

Brief Report

Bevonescein—A Peptide Dye Conjugate for Visualization of Peripheral Nerves in Patients During Surgery

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Abstract

Background/Objectives: The identification of peripheral nerves is critical for their preservation during surgery, as accidental transection or injury can lead to significant patient morbidity. Current methods for identifying nerves typically rely on qualitative white-light visualization of anatomy, texture, and color. To improve nerve identification during surgical procedures, we developed a novel nerve imaging agent, “bevonescein,” a derivative of the peptide–dye conjugate FAM-HNP401. **Methods:** Variants of FAM-HNP401 were designed to be synthesized completely on solid phase to enable the efficient generation of GMP (Good Manufacturing Practice)-qualified bevonescein. We determined the nerve binding affinity for each variant, CPC-17, CPC-18, CPC-19 (bevonescein), and CPC-20, using mean fluorescent intensity measurements after binding the agents to human sural nerve sections. **Results:** Bevonescein (CPC-19) demonstrated significantly superior nerve binding compared to other variants and controls. Bevonescein-labeled nerves exhibited a mean fluorescent intensity of 562 ± 34.7 , compared to 252 ± 41.7 for CPC-17, 344 ± 34.7 for CPC-18, and 270 ± 41.7 for CPC-20. The dye-alone control, 5-carboxyfluorescein, showed a fluorescent intensity of 168 ± 41.4 . **Conclusions:** Bevonescein represents a first-in-class molecule that can improve the visualization of peripheral nerves during surgery, potentially reducing nerve injury and associated morbidity. It has been successfully tested in a Phase 1/2 clinical trial demonstrating safety and efficacy at a 500 mg dose and is currently in Phase 3 clinical testing.

Keywords: bevonescein; peripheral nerve; fluorescence; peptide–dye conjugate; intraoperative imaging; fluorescence-guided surgery; human nerve targeting

1. Introduction

Peripheral nerve identification is vital for its preservation during surgery. Accidental transection or injury can lead to significant patient morbidity, including chronic post-operative pain, numbness, paralysis, urinary incontinence, and erectile dysfunction [1,2]. Nerve damage can occur during many types of surgeries, with the highest documented injury rates in those involving the intercostobrachial nerve (axillary dissections), recurrent laryngeal nerve (thyroid surgery), facial nerve (ear and facial surgeries), and cavernosal nerves (prostate surgery) [3–8]. Current methods of identifying nerve tissue during surgery rely primarily on qualitative criteria such as anatomy, texture, color, and relationship to surrounding structures to distinguish them from non-neural tissue. These subjective criteria become even less reliable in the presence of trauma, tumor invasion, or infection,

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further complicating accurate nerve identification. To enable better identification of nerves during surgery, we developed a novel nerve imaging agent, bevonescein which was recently studied in a Phase 1/2 clinical trial for safety and the ability to highlight nerves (ClinicalTrials.gov, NCT04420689). Bevonescein is a peptide-based agent that facilitates the targeted delivery of a fluorescent moiety (5-carboxyfluorescein) to nerves after intravenous (IV) administration. In animal models, IV administration of bevonescein prior to fluorescent imaging improved the identification of degenerated facial nerves [9,10]. In a Phase 1/2 human clinical trial, bevonescein showed no dose-limiting toxicity and achieved significantly higher signal-to-background ratio for nerve tissue compared to white light visualization alone [11]. In this trial, the optimal human dose for bevonescein was determined to be 500 mgs with imaging conducted 1–5 h after IV administration [11].

The clinical need for real-time nerve visualization during surgery has driven the development of multiple detection strategies. Significant technical and practical barriers have limited their translation for routine surgical use. Though widely adopted, neurophysiological methods, including electromyographic monitoring [12–14] and dermatomal somatosensory evoked potential [15,16], rely solely on functional assessment rather than direct visualization. They remain susceptible to interference from anesthesia and nerve blocks that are routinely used during surgeries [17]. Optical techniques such as coherence tomography [18], confocal laser microscopy [19], and collimated polarized light [20] imaging can provide exceptional resolution for structural imaging of superficial nerves, but are hard to adapt to most open surgeries. These are mostly relevant for optical biopsy applications versus nerve sparing during surgery. Neuronal tracers typically require transport periods of 24–72 h for retrograde tracers or 7–14 days for anterograde tracers, making them fundamentally incompatible with intraoperative use [21–25]. Ultrasound has been clinically established for guiding needle placement during peripheral nerve blocks and injections, but it typically uses known anatomy to guide injections and is not functioning to directly visualize nerve tissue. Myelin-targeting agents have shown promise in preclinical nerve imaging studies. These include distyrylbenzene derivatives [26] that bind myelin basic protein and anti-ganglioside antibodies [27] used diagnostically in autoimmune neuropathies. However, these agents face significant limitations, including the requirement for extensive formulation to achieve adequate water solubility and their tendency for non-specific labeling of other fatty tissues [27–34] which are often adjacent to nerves. Near-infrared (NIR) nerve-specific small molecule contrast agents (LGW16-03 and LGW01-08) have recently been shown to highlight nerves in vivo in animal models and ex vivo on human tissues. New formulation methods may enable their clinical translation [35–38]. Peptide-based imaging agents offer a promising alternative, combining target specificity and favorable pharmacokinetic properties (peak signal between 4 and 6 h with near complete washout at 24 h) that are compatible with real-time surgical workflows.

Bevonescein is a variant of a previously reported molecule, FAM-HNP401, a peptide-dye conjugate [39] that selectively highlights nerves both in vitro and in vivo. HNP401 was identified using an unbiased selection strategy in combination with phage displaying a highly diverse 16-amino acid peptide library. This selection discovered specific peptide sequences that had affinity for resected human nerve tissue compared to other tissues, such as muscle. The HNP401 peptide was then modified off-resin by conjugation of 5,6-carboxyfluorescein (FAM) at a C-terminal cysteine using 5,6-FAM-maleimide. Co-labeling experiments with neurofilament antibody SMI312 (Abcam, Waltham, MA, USA) showed that the fluorescent signal from FAM-HNP401 did not colocalize with axons. FAM-HNP401 instead colocalized with the perineurium, suggesting it bound connective tissue rather than axons [9,10,39]. Studies conducted with FAM-HNP401 demonstrated that it could enhance in vivo contrast for nerve versus muscle by 3.03 ± 0.57 . In animal models,

FAM-HNP401 could bind and highlight multiple human peripheral nerves, including the lower leg sural, the upper arm medial antebrachial, as well as autonomic nerves isolated from human prostate [39].

2. Materials and Methods

2.1. Human Tissue Samples

Human sural nerve tissue was obtained from the UCSD Department of Surgery under Institutional Review Board (IRB) protocol number 130837. Sample tissues were de-identified so the sex and age of donors were unknown. Frozen nerve tissue was embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound, snap-frozen in liquid nitrogen, cryosectioned to a thickness of 10 μm , and mounted on glass slides. Sections were placed at room temperature (RT) in a humidified chamber for 30 min prior to the application of test articles.

2.2. Peptide Preparation

Peptide variants (CPC-17, CPC-18, CPC-19 (bevonescein), and CPC-20) were designed for solid-phase synthesis and purchased from CPC Scientific (Rocklin, CA, USA). Peptide stock solutions were prepared at 50 mg/mL and diluted to a working concentration of 100 μM (based on peptide molecular weight) in 0.5 $\times$ Hanks' Balanced Salt Solution (HBSS) prior to topical application. A control solution of 5-carboxyfluorescein (100 μM) was prepared in 0.5 $\times$ HBSS to serve as a control for non-specific binding.

2.3. Ex Vivo Nerve Binding Assay

To evaluate peptide binding, a hydrophobic barrier was drawn around the tissue sections using a PAP pen. A 50 μL volume of the peptide or control solution (100 μM) was applied directly to the nerve tissue and incubated for 30 min at room temperature in a humidified chamber. Following incubation, sections were washed twice by submerging the slides for 5 min in 0.5 $\times$ HBSS, followed by a single wash in 1 $\times$ phosphate-buffered saline (PBS). Sectioning and staining were done on a single dissected nerve with replicate samples on 4 different days. Each separate day replicate sections ($n = 3$ or 4 per test compound) were treated with either CPC-17, CPC-18, CPC-19, CPC-20, or 5-carboxyfluorescein, followed by washing, imaging, and analysis.

2.4. Imaging and Analysis

Nerve sections were imaged using a Zeiss V16 stereoscope equipped with an enhanced green fluorescent protein (eGFP) filter set. To correct for background signal, an image of a blank slide was acquired under identical imaging parameters. For each experimental set involving probe comparisons, acquisition parameters were held constant to allow direct comparison of the resulting data. Quantitative binding analysis was performed using ImageJ software version 1.54r. All raw image files for each experimental cohort were simultaneously loaded into ImageJ as 16-bit TIFF images. Once established, these settings were uniformly applied to all images in the cohort using ImageJ. The brightest image was selected as the reference to prevent saturation during level propagation. Regions of interest (ROIs) were drawn around each nerve bundle to calculate the average fluorescent intensity (details on ROI selection are provided in Supplementary Figure S1). A similar ROI shape and size was used to calculate the mean background fluorescent intensity, which was subtracted from the treatment values.

2.5. Statistical Analysis

Fluorescence intensity data were analyzed using a two-way analysis of variance (ANOVA) with peptide treatment as the primary factor of interest and experiment as a blocking factor to account for batch-to-batch variability across independent experimental replicates. For each tissue section, mean fluorescence intensity was calculated by averaging measurements across five regions of interest drawn around individual nerve bundles (Supplementary Figure S1). Following a significant omnibus ANOVA, post hoc pairwise comparisons between treatment groups were performed using Tukey's test. Statistical significance was set at $p < 0.05$ for all analyses. The pairwise comparisons revealed that CPC-19 exhibited significantly higher fluorescence intensity than all other peptide conjugates and the dye-only control (all $p < 0.001$).

3. Results

As FAM-HNP401 progressed from discovery to clinical development, chemical modifications were required to produce a peptide–dye conjugate at the large scale required for clinical use. To enable efficient generation of GMP (Good Manufacturing Practice) material, variants of FAM-HNP401 were designed that could be synthesized completely on the solid phase. These four variants were made with and without a 2-amino acid deletion on the N-terminus of the HN401 peptide, each with fluorescein being attached to either the N or C terminus (Table 1). A single isomer of fluorescein (5-carboxyfluorescein) was used, which is customary for compounds synthesized under GMP standards.

Table 1. List of FAM-HNP401 peptide variants. HPLC and LC-MS analysis of peptides are provided in Supplementary Materials. HPLC and LC-MS traces for CPC-19 (bevonescien) (Supplementary Figure S2). HPLC traces for CPC-18 and CPC-20 (Supplementary Figure S3). LC-MS traces for CPC-18 and CPC-20 (Supplementary Figure S4). Abbreviations: 5-FAM = 5-carboxyfluorescein, ID = identity, HNP = human nerve peptide, N2 = N-terminal 2 amino acid deletion.

Compound ID	Peptide ID	Peptide Sequence	Molecular Weight
CPC-17	5-FAM-HNP401	NH ₂ -SGQVPWEEPPYYVVKSSGG-CONH ₂	2480.1
CPC-18	HNP401-K-(5-FAM)-CONH ₂	Acetyl-SGQVPWEEPPYYVVKSSGGK-CONH ₂	2624.8 Purity = 96.2%
CPC-19 (bevonescien)	5-FAM-(N2)-HNP401-CONH ₂	NH ₂ -QVPWEEPPYYVVKSSGG-CONH ₂	2310.5 Purity = 97.2%
CPC-20	(N2)-HNP401-K-(5-FAM)-CONH ₂	Acetyl-QVPWEEPPYYVVKSSGGK-CONH ₂	2454.6 Purity = 97.0%

To confirm affinity for human nerves, each variant was tested for binding to sections of human tissue as previously described and detailed in methods [39]. A single assay (in vitro binding to human sural nerve) was used to evaluate the nerve binding activity of these peptide variants, as they all had the identical binding sequence of HNP-401 with only modifications in the peptide length and the positioning and method of the 5-carboxyfluorescein attachment. These peptide variants were topically applied to mounted tissue sections of human sural nerves. Sections were washed and imaged for fluorescence intensity (Ex 480 nm, Em 530 nm using a Chroma eGFP filter set) (Figure 1A). CPC-19 (bevonescien) exhibited the highest nerve binding as measured by average fluorescence intensity (Figure 1B). Estimated marginal mean fluorescent intensity values were calculated as: CPC-17 = 252 ± 41.7 , CPC-18 = 344 ± 34.7 , CPC-19 (bevonescien) = 562 ± 34.7 , CPC-20 = 270 ± 41.7 , 5-carboxyfluorescein = 168 ± 41.4 . Following a significant omnibus ANOVA, post hoc pairwise comparisons were conducted using Tukey's test. Statistical significance was defined as $p < 0.05$ for all analyses. These comparisons demonstrated that CPC-19 produced significantly higher fluorescence intensity than all other peptide conjugates and the dye-only control (all $p < 0.001$). CPC-19 (bevonescien) was also the most cost-efficient

peptide conjugate to synthesize, given it was 2 amino acids shorter than CPC-17, and the N-terminal conjugation of 5-carboxyfluorescein could be done on resin using 5-carboxyfluorescein (Thermo-Fisher Scientific, CAS: 76823-03-5) without the use of fmoc-Lys(5-FAM) from Santa Cruz (Biotech, CAS 1242933-88-5). It was somewhat unexpected that CPC-19 (bevonescein) had such a significant increase in nerve binding, given the structural similarities to the other peptides. Because the molecular binding target for CPC-19 (bevonescein) is unknown, one can only speculate how the 2-amino-acid N-terminal deletion, combined with the N-terminal conjugation of fluorescein, significantly increased nerve binding.

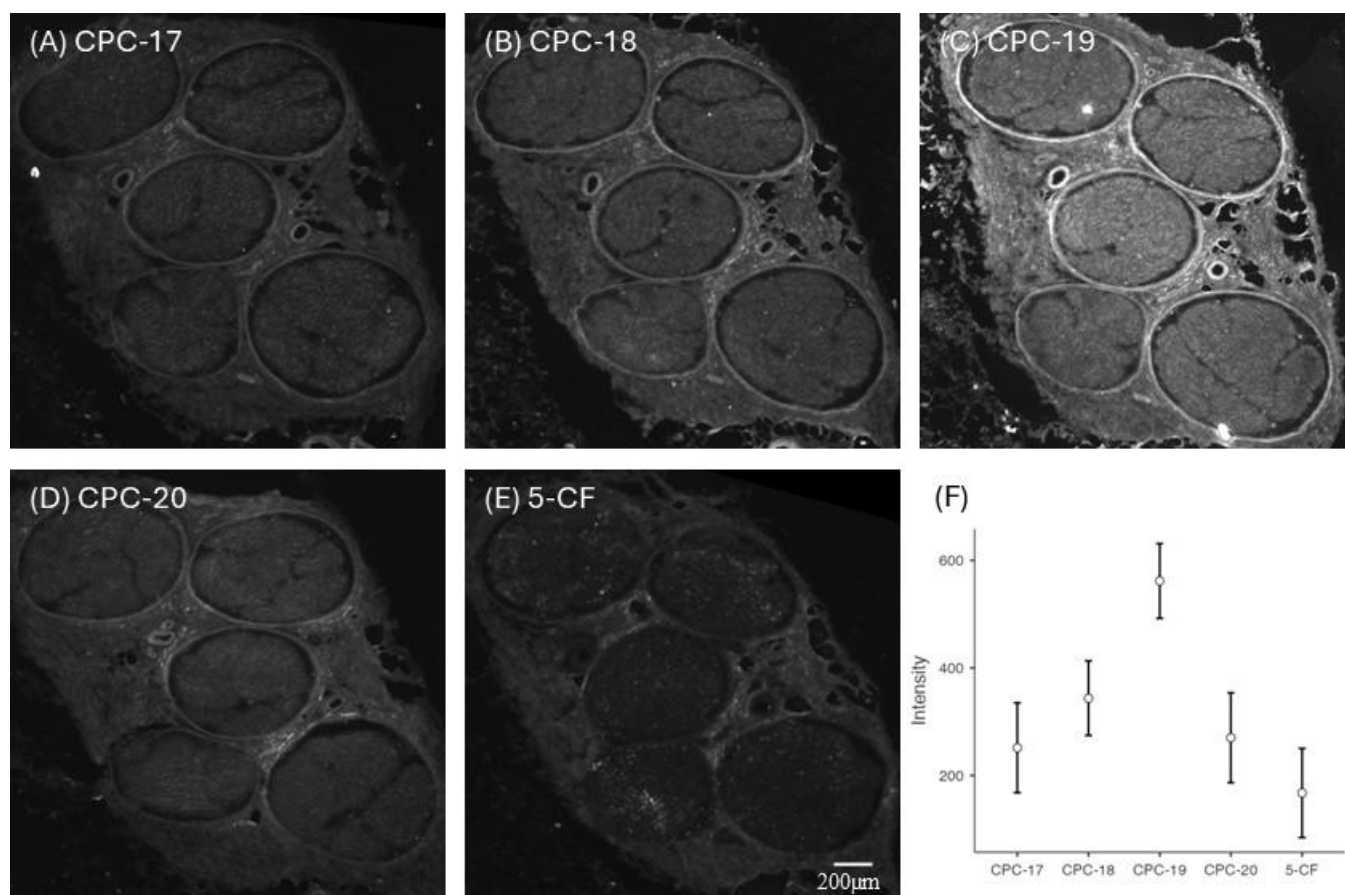


Figure 1. Representative fluorescence images of human peripheral nerves topically treated with different variants of the HNP401 parent peptide (A). CPC-17, (B). CPC-18, (C). CPC-19 (bevonescein), (D). CPC-20, and (E). 5-carboxyfluorescein. (F). Quantification of average fluorescence intensity for each test compound. Four sections per compound were imaged on 4 separate days. The pairwise comparisons revealed that CPC-19 exhibited significantly higher fluorescence intensity than all other peptide conjugates and the dye-only control (all $p < 0.001$). Details of statistical analysis are provided in Supplementary Table S1.

4. Discussion

In conclusion, bevonescein represents a novel peptide–dye conjugate that has advanced to preclinical and clinical testing for in vivo labeling of peripheral nerves in patients during surgery after intravenous injection. Anti-ganglioside antibody conjugates and free dyes have been previously reported for the detection of peripheral nerves in animal models [26–28,38]. However, free dyes lack a selective binding mechanism for nerve targeting and instead tend to accumulate in myelin due to its hydrophobic makeup. Accumulation of free dyes in myelin may also potentially disrupt nerve function. In addition,

because myelin is present at a low level or absent in autonomic nerves, the use of free dyes is further limited for visualizing these fine yet critical nerve structures. In vivo testing has also shown that bevonescein can be used to identify degenerate nerves, with parallel testing demonstrating that degenerative nerves were not labeled with oxazine-4 [9,10]. Anti-ganglioside antibodies offer more specific targeting but have long blood half-lives, requiring administration days before surgery and carrying a higher risk of eliciting immune responses. Although antibodies can be delivered intravenously or topically and provide advantages such as high affinity and well-defined targets, their prolonged circulation times necessitate extended periods for both accumulation and washout to achieve optimal nerve contrast. In contrast, systemic injection of fluorescently labeled peptides like bevonescein overcomes many of these limitations by enabling rapid (4–6 h), uniform labeling of all nerves throughout the body with a single peptide–dye conjugate injection with near complete washout at 24 h. Bevonescein has recently been successfully tested in a Phase 1/2 clinical trial that has demonstrated safety and efficacy at a dose of 500 mg [11] (ClinicalTrials.gov: NCT04420689). Clinical testing of bevonescein showed no dose-limiting toxicity and achieved a significantly higher signal-to-background ratio for nerve tissue compared to white light visualization alone. The blood half-life of bevonescein was 29–72 min, and the optimal dose was 500 mg with imaging done 1–5 h after administration of the drug [11]. Bevonescein is currently in Phase 3 clinical testing for intraoperative use in head and neck surgeries (ClinicalTrials.gov: NCT05377554). Clinical testing is also currently ongoing to determine the feasibility of bevonescein-assisted nerve visualization using a REVEAL 475 fluorescence imaging device [40] (Design for Vision, Bohemia, NY, ClinicalTrials.gov: NCT06227585) and to test the feasibility of bevonescein for intraoperative nerve and ureter visualization in patients undergoing minimally invasive abdominal pelvic surgery (ClinicalTrials.gov: NCT06662097). If approved, bevonescein would represent a first-in-class molecule that can improve visualization of peripheral nerves during surgery, potentially reducing nerve injury and associated morbidity.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/futurepharmacol6010013/s1>, Figure S1: Schematic of ROI selection. Figure S2: HPLC and Mass determination for tested CPC-19 (bevonscein). Figure S3: Purity determination for other tested compounds. Figure S4: Mass determination for other tested compounds. Table S1: Comparison/Statistical Analysis of nerve binding for tested compounds. References [41–44] are cited in the supplementary materials.

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Institutional Review Board Statement: All procedures involving human participants were conducted in accordance with the ethical principles outlined in the Declaration of Helsinki (1975, revised in 2013). In compliance with point 23 of the Declaration, all human tissue samples were obtained through a study protocol that was reviewed and approved by the Institutional Review Board (IRB), University of California, San Diego (UCSD) Department of Surgery. The approved IRB protocol #130837 was approved on 17 January 2017. This approval ensured compliance with applicable national and international ethical standards for research involving human subjects.

Informed Consent Statement: Informed consent was obtained for all human tissues used in this study.

Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

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Conflicts of Interest: Michael A. Whitney is a founder of Alume Biosciences, Inc., which currently holds a license for this technology from The Regents of the University of California, San Diego. Alume Biosciences Inc. had no role in the design of this study; in the collection, analysis, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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