions of the intestine and colon (Figure 6). Chlorotoxin based imaging technology may provide a much needed tool that would help gastroenterologists identify and remove difficult to detect flat polyps while improving detection of neoplastic polyps and distinguishing them from non neoplastic tissue.

Re-engineered and Chlorotoxin-like Peptides as Fluorescent Imaging Agents

As described above, native chlorotoxin is a 36 amino acid peptide with four disulfide linkages. We conjugated Cy5.5 and other cyanine based NIR fluorophores to chlorotoxin using standard *N*-hydroxysuccinimide ester (NHS-ester) chemistry. Chlorotoxin has three lysines at positions 15, 23, and 27, and an *N*-terminal primary amine that can act as potential sites for conjugation. The synthesis was optimized by controlling the pH, the dye to peptide molar ratio, and the reaction time, so the final bioconjugate would be predominantly mono-labeled [3]. However, since four sites exist that have the potential for conjugation, even under optimal conditions small amounts of multi-labeled product could be generated. Batch to batch variations in the final composition were slight but did vary even when reaction conditions were identical. A purified mono-labeled product could be obtained by reversed-phase HPLC but a 50% loss in final yield was not unusual and made the synthesis cost prohibitive. Our immediate goal is to get US Food and Drug Administration (FDA) approval and bring the final product to clinical trial as soon as possible. Delivering a final synthetic product that gives consistent and reproducible results would improve the chances of getting FDA approval. In addition, a final product that has improved serum half-life and stability would be highly desirable. We recently reported that by substituting Lys15 and Lys23 with either alanine or arginine in chlorotoxin eliminated the generation of multi-labeled bioconjugates while retaining its tumor targeting specificity [47]. We also synthesized a cyclized version of chlorotoxin that exhibited increased serum stability and yielded only a mono-labeled Cy5.5 product (Figure 7). Based on these findings, we hope to progress these chemically re-engineered peptides as tumor imaging agents and advance them to human clinical trials.

Chlorotoxin shares sequence homology and contains a number of highly conserved amino acids with various chlorotoxin-like peptides isolated from the venom of a number of different scorpion species [48, 50–57] (Figure 7). *Buthus martensii* Karsch chlorotoxin (BmKCT) is a chlorotoxin-like peptide isolated from the venom of the Chinese scorpion indigenous to regions of China and the Korean peninsula. BmKCT is similar to chlorotoxin and shares 68% sequence homology with chlorotoxin isolated from *Leiurus quinquestriatus*, the Israeli death scorpion. BmKCT is a 35 amino acid protein and, like chlorotoxin, contains four disulfide bridges [49]. Studies by the Li group have shown that BmKCT conjugated to Cy5.5 specifically targeted glioma tumors in a rat xenograft model. In addition, both BmKCT and chlorotoxin inhibited glioma cell growth when expressed as a GST fusion protein [58]. Another novel chlorotoxin-like peptide was recently purified from the venom of the scorpion *Androctonus australis* (AaCtx) and shown to have 70% amino acid sequence

homology with chlorotoxin, differing by only twelve amino acids [48]. A synthetic version of this peptide showed weak activity on inhibiting glioma cell migration and invasion when compared to chlorotoxin. The absence of negatively charged amino acids on the α -helix and N-terminal loop may be responsible for this weak activity. Direct comparison of chlorotoxin-based bioconjugates with the chlorotoxin-like peptide conjugates could determine which amino acids are candidates for further modification and potentially improve the binding characteristics, serum stability, and half-life of these tumor imaging agents.

Chlorotoxin Functionalized Nanoparticles as Multi-modal Imaging Agents

The use of nanoparticles in preclinical and clinical practice has recently been reviewed [59]. Non-functionalized iron oxide nanoparticles like Feridex I.V.® are currently in use in human clinical practice. Feridex I.V.® is a widely used MRI contrast reagent consisting of superparamagnetic iron oxide particles. This contrast agent is not targeted and non-specifically taken up by the reticuloendothelium system of the liver [60]. These properties make Feridex I.V.® useful as a MRI contrast enhancement agent for lesions of the liver but with limited utility for imaging tumors in the central nervous system. Gadolinium-based contrast media are routinely used to detect tumors of the central nervous system. The use of contrast agents with MRI was extensively reviewed by Marco Essig et al [61]. Gadolinium-based imaging agents are very effective in delineating brain tumors but cannot cross the blood brain barrier and therefore detection is limited to tumors or areas within neoplastic lesions where the blood brain barrier has been disrupted. Alterations in the blood brain barrier are common in brain tumors but is often heterogeneous within a tumor and dependent on the size and shape of the lesion [61]. Contrast agents that cross the intact blood brain barrier and specifically bind to tumor cells have the potential to improve MRI detection and evaluation of central nervous system lesions. In addition, NIR fluorescent molecules can be linked to the nanoparticle allowing both MRI contrast enhancement and intra-operative optical imaging capabilities.

Chlorotoxin conjugated nanoparticles have been shown to be effective compounds for use as MRI contrast agents, optical imaging reagents, and carriers for DNA and chemotherapeutic agents in preclinical studies using mouse models of cancer. These nano-scale materials are composed of a myriad of molecules (Table 1) and have varying functions and properties (Figure 8). Since these nanoparticles are functionalized with a tumor targeting molecule, are small in size, and have a surface coating that enable them to cross the blood brain barrier, they have utility as imaging agents in tumors of the central nervous system. Three distinct families of nanoparticles have been explored as chlorotoxin-functionalized particle-based imaging agents. Of these, iron oxide based multi-modal nanoparticles have been extensively developed and advanced in preclinical studies using mouse models of cancer. In addition, two new classes of nanoparticles, semi-conducting polymer dots (Pdots), and rare-earth upconverting nanoparticles (UNCP) have recently been reported as functionalized fluorescent probes with physical properties that make them attractive for use as imaging agents.

Iron oxide based nanoparticles that have been functionalized with chlorotoxin have been generated and extensively studied with the purpose of imaging brain tumors using MRI and optical imaging [63–65]. This multi-modal approach has the added benefit of being able to take advantage of non-invasive MRI technology as well as fluorescent-based intra-operative technology. These nanoparticles are composed of an iron oxide core coated with PEG or a combination of chitosan and PEG co-polymers (NPCP) that act as a scaffold for the attachment of targeting molecules and fluorescent dyes. The polymer coating also functions to stabilize the particles, increase serum half-life, and prevent agglomeration. Preclinical studies in mice have demonstrated the effectiveness of this multi-modal imaging approach.

Mice injected with chlorotoxin functionalized nanoparticles had a 38% increase in contrast enhancement in MRI scans when compared to non-functionalized nanoparticles in a mouse model of medulloblastoma [63]. The same chlorotoxin functionalized probe could be used to visualize the tumor using fluorescent-based optical imaging technology, showing the multi-modal capabilities of these probes [63]. Chlorotoxin functionalized nanoparticles have also been shown to accumulate in glioma using both MR and optical imaging [64].

Studies have demonstrated that chitosan/PEG co-polymer coated iron oxide nanoparticles functionalized with chlorotoxin exhibited favorable biodistribution profiles [62]. These nanoparticles distribute mainly to the liver, spleen, and kidney while having the ability to cross the blood brain barrier making it ideal for imaging cancers of the brain [63]. Toxicity studies in mice at a dose of 9 mg/kg appreciated no discernable toxic effects or lesions specifically in the liver, spleen, and kidney, the main clearance organs for these nanoparticles. Studies indicate that the chlorotoxin-targeted particles circulate in the blood for an extended period of time, having a serum half-life of approximately eight hours in mice [62].

Semiconducting polymer dots (Pdots) are a novel family of fluorescent probes that are of great interest for fluorescent-based tumor targeting applications. Pdot probes, like quantum dots (Qdots), are small and extremely bright, making them appealing for use as targeted imaging probes. Unlike quantum dots, Pdots are made from biologically non-toxic materials making them an attractive candidate as a fluorescent imaging probe for human clinical applications. Recent advances in Pdot technology demonstrated targeted delivery of chlorotoxin functionalized Pdot probes [67]. These probes were on average 15 nm in size, 15 times brighter than Qdots, resistant to photo-bleaching, and stable in serum for over seven days [67].

Rare-earth metals represent a class of nanoparticle exhibiting unique properties for optical imaging and have recently been used for tumor targeting and imaging in live animals. These particles absorb low energy NIR (930 nm) wavelengths of light and “upconvert” to emit in the red visible spectrum (660 nm). This allows deep tissue penetration of NIR excitation wavelengths without any autofluorescent signal resulting in a high signal to noise ratio. These nanoprobe are soluble in aqueous solutions, are extremely photo stable, and fluoresce over long periods of time [69]. Recently, small nanoprobe (55 nm × 25 nm) of polyethylenimine coated hexagonal-phase NaYF₄:Yb, Er/Ce nanorods were functionalized with chlorotoxin and used to directly visualize C6 glioma xenografted tumors *in vivo* [68]. The chlorotoxin nanoprobe effectively targeted tumor tissue after 24 hours without any appreciable signs of toxicity [68].

Challenges and Perspectives

While it is clear that advances in imaging technology are needed to improve the quality of patient care, it remains to be seen how functionalized imaging agents will improve patient outcomes in a clinical setting. Preclinical studies in mouse models indicate that these types of imaging agents may be a promising step in filling the gap of current imaging technology. Optical imaging probes may provide surgeons with an additional tool in an integrated approach to image guided surgery where NIR fluorescently tagged peptides are used in combination with MRI, positron emission tomography, neuronavigation platforms, intra-operative MRI, and high resolution ultrasound [70]. This integrated approach is particularly important due to the limited depth of tissue penetration associated with fluorescent imaging and is therefore not suitable for deep tissue imaging.

In addition to advances in optical imaging agents, advances in surgical instrumentation are required in order to bring this technology into clinical practice. The type of intra-operative

device must be customized based on the surgical procedure. Intra-operative microscopes modified with a fluorescent light source and detection filters have been used to detect fluorescein [71] and protoporphyrin-IX [72] fluorescence during resection of malignant glioma. Additional modifications would enable surgeons to detect fluorescence in the NIR range. Fiber optic based endoscopic imaging systems have also been developed to detect 5-aminolevulinic acid (5-ALA)-induced protoporphyrin IX during glioma resections [73]. Endoscopic imaging systems could be tailored for use in open-field surgical procedures such as tumor margin delineation or positive lymph nodes detection or modified for use as early detection of intestinal lesions in a luminal space. Neurosurgical fluorescent-based imaging is extensively discussed in a review by Brian W. Progue et al [74].

It is clear that the extent of resection has a significant impact on the progression free survival of patients with brain cancer. In children with medulloblastoma, the five-year survival rate for near complete resection is 80% compared to a 50%–65% survival rate if significant residual post-operative tumor tissue remains [2]. In retrospective studies of children with malignant glioma, patients with gross total tumor resection survived over 60 months while patients with subtotal or limited resection survived 25 months and 10.5 months respectively [75, 76]. The extent of surgical resection is also the most predictive prognostic indicator in adult cases of malignant glioma [77, 78]. These studies found that 80% of recurrences developed from post-operative residual tumor [77]. Studies performed by the Radiation Therapy Oncology Group on the 5-ALA fluorescence-guided surgery on adult patients with glioma showed an influence on the extent of resection on survival in some groups of patients [79]. This study also showed that patients who underwent surgery using 5-ALA fluorescence guided technology had a more complete tumor resection [80] indicating that fluorescence guided surgery can improve the quality of patient care. The extent of tumor resection is not the only significant prognostic indicator and factors such as age, tumor location, and the degree of functional performance measured by Karnofsky Performance Status also contribute to survival statistics. Additionally, even surgical resections that show no residual tumor by post-operative MRI may not confer a cure for patients with malignant glioma due to the infiltrative nature of the disease.

CONCLUSION

Despite advances in surgical guidance tools, clear delineation between tumor and normal tissue continues to rely on visual differences that are often too subtle for the neurosurgeon to differentiate. This lack of visual cues often results in unacceptable morbidity and increased potential of recurrence and cancer progression. As this field of research continues to advance, the discovery and synthesis of safe and novel compounds with superior optical properties will allow the design of a new generation of *in vivo* targeted imaging agents for real-time surgical biophotonic imaging. For cancers in which the extent of surgical resection directly correlates with patient survival, the advancement of these targeted fluorescent probes to human clinical trials offers an opportunity to fundamentally improve cancer survivorship.

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References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60:277–300. [PubMed: 20610543]
2. Kieran MW, Walker D, Frappaz D, Prados M. Brain tumors: from childhood through adolescence into adulthood. *J Clin Oncol.* 2010; 28:4783–9. [PubMed: 20458039]
3. Veisheh M, Gabikian P, Bahrami SB, et al. Tumor paint: a chlorotoxin: Cy5. 5 bioconjugate for intraoperative visualization of cancer foci. *Cancer Res.* 2007; 67:6882–8. [PubMed: 17638899]
4. DeBin JA, Strichartz GR. Chloride channel inhibition by the venom of the scorpion *Leiurus quinquestriatus*. *Toxicon.* 1991; 29:1403–8. [PubMed: 1726031]
5. DeBin JA, Maggio JE, Strichartz GR. Purification and characterization of chlorotoxin, a chloride channel ligand from the venom of the scorpion. *Am J Physiol.* 1993; 264:C361–9. [PubMed: 8383429]
6. Drenth J. The structure of neurophysin. *J Biol Chem.* 1981; 256:2601–2. [PubMed: 7204368]
7. Lippens G, Najib J, Wodak SJ, Tartar A. NMR sequential assignments and solution structure of chlorotoxin, a small scorpion toxin that blocks chloride channels. *Biochemistry.* 1995; 34:13–21. [PubMed: 7819188]
8. Ullrich N, Gillespie GY, Sontheimer H. Human astrocytoma cells express a unique chloride current. *Neuroreport.* 1996; 7:1020–4. [PubMed: 8804043]
9. Ullrich N, Sontheimer H. Biophysical and pharmacological characterization of chloride currents in human astrocytoma cells. *Am J Physiol.* 1996; 270:C1511–21. [PubMed: 8967454]
10. Maertens C, Wei L, Tytgat J, Droogmans G, Nilius B. Chlorotoxin does not inhibit volume-regulated, calcium-activated and cyclic AMP-activated chloride channels. *Br J Pharmacol.* 2000; 129:791–801. [PubMed: 10683204]
11. Soroceanu L, Gillespie Y, Khazaeli MB, Sontheimer H. Use of chlorotoxin for targeting of primary brain tumors. *Cancer Res.* 1998; 58:4871–9. [PubMed: 9809993]
12. Lyons SA, O'Neal J, Sontheimer H. Chlorotoxin, a scorpion-derived peptide, specifically binds to gliomas and tumors of neuroectodermal origin. *Glia.* 2002; 39:162–73. [PubMed: 12112367]
13. Hockaday DC, Shen S, Fiveash J, et al. Imaging glioma extent with ¹³¹I-TM-601. *J Nucl Med.* 2005; 46:580–6. [PubMed: 15809479]
14. Mamelak AN, Rosenfeld S, Bucholz R, et al. Phase I single-dose study of intracavitary-administered iodine-131-TM-601 in adults with recurrent high-grade glioma. *J Clin Oncol.* 2006; 24:3644–50. [PubMed: 16877732]
15. Deshane J, Garner CC, Sontheimer H. Chlorotoxin inhibits glioma cell invasion via matrix metalloproteinase-2. *J Biol Chem.* 2003; 278:4135–44. [PubMed: 12454020]
16. Puyraimond A, Fridman R, Lemesle M, Arbeille B, Menashi S. MMP-2 colocalizes with caveolae on the surface of endothelial cells. *Exp Cell Res.* 2001; 262:28–36. [PubMed: 11120602]
17. McFerrin MB, Sontheimer H. A role for ion channels in glioma cell invasion. *Neuron Glia Biol.* 2006; 2:39–49. [PubMed: 16520829]
18. Basbaum CB, Werb Z. Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. *Curr Opin Cell Biol.* 1996; 8:731–8. [PubMed: 8939664]
19. Tournier JM, Polette M, Hinnrasky J, Beck J, Werb Z, Basbaum C. Expression of gelatinase A, a mediator of extracellular matrix remodeling, by tracheal gland serous cells in culture and in vivo. *J Biol Chem.* 1994; 269:25454–64. [PubMed: 7929245]
20. Bode W, Grams F, Reinemer P, et al. The metzincin-superfamily of zinc-peptidases. *Adv Exp Med Biol.* 1996; 389:1–11. [PubMed: 8860988]
21. Kahari VM, Saarialho-Kere U. Matrix metalloproteinases and their inhibitors in tumour growth and invasion. *Ann Med.* 1999; 31:34–45. [PubMed: 10219712]
22. Salo T, Liotta LA, Tryggvason K. Purification and characterization of a murine basement membrane collagen-degrading enzyme secreted by metastatic tumor cells. *J Biol Chem.* 1983; 258:3058–63. [PubMed: 6298220]
23. McLean TW. Medulloblastomas and central nervous system primitive neuroectodermal tumors. *Curr Treat Options Oncol.* 2003; 4:499–508. [PubMed: 14585230]

24. Kesavan K, Ratliff J, Johnson EW, et al. Annexin A2 is a molecular target for TM601, a peptide with tumor-targeting and anti-angiogenic effects. *J Biol Chem*. 2010; 285:4366–74. [PubMed: 20018898]
25. Frangioni JV. In vivo near-infrared fluorescence imaging. *Curr Opin Chem Biol*. 2003; 7:626–34. [PubMed: 14580568]
26. Weissleder R, Pittet MJ. Imaging in the era of molecular oncology. *Nature*. 2008; 452:580–9. [PubMed: 18385732]
27. Butte PV, Mamelak AN, Nuno M, Bannykh SI, Black KL, Marcu L. Fluorescence lifetime spectroscopy for guided therapy of brain tumors. *Neuroimage*. 2011; 54 (Suppl 1):S125–35. [PubMed: 21055475]
28. Kantelhardt SR, Leppert J, Krajewski J, et al. Imaging of brain and brain tumor specimens by time-resolved multiphoton excitation microscopy ex vivo. *Neuro Oncol*. 2007; 9:103–12. [PubMed: 17325340]
29. Leppert J, Krajewski J, Kantelhardt SR, et al. Multiphoton excitation of autofluorescence for microscopy of glioma tissue. *Neurosurgery*. 2006; 58:759–67. discussion 759–67. [PubMed: 16575340]
30. Yong WH, Butte PV, Pikul BK, et al. Distinction of brain tissue, low grade and high grade glioma with time-resolved fluorescence spectroscopy. *Front Biosci*. 2006; 11:1255–63. [PubMed: 16368511]
31. Kuiper T, van Den Broek FJ, Naber AH, et al. Endoscopic Trimodal Imaging Detects Colonic Neoplasia as Well as Standard Video Endoscopy. *Gastroenterology*. 2011
32. Haglund MM, Berger MS, Hochman DW. Enhanced optical imaging of human gliomas and tumor margins. *Neurosurgery*. 1996; 38:308–17. [PubMed: 8869058]
33. Kremer P, Wunder A, Sinn H, et al. Laser-induced fluorescence detection of malignant gliomas using fluorescein-labeled serum albumin: experimental and preliminary clinical results. *Neurol Res*. 2000; 22:481–9. [PubMed: 10935221]
34. Stummer W, Novotny A, Stepp H, Goetz C, Bise K, Reulen HJ. Fluorescence-guided resection of glioblastoma multiforme by using 5-aminolevulinic acid-induced porphyrins: a prospective study in 52 consecutive patients. *J Neurosurg*. 2000; 93:1003–13. [PubMed: 11117842]
35. Stummer W, Stocker S, Wagner S, et al. Intraoperative detection of malignant gliomas by 5-aminolevulinic acid-induced porphyrin fluorescence. *Neurosurgery*. 1998; 42:518–25. discussion 525–6. [PubMed: 9526986]
36. Sampath L, Kwon S, Hall MA, Price RE, Sevcik-Muraca EM. Detection of Cancer Metastases with a Dual-labeled Near-Infrared/Positron Emission Tomography Imaging Agent. *Transl Oncol*. 2010; 3:307–217. [PubMed: 20885893]
37. Tanaka E, Choi HS, Fujii H, Bawendi MG, Frangioni JV. Image-guided oncologic surgery using invisible light: completed pre-clinical development for sentinel lymph node mapping. *Ann Surg Oncol*. 2006; 13:1671–81. [PubMed: 17009138]
38. Marshall MV, Draney D, Sevcik-Muraca EM, Olive DM. Single-dose intravenous toxicity study of IRDye 800CW in Sprague-Dawley rats. *Mol Imaging Biol*. 2010; 12:583–94. [PubMed: 20376568]
39. Greenberg NM, DeMayo F, Finegold MJ, et al. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci U S A*. 1995; 92:3439–43. [PubMed: 7724580]
40. Hyndman ME, Mullins JK, Pavlovich CP. Pelvic node dissection in prostate cancer: extended, limited, or not at all? *Curr Opin Urol*. 2010; 20:211–7. [PubMed: 20224412]
41. Heidenreich A, Varga Z, Von Knobloch R. Extended pelvic lymphadenectomy in patients undergoing radical prostatectomy: high incidence of lymph node metastasis. *J Urol*. 2002; 167:1681–6. [PubMed: 11912387]
42. Seeff LC, Richards TB, Shapiro JA, et al. How many endoscopies are performed for colorectal cancer screening? Results from CDC's survey of endoscopic capacity. *Gastroenterology*. 2004; 127:1670–7. [PubMed: 15578503]
43. Wallace MB, Kiesslich R. Advances in endoscopic imaging of colorectal neoplasia. *Gastroenterology*. 2010; 138:2140–50. [PubMed: 20420951]

44. van Rijn JC, Reitsma JB, Stoker J, Bossuyt PM, van Deventer SJ, Dekker E. Polyp miss rate determined by tandem colonoscopy: a systematic review. *Am J Gastroenterol*. 2006; 101:343–50. [PubMed: 16454841]
45. Soetikno RM, Kaltenbach T, Rouse RV, et al. Prevalence of nonpolypoid (flat and depressed) colorectal neoplasms in asymptomatic and symptomatic adults. *JAMA*. 2008; 299:1027–35. [PubMed: 18319413]
46. Fodde R, Edelmann W, Yang K, et al. A targeted chain-termination mutation in the mouse *Apc* gene results in multiple intestinal tumors. *Proc Natl Acad Sci U S A*. 1994; 91:8969–73. [PubMed: 8090754]
47. Akcan M, Stroud MR, Hansen SJ, et al. Chemical Re-engineering of Chlorotoxin Improves Bioconjugation Properties for Tumor Imaging and Targeted Therapy. *J Med Chem*. 2011
48. Rjeibi I, Mabrouk K, Mosrati H, et al. Purification, synthesis and characterization of AaCtx, the first chlorotoxin-like peptide from *Androctonus australis* scorpion venom. *Peptides*. 2011; 32:656–63. [PubMed: 21262299]
49. Zeng XC, Li WX, Zhu SY, et al. Cloning and characterization of a cDNA sequence encoding the precursor of a chlorotoxin-like peptide from the Chinese scorpion *Buthus martensii* Karsch. *Toxicon*. 2000; 38:1009–14. [PubMed: 10708793]
50. Adadj E, Naudat V, Quiniou E, Wouters D, Sautiere P, Craescu CT. Solution structure of Lqh-8/6, a toxin-like peptide from a scorpion venom--structural heterogeneity induced by proline cis/trans isomerization. *Eur J Biochem*. 1997; 246:218–27. [PubMed: 9210487]
51. Ali SA, Stoeva S, Schutz J, et al. Purification and primary structure of low molecular mass peptides from scorpion (*Buthus indicus*) venom. *Comp Biochem Physiol A Mol Integr Physiol*. 1998; 121:323–32. [PubMed: 10048185]
52. Fazal A, Beg OU, Shafqat J, Zaidi ZH, Jornvall H. Characterization of two different peptides from the venom of the scorpion *Buthus indicus*. *FEBS Lett*. 1989; 257:260–2. [PubMed: 2583272]
53. Fu YJ, Yin LT, Wang W, Chai BF, Liang AH. Synthesis, expression and purification of a type of chlorotoxin-like peptide from the scorpion, *Buthus martensii* Karsch, and its acute toxicity analysis. *Biotechnol Lett*. 2005; 27:1597–603. [PubMed: 16245180]
54. Rosso JP, Rochat H. Characterization of ten proteins from the venom of the Moroccan scorpion *Androctonus mauretanicus mauretanicus*, six of which are toxic to the mouse. *Toxicon*. 1985; 23:113–25. [PubMed: 3992595]
55. Tytgat J, Debont T, Rostoll K, et al. Purification and partial characterization of a 'short' insectotoxin-like peptide from the venom of the scorpion *Parabuthus schlechteri*. *FEBS Lett*. 1998; 441:387–91. [PubMed: 9891977]
56. Wu JJ, Dai L, Lan ZD, Chi CW. The gene cloning and sequencing of Bm-12, a chlorotoxin-like peptide from the scorpion *Buthus martensi* Karsch. *Toxicon*. 2000; 38:661–8. [PubMed: 10673158]
57. Wudayagiri R, Inceoglu B, Herrmann R, Derbel M, Choudary PV, Hammock BD. Isolation and characterization of a novel lepidopteran-selective toxin from the venom of South Indian red scorpion, *Mesobuthus tamulus*. *BMC Biochem*. 2001; 2:16. [PubMed: 11782289]
58. Fan S, Sun Z, Jiang D, et al. BmKCT toxin inhibits glioma proliferation and tumor metastasis. *Cancer Lett*. 2010; 291:158–66. [PubMed: 19906483]
59. Kim BY, Rutka JT, Chan WC. Nanomedicine. *N Engl J Med*. 2010; 363:2434–43. [PubMed: 21158659]
60. Morana G, Salviato E, Guarise A. Contrast agents for hepatic MRI. *Cancer Imaging*. 2007; 7(Spec No A):S24–7. [PubMed: 17921081]
61. Essig M, Weber MA, von Tengg-Kobligk H, Knopp MV, Yuh WT, Giesel FL. Contrast-enhanced magnetic resonance imaging of central nervous system tumors: agents, mechanisms, and applications. *Top Magn Reson Imaging*. 2006; 17:89–106. [PubMed: 17198225]
62. Lee MJ, Veisoh O, Bhattarai N, et al. Rapid pharmacokinetic and biodistribution studies using chlorotoxin-conjugated iron oxide nanoparticles: a novel non-radioactive method. *PLoS One*. 2010; 5:e9536. [PubMed: 20209054]

63. Veiseh O, Sun C, Fang C, et al. Specific targeting of brain tumors with an optical/magnetic resonance imaging nanoprobe across the blood-brain barrier. *Cancer Res.* 2009; 69:6200–7. [PubMed: 19638572]
64. Sun C, Veiseh O, Gunn J, et al. In vivo MRI detection of gliomas by chlorotoxin-conjugated superparamagnetic nanoprobes. *Small.* 2008; 4:372–9. [PubMed: 18232053]
65. Veiseh O, Sun C, Gunn J, et al. Optical and MRI multifunctional nanoprobe for targeting gliomas. *Nano Lett.* 2005; 5:1003–8. [PubMed: 15943433]
66. Huang R, Ke W, Han L, Li J, Liu S, Jiang C. Targeted delivery of chlorotoxin-modified DNA-loaded nanoparticles to glioma via intravenous administration. *Biomaterials.* 2011; 32:2399–406. [PubMed: 21185076]
67. Wu C, Hansen SJ, Hou Q, et al. Design of highly emissive polymer dot bioconjugates for in vivo tumor targeting. *Angew Chem Int Ed Engl.* 2011; 50:3430–4. [PubMed: 21381164]
68. Yu XF, Sun Z, Li M, et al. Neurotoxin-conjugated upconversion nanoprobes for direct visualization of tumors under near-infrared irradiation. *Biomaterials.* 2010; 31:8724–31. [PubMed: 20728213]
69. Mader HS, Kele P, Saleh SM, Wolfbeis OS. Upconverting luminescent nanoparticles for use in bioconjugation and bioimaging. *Curr Opin Chem Biol.* 2010; 14:582–96. [PubMed: 20829098]
70. Nejat F, El Khashab M, Rutka JT. Initial management of childhood brain tumors: neurosurgical considerations. *J Child Neurol.* 2008; 23:1136–48. [PubMed: 18952580]
71. Kuroiwa T, Kajimoto Y, Ohta T. Development of a fluorescein operative microscope for use during malignant glioma surgery: a technical note and preliminary report. *Surg Neurol.* 1998; 50:41–8. discussion 48–9. [PubMed: 9657492]
72. Stummer W, Stepp H, Moller G, Ehrhardt A, Leonhard M, Reulen HJ. Technical principles for protoporphyrin-IX-fluorescence guided microsurgical resection of malignant glioma tissue. *Acta Neurochir (Wien).* 1998; 140:995–1000. [PubMed: 9856241]
73. Tamura Y, Kuroiwa T, Kajimoto Y, Miki Y, Miyatake S, Tsuji M. Endoscopic identification and biopsy sampling of an intraventricular malignant glioma using a 5-aminolevulinic acid-induced protoporphyrin IX fluorescence imaging system. Technical note. *J Neurosurg.* 2007; 106:507–10. [PubMed: 17367078]
74. Pogue BW, Gibbs-Strauss S, Valdes PA, Samkoe K, Roberts DW, Paulsen KD. Review of Neurosurgical Fluorescence Imaging Methodologies. *IEEE J Sel Top Quantum Electron.* 2010; 16:493–505. [PubMed: 20671936]
75. Campbell JW, Pollack IF, Martinez AJ, Shultz B. High-grade astrocytomas in children: radiologically complete resection is associated with an excellent long-term prognosis. *Neurosurgery.* 1996; 38:258–64. [PubMed: 8869052]
76. Pollack IF. The role of surgery in pediatric gliomas. *J Neurooncol.* 1999; 42:271–88. [PubMed: 10433110]
77. Albert FK, Forsting M, Sartor K, Adams HP, Kunze S. Early postoperative magnetic resonance imaging after resection of malignant glioma: objective evaluation of residual tumor and its influence on regrowth and prognosis. *Neurosurgery.* 1994; 34:45–60. discussion 60–1. [PubMed: 8121569]
78. Stummer W, van den Bent MJ, Westphal M. Cytoreductive surgery of glioblastoma as the key to successful adjuvant therapies: new arguments in an old discussion. *Acta Neurochir (Wien).* 2011
79. Pichlmeier U, Bink A, Schackert G, Stummer W. Resection and survival in glioblastoma multiforme: an RTOG recursive partitioning analysis of ALA study patients. *Neuro Oncol.* 2008; 10:1025–34. [PubMed: 18667747]
80. Stummer W, Pichlmeier U, Meinel T, Wiestler OD, Zanella F, Reulen HJ. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol.* 2006; 7:392–401. [PubMed: 16648043]

Figure 1. Model of the three-dimensional structure of chlorotoxin with corresponding amino acid sequence shown below. Disulfide bonds are shown in orange ball and stick format. Disulfide connectivities are shown by solid blue lines[7].

Figure 2.

Autofluorescence in the brain. Fluorescent signal in mouse brains were measured on the IVIS Spectrum imaging system (Caliper LifeSciences) using three excitation wavelengths (465 nm, 675 nm, 745 nm). Signal was detected using the indicated emission filters. A representative image of the brain from each excitation wavelength is shown and represents the expected background autofluorescence signal that would be produced when imaging using the wavelengths required for fluorescein (465 Ex/520 Em), Cy5.5 (675 Ex/720 Em), and IRDye800/ICG (745 Ex/820 Em). Each biophotonic brain image is on an identical pseudo-color scale in units of (p/sec/cm²/sr)/(μW/cm²).

Figure 3.
Near-infrared dyes: (A) structure of Cy5.5 (B) structure of IRDye 800CW (C) Fluorescent properties of selected near-infrared dyes.

Figure 4.

Ctx:Cy5.5 detects tumor cells in the SmoA1 mouse medulloblastoma model. Wild type, asymptomatic, and symptomatic mice were injected with Ctx:Cy5.5 and then euthanized three days later. The brains were frozen, sliced on a cryostat, and scanned on the Odyssey infrared imaging system (LiCor Biosciences). Slices were then stained with hemotoxylin and eosin (H&E). Wild type mice (left) injected with Ctx:Cy5.5 show limited labeling of conjugate in normal tissue. Mice without clinical symptoms of disease (middle) exhibit fluorescent signal in a small foci of cells which is shown to be tumor tissue (long arrow) in the cerebellum on H&E stained slides. Ctx:Cy5.5 labeled tumor cells (long arrow) in mice with clinical symptoms of disease (right) while no signal is detected in the normal cerebellar structure (short arrow). Signal in the fluorescent images precisely match tumor tissue visible in the H&E stained sections while signal in normal tissue was minimal.

Figure 5.

Specificity of Ctx:Cy5.5 for mouse prostate cancer: (A) Biophotonic image of prostate and other organs from TRAMP mouse. CAP, cancerous prostate. (B) Wild-type control shows specific signal in TRAMP prostate and lung. Representative image from three animals in each group. Images were obtained five days after injection of 0.1 mL of 10 μ mol/L probe. Top row, brain, heart, kidney, liver, lung, and spleen. Bottom row, NP, normal prostate. (C) Intra-operative images of control (left) and TRAMP (right) mouse abdomen following removal of intestine, prostate, and liver. Multiple lymph nodes (N) were positive by biophotonic imaging and histology. A 1.5-mm-diameter tissue nodule that was very bright on imaging revealed a small focus of prostate cancer cells in lymph (L) channels surrounded by fat. Lower amounts of signal were detected in fat (F) and testes (T). (D) H&E stain showing cancer focus in lymph channel and cancer packed lymph node. Figure 5 is a reproduction first published by our lab in 2005 [3].

Figure 6. Ctx:Cy5.5 imaging of the *Apc*^{I638N} mouse model of adenomatous polyposis (FAP) intestinal cancer: (A and C) Histological confirmation of adenocarcinoma and adenoma respectively. (B) Biophotonic image of adenocarcinoma (left) and adenoma (right) shows clear delineation from normal intestine. This figure is a reproduction published by our lab in 2005 [3].

Figure 7.

Model of the three-dimensional structure of cyclized chlorotoxin and sequences of the synthesized and chlorotoxin-like peptides: (A) the model of the three-dimensional structure of cyclized chlorotoxin showing the disulfide bonds in yellow ball and stick format, the seven amino acid linker in red and the lysine residues in blue. (B) amino acid sequence of native chlorotoxin (Ctx), alanine- and arginine-substituted chlorotoxin, *Buthus martensii* Karsch chlorotoxin (BmKCT), and *Androctonus australis* chlorotoxin (AaCtx) [5, 47–49]. Disulfide connectivities for Ctx [7] and AaCtx [48] are shown with solid green lines above and below the amino acid sequence respectively. Bonds that are identical between Ctx and AaCtx are shown in light green and bonds that differ are indicated by dark green lines. Note that disulfide connectivities for BmKCT are not available.

Figure 8. General configuration of functionalized nanoparticles. These compounds consist of a particle core, a polymer coating surface modification, fluorophore, and chlorotoxin for tumor targeting.

Table 1

Chlorotoxin-based imaging probes.

Probe	Platform	Imaging Modality	Tumor Types Tested	References
Ctx: Cy5.5	Direct conjugate	Optical	Medulloblastoma Prostate Cancer Intestinal Cancer Glioma Rhabdomyosarcoma	[3]
NPCP-Cy5.5-Ctx	Iron oxide PEGylated- Chitosan copolymer	MRI Optical	Medulloblastoma	[62, 63]
NP-PEG- Ctx and NP-PEG- Cy5.5-Ctx	Iron Oxide PEG coated	MRI Optical	Glioma	[64, 65]
PAMAM-PEG-Ctx	Polyamidoamine dendrimer PEG coated	DNA delivery	Glioma	[66]
Pdot:Ctx	Polymer-Blend nanoparticle (PFBT/PF-DBT5)	Optical	Medulloblastoma	[67]
Ctx:UCNP	Rare earth upconversion nanoparticles (NaYf4:Yb,Er/Ce)	Optical	Glioma	[68]