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Nerve Tissue Targeted Fluorophore Synthesis: From Scheme to Administration

Cassandra Mathieson

Abstract

Nerve tissue damage as a result of surgical injury is a common negative outcome during many surgical interventions. Depending on the site of the injury and the amount of cellular damage, nerve tissue injury may potentially have a life altering effect on the surgical patient.
Post-surgical nerve pain is often a chronic problem which persists long after injuries have healed, and can severely infringe upon quality of life for individuals.
Surgery that is fluorescence guided will aid in the visualization of nerve structure patterns, and as a result may significantly improve patient outcomes. Small molecule fluorophores may potentially cross the blood brain barrier and blood nerve barrier, allowing unparalleled future application in surgical interventions involving the central and peripheral nervous systems. In this research, work was done to synthesize a small set of fluorescent contrast agents which may have intraoperative application to highlight nerve tissue in real time, with great sensitivity and in high contrast against background tissues. Though work is ongoing, absorption and fluorescence spectra will be observed as a way to evaluate each synthesized agent. Systemic administration of the novel agents in rodents will help define nerve tissue uptake specificity. In order to evaluate nerve signal to background ratios, images of surgical sites will be collected and quantified. The knowledge garnered from this study will greatly help inform nerve tissue specific, clinical grade, contrast agent development.

Introduction

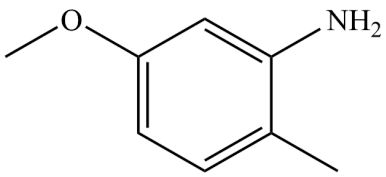
Nerve tissue damage as a result of surgical injury is a common negative outcome during many surgical interventions (1). Neuropathic pain is currently experienced by an estimated three percent of the developed world population (2). Due to its persistence well beyond healing time, post-surgical nerve tissue injury may potentially have a life altering effect on the patient. Depending on the site of the injury and the amount of cellular damage, neuropathic pain resulting from surgical injury may cause severe debilitation and interfere greatly with quality of life (2). Surgical training involves mapping typical anatomical planes in order to plan avoidance of major nerves during surgical intervention. Individual interior landscapes may vary greatly however, as a result of previous trauma, past surgery, or myriad other conditions. When anatomical structures are atypical, the risk of nerve injury during surgery is additionally heightened. Indeed some nerve groups are slight enough that they prove hard to see even when optically magnified (4).
In order to substantially reduce the amount of surgical injury to nerve tissue and associated chronic postoperative nerve pain, clinical supports for greater visualization during surgical intervention are imperative. Though the benefit of more highly visible nerve tissue is clear, previous approaches to improving visualization during surgery have been limited in their resolution, specificity, and real-time applications (4).Surgery that is fluorescence guided may highlight complex nerve structure patterns with high-contrast against background tissues, and with high sensitivity. This real-time intraoperative visual assistance may significantly improve patient outcomes, by helping surgical staff to recognize delicate nerve structures, and thus avoid inflicting surgical injury.

The purpose of this study was to synthesize a small set of fluorescent contrast agents, in order to evaluate them for eventual potential clinical application in fluorescence guided surgery. Optical properties including absorption and fluorescence were observed as a way to evaluate each agent. The near-infrared (NIR) window (650-900 nanometers) is the ideal wavelength range for these fluorophores, as it offers the highest contrast in imaging and also minimal light scattering and endogenous tissue fluorescence (4). Systemic administration of the novel agents in rodents was used to evaluate successful uptake in nerve tissue compared to surrounding tissues. In order to observe nerve signal to background ratios, images of the surgical sites were then collected and quantified.

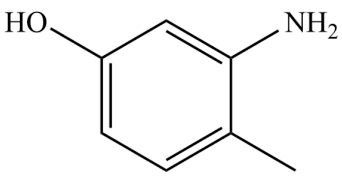
Methods

Synthesis

A chemical synthesis scheme was designed which allowed sequential progression from chemical compound precursors to two final fluorophores (LGW18-42 and LGW18-44) with near infrared emission (650-900 nm). Each precursor was synthesized via single step reactions that involved manipulation of either 5-methoxy-2-methylaniline or 3-amino-4-methylphenol, or a previously synthesized precursor originating from those aforementioned compounds.



5-methoxy-2-methylaniline



3-amino-4-methylphenol

Synthesis of each precursor in the scheme preceding synthesis of the final compounds included chemical manipulations of commercially available oxazine fluorophores. The nature of the small molecule fluorophore is such that it has naturally high specificity of uptake in the nerve tissue. One challenge to the use of such fluorophores is that they may offer low quantum yield, that is, that absorbed photons may not equate to acceptable light emission. This presents obvious problems when considering their use in image guided surgery. Additional problems with solubility and stability also occur. The chief charge of this research is to discover chemical variance in the molecules which will retain nerve specificity while correcting the aforementioned challenges.

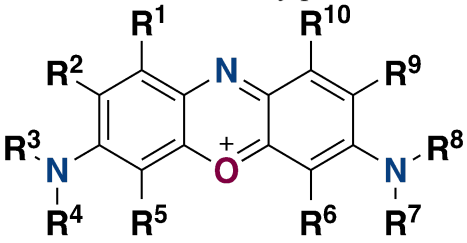


Fig.1 shows chemical structures for 5-methoxy-2-methylaniline, 3-amino-4-methylphenol, and a general structure of oxazine fluorophores. R^1, R^2 = H, or CH_3; R^3, R^4 = CH_3, or CH_2CH_3; R^5, R^6 = H, or CH_3; R^7, R^8 = CH_3, or CH_2CH_3; R^9, R^{10} = H, or CH_3;

Verification of Content

Following synthesis of each precursor, samples of the product are evaluated for purity in the lab via a multistep method, before progressing forward with the scheme. Thin layer chromatography (TLC) is used as a first step in confirming purity of each reaction product, by revealing the presence of product versus starting material. TLC plates are coated in a silica gel stationary phase. The process of using TLC involves comparing a deposited sample of a reaction's starting material, with a deposited sample of the synthesized product of the reaction. Bands will develop according to each compound's affinity with the stationary phase (5).

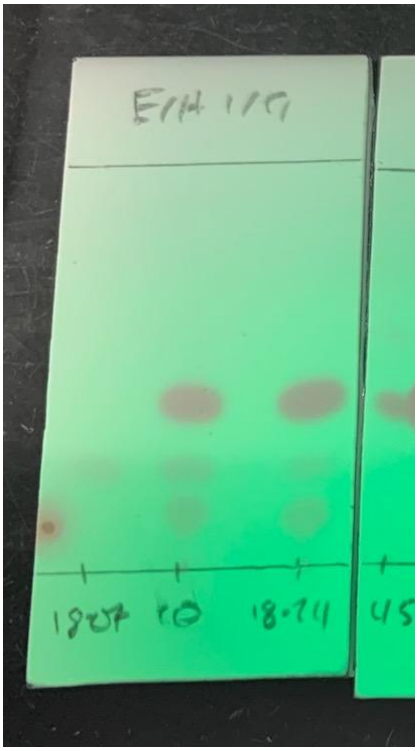


Fig 3. shows TLC after samples of starting material (18-07) and intended product (18-24) have been deposited and processed. There is a low concentration of 18-07 remaining in the 18-24 sample, revealed by the presence of a band in the 18-24 sample section which runs parallel with the 18-07 band. The highest concentration band is most likely that of the intended product, 18-24, but a third lower concentration band not corresponding to the starting material provokes the need for further testing.

Absence of the band corresponding with the starting material in the product area, in addition to a newly developed band in the product area of the plate, indicates a complete reaction.
TLC is a valuable tool for quick evaluation of a reaction's progress, as the process is relatively inexpensive, and can be completed in approximately five minutes in its entirety (5).

Fig 2. shows two precursor compounds from the LGW 18-42 and LGW 18-44 scheme.

The second step in verifying
product purity is to process product samples via liquid chromatography mass spectrometry (LC/MS). LC/MS uses mass to charge ratios (m/z) to identify and delineate between chemical compounds (6). It is a valuable tool for evaluating the success of chemical reactions, by using associated masses to identify remaining reagents in a solution versus intended synthesized product. Liquid chromatography with photodiode array detection (DAD) offers additional data, related to the classification of compounds and their absorbance, respectively. M/z when paired with DAD is especially valuable for the purposes of this experiment, as the intended product of each reaction will have not only a specific m/z ratio, but also substantial fluorescent properties.

Location of peaks in the readings for both m/z and DAD is an excellent way to narrow down the location of highest product concentration within the product sample as an analytical step, to then be able to anticipate its location in the final step of verification.
Once LC/MS has been completed, each product is isolated via automated flash chromatography. Flash chromatography uses affinity with a stationary phase to isolate and separate compounds into fractions, as they are pushed through the stationary phase column (7). Digital readings related to product UV absorbance peaks are generated while samples are processed via this method. This absorbance data paired with the anticipated location of peaks relating to the intended synthesized product from previous steps in this method, inform the general location of the intended product within the column tubes. Once this final step in the verification process is complete, the fractions which are likely to hold the purest target product may be collected. The sample is then dried to remove any added solvent, and product purification is complete.

Optical Property Collection

Following successful synthesis of 18-42 and 18-44, optical properties of fluorescence and absorption are evaluated by digital processing of the isolated product sample. Near infrared emission is ideal (650-900 nm) for the purposes of this research. Alternate emissions may be absorbed by hemoglobin, water, and dietary chlorophyll in the human body, reducing nerve tissue specificity (8) or present solubility and stability challenges. Fluorophores with emission within the NIR window have minimal light scattering and the lowest absorbance in muscle and adipose tissues, when compared to ultraviolet or visible white light, allowing them to penetrate tissues to greater depth (9). Additionally NIR fluorophores have been shown to highlight some of the most delicate nervous tissue in the peripheral nervous system of mammals, with high-contrast against background tissues (4). Nerves which would be invisible in white light, become brightly illuminated.

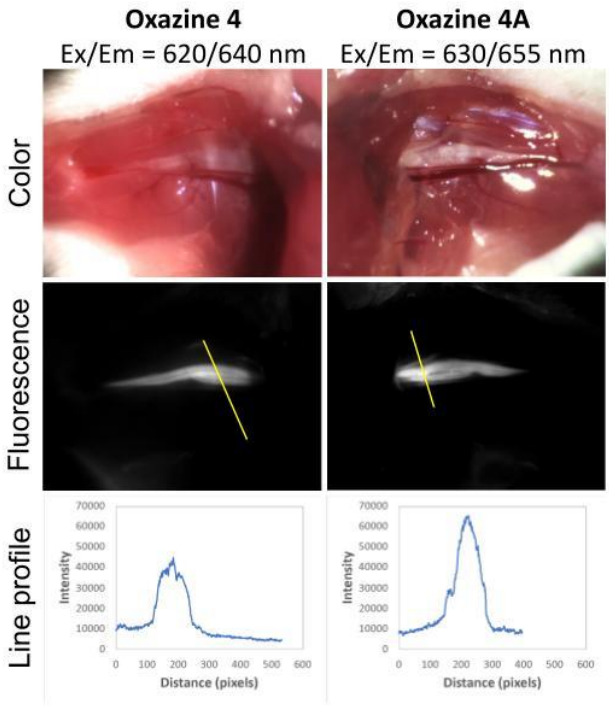


Figure 4 shows photographs in conventional white-light, fluorescence images, and corresponding fluorescence intensity cross-sectional profiles for visible emissive Oxazine 4 (left column) and NIR emissive Oxazine 4A (right column). This demonstrates NIR Oxazine 4A's superior *in vivo* nerve-specific contrast. (*)

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In Vivo Systemic Administration & Imaging

Prior to consideration for clinical testing in humans, LGW18-42 and LGW 18-44 will be tested for biocompatibility and evaluation of optical properties in rodent test subjects, via systemic administration through the major tail vein. Preceding injection, each compound is first dissolved in a solution composed of 10% dimethyl sulfoxide (DMSO), 5% Kolliphor, 65% fetal bovine serum (FBS) and 20% phosphate-buffered saline (PBS). This specific formulation has previously been shown to support solubility of lipophilic compounds (10,12). The brachial plexus and sciatic nerve of subjects are exposed via surgical removal of muscle and adipose tissue layers, four hours post injection. This time frame was selected as previous work has shown it to be the point of highest contrast between nervous tissue and background tissues, for fluorophores which are nerve specific (10).

A previously developed small animal image capturing device, a fluorescence macroscope, was used to generate color and fluorescence images after nerve exposure. Simplified, this technology captures color images in real time by collecting video and then modifying frames into still shot format. Fluorescence images are captured by fitting LED lights within the device with filters capable of supporting excitation and capturing emission. (10).

Looking Forward

The low molecular weight belonging to small molecule fluorophores, may allow them to bypass the blood brain barrier (BBB) for successful uptake in central nervous system (CNS) tissue. Additionally, uptake may be successful in the blood nerve barrier (BNB) dividing the nerve axons of the peripheral nervous system, from the bloodstream. This quality provides exciting prospects for future clinical applications in both the PNS and CNS,

which is lacking in larger contrast agents, such as nerve-specific peptides. LGW18-42 and LGW18-44 will both be evaluated for successful uptake in both the peripheral and central nervous system tissues. Successful nerve tissue specific uptake with high-contrast compared to background tissue in the CNS, could improve intraoperative vision and avoid neuronal damage in surgical interventions. This support may reduce or eliminate morbidities associated with those injuries.

Previously developed small molecule fluorophores may have molecular weights which allow them to bypass the BBB, but face challenges around specificity of uptake. Fluorescing in natural light causes widespread uptake in surrounding adipose tissues due to the lipophilicity of such molecules (10), and reduces contrast of nerve tissue as a result.

Fig. 5 shows fluorophores in solution in natural light (top) and with laser excitation (bottom) (*).

Chemical manipulation as described in this report, may alter fluorescence of small molecule fluorophores into the NIR window, in order to target uptake in nerve-tissue specifically with high contrast compared to background tissues.

At the time of this report, this project is ongoing. Synthesis has been successfully completed for LGW18-42 and LGW18-44. In the time to come, data as outlined previously will be collected for each compound. In the case that either shows traits which highlight them as potential candidates for future clinical application in image guided surgery, research will progress forward with systemic administration.

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(*) Images and data from OHSU Gibbs Lab.