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Fluorescence Imaging of Peripheral Nerves by a Na_v1.7-Targeted Inhibitor Cystine Knot Peptide

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Abstract

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Supporting Information

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IgG staining in human vagus nerves; H&E and Na_v1.7 expression in mouse sciatic nerves; tryptic digestion experiments for Hsp1a and Hsp1a-FL; epifluorescence images of sciatic nerves injected with Hsp1a-FL, block, or PBS, and the fluorescence quantification; fresh tissue confocal fluorescence microscopy (PDF)

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Twenty million Americans suffer from peripheral nerve injury caused by trauma and medical disorders, resulting in a broad spectrum of potentially debilitating side effects. In one out of four cases, patients identify surgery as the root cause of their nerve injury. Particularly during tumor resections or after traumatic injuries, tissue distortion and poor visibility can challenge a surgeon's ability to precisely locate and preserve peripheral nerves. Intuitively, surgical outcomes would improve tremendously if nerves could be highlighted using an exogenous contrast agent. In clinical practice, however, the current standard of care-visual examination and palpation-remains unchanged. To address this unmet clinical need, we explored the expression of voltage-gated sodium channel $\text{Na}_v1.7$ as an intraoperative marker for the peripheral nervous system. We show that expression of $\text{Na}_v1.7$ is high in peripheral nerves harvested from both human and mouse tissue. We further show that modification of a $\text{Na}_v1.7$ -selective peptide, Hsp1a, can serve as a targeted vector for delivering a fluorescent sensor to the peripheral nervous system. Ex vivo, we observe a high signal-to-noise ratio for fluorescently labeled Hsp1a in both histologically prepared and fresh tissue. Using a surgical fluorescent microscope, we show in a simulated clinical scenario that the identification of mouse sciatic nerves is possible, suggesting that fluorescently labeled Hsp1a tracers could be used to discriminate nerves from their surrounding tissues in a routine clinical setting.

Graphical Abstract

INTRODUCTION

Injuries to the peripheral nervous system represent a significant concern in surgical practice, and can occur during virtually any type of intervention.¹⁻⁵ While the majority of peripheral nerve injuries occur in the upper limbs and are of traumatic origin, around 25% of patients suffering from neuropathic pain identify surgical morbidity as the originating cause.⁶ In particular, oncologic surgery carries a risk of peripheral nerve damage because of the distorted physiology around a malignant lesion and the need to achieve complete resection of tumors. In head and neck cancers, this often manifests in facial nerve paralysis, which can result in significant disfigurement, impacting a patient's quality of life on both an emotional and physiological level.⁷ During thyroidectomies, the laryngeal nerve is frequently injured, resulting in changes or loss of voice or respiratory issues.⁸⁻¹⁰ Common side effects of prostatectomies are injuries to the prostatic plexus which can result in partial or complete incontinence or erectile dysfunction.^{11,12} Many other nerves are at risk of damage during surgery, including the brachial plexus during breast cancer intervention.¹³

Clearly, these unintended side effects could be reduced if an operating surgeon were more aware of the exact location of these vital nerves. While preoperative technologies to improve delineation and planning for larger nerve bundles do exist,^{14–16} a surgeon's intraoperative arsenal to avoid iatrogenic nerve injury during an operation consists mainly of loupes, surgical light microscopes, and electromyography electrodes which, though useful during some procedures,¹⁷ require nerves to be close to the tissue surface for stimulation, and cannot be used for all types of interventions.¹⁸ Intuitively, the development of a fluorescent nerve agent would ameliorate this situation, but despite the clear medical need and its importance, peripheral nerves have proven to be a difficult target. Some researchers, including the groups of Gibbs,¹⁹ Frangioni,²⁰ and Nguyen,²¹ among others, have developed tracers for peripheral nerve imaging, and two recent reviews provide an overview of the field.^{22,23} However, more development has to occur to advance nerve imaging agents to a point where routine clinical use is feasible.

It is with this need in mind that we turned our attention to the voltage-gated sodium channel (Na_v) subtype 1.7, a sodium channel expressed on peripheral neurons that has received a tremendous amount of attention as a potential target for analgesics.²⁴ We recently isolated the peptide Hsp1a from the venom of the Peruvian tarantula *Homeomma* spec, which features a classic inhibitor cystine knot (ICK) motif. Hsp1a showed that it is a highly potent and subtype-selective inhibitor of Na_v1.7. We hypothesized that a fluorescently labeled version of Hsp1a could be used as a targeted vector for delivery of an optical sensor to peripheral nerves in vivo. We show here that labeling of Hsp1a with BODIPY-FL *N*-hydroxy succinimide (NHS) ester yields a fluorescent version of the peptide, Hsp1a-FL, while retaining nanomolar potency for inhibition for Na_v1.7.

We show that both human and mouse peripheral nerves express Na_v1.7, and that Hsp1a-FL highlights mouse sciatic nerves both ex vivo as well as in vivo, generating contrast using preclinical and clinical imaging instruments. In vivo imaging was possible without causing any visible unintended pharmacodynamic effects. We anticipate that this first-generation Hsp1a tracer will inform further, clinically translatable imaging agent designs, which will have the potential to directly impact the surgical standard of care by lending contrast to nerves, thereby reducing iatrogenic injury and surgical morbidity.

RESULTS

Na_v1.7 Biomarker Validation in Human Vagus Nerves.

Na_v1.7 is a highly conserved sodium channel that is expressed at high levels in peripheral neurons.^{25–27} To corroborate this data, we examined Na_v1.7 expression in human vagus nerves from the cervical region (Figure 1a). All nerves were donated by the Fusion Solution Bioskills Laboratory autopsy program. The biospecimens were about 5 cm long ($n = 10$ nerves from $n = 5$ individuals, Figure 1b). The nerves were frozen using optimal cutting temperature (OCT) compound directly after surgical resection, and sectioned at 10 μ m thickness for staining. Using an anti-Na_v1.7 antibody, we showed that this sodium channel is expressed throughout the axons. Isotype control staining was negative, confirming the antibody's specificity. Hematoxylin & Eosin (H&E) staining enabled visualization of the nerve structure, showing the nerve axons and Schwann cells (Figure 1c).

Na_v1.7 Expression in Mouse Sciatic Nerves.

To evaluate the potential of Na_v1.7 as a biomarker target for imaging the peripheral nervous system, we examined the Na_v1.7 content in the sciatic nerve of female athymic nude mice (Figure 1d). Similar to the human vagus nerves, the sciatic nerves were surgically removed and embedded in OCT, sectioned at 10 μ m thickness and immunohistochemically stained with anti-Na_v1.7 antibody. Analogous to the data obtained for human peripheral nerves, staining highlighted the axonal bundles (Figure S1). H&E staining of adjacent slides highlighted the Schwann cells within the nerve. Here, too, no staining was observed when using isotype control antibodies, confirming specificity (Figure S2 a,b)

Design of the Fluorescence Hsp1a Conjugate Hsp1a-FL.

Figure 1e shows a stepwise, schematic overview for developing Hsp1a-FL. The basis of this tracer is Hsp1a, a peptide isolated from spider venom (Step 1). The structure of Hsp1a was recently determined, a chemical synthesis protocol developed, and its potent and specific inhibition of human Na_v1.7 identified (Step 2). We modified this naturally occurring venom peptide with a fluorophore, generating the fluorescent, targeted tracer Hsp1a-FL (Step 3). The targeted tracer was injected into mice (Step 4), and found to be retained in their sciatic nerves, which become fluorescent (Step 5).

Chemical Synthesis, Target Binding, and Affinity.

We chose to modify Hsp1a via nucleophilic substitution, analogous to our previous work with similarly sized bioactive peptides.^{28,29} Hsp1a was modified with BODIPY-FL due to the small size and relatively high stability of this fluorophore (Figure 2a,b). The chemical transformation was performed under basic conditions in a mixture of water and acetonitrile, yielding Hsp1a-FL in 55% yield and 83% purity. The retention time (t_r) shifted from 21 min for Hsp1a to 24 min for Hsp1a-FL (Figure 2c and Figure S3a). The major impurity was the partially reduced peptide, 9% (t_r 24.2 min), which was also present in the starting material (t_r 21 min, 90%; and t_r 21.2 min, 10% for Hsp1a and reduced Hsp1a, respectively). LC/MS spectra for both Hsp1a and Hsp1a-FL showed clean peak families confirming the peptides' calculated masses, 3389 and 3663 Da for Hsp1a and Hsp1a-FL, respectively (Figure 2d and Figure S3b–d). A tryptic digest was performed to determine the location of the BODIPY-FL conjugation. For the unmodified Hsp1a, we found ions that correspond to the fragment F5-R22 (2114.90 Da, Figure S3d). This fragment was not seen for digested Hsp1a-FL; instead, the conjugated peptide gave rise to the novel fragment Y1-R22 plus the mass of the BODIPY scaffold without BF₂ (2864.24 Da, Figure S3c). Observation of this peak suggests that conjugation occurred at K4. If the fluorophore were not conjugated at K4, the fragment would have been digested by trypsin into two smaller fragments. In principal, Hsp1a could have been modified at three principal nucleophilic positions (the N-terminal amine, K4 and K26) and we do indeed observe a small amount of fluorescent side products, including Hsp1a with two conjugated dyes, which were not further characterized. We presume that the predominant modification of Hsp1a at K4 is likely electronically or sterically favored.

We examined the inhibition of human Na_v1.7 by Hsp1a and Hsp1a-FL using automated whole-cell patch-clamp electrophysiology. Hsp1a is an extremely potent inhibitor of Na_v1.7 (IC₅₀ = 13 nM), and its potency is only marginally reduced by addition of the BODIPY-FL

label in Hsp1a-FL ($IC_{50} = 62$ nM) (Figure 2e,f). We conclude that fluorescent modification of Hsp1a marginally reduces its potency, but it remains a potent nanomolar inhibitor of $Na_v1.7$ that should be suitable for targeting peripheral neurons.

Histological Correlation and Validation.

To correlate the mesoscopic and cellular distribution of Hsp1a-FL in peripheral nerves, we performed histological analysis in mice. Mice were injected with Hsp1a-FL (7 nmol, 68 μ M in 100 μ L of PBS), sacrificed after 30 min, and then their sciatic nerves were removed and sectioned for imaging. Fluorescence signal was obtained in the green channel (488 nm excitation), showing circular patterns when nerves were sliced transversely, and linear patterns when nerves were sliced longitudinally (left column, Figure 3). Both patterns resembled the tubular structure of nerve bundles and axons. Longitudinal sections were performed close to the surface, attempting to visualize the epineurium and perineurium, as well as more centrally, showing endoneurium and nerve bundles. We also show the adjacent H&E and anti- $Na_v1.7$ staining, resembling the histological structures obtained with Hsp1a-FL (center and right column for H&E and anti- $Na_v1.7$, respectively). We tested the specificity of Hsp1a-FL by coinjecting a 3-fold excess of the original, unmodified peptide (7 nmol, 68 μ M in 100 μ L of PBS for Hsp1a-FL and 21 nmol, 210 μ M in 100 μ L for Hsp1a). Coinjection of a molar excess of unlabeled Hsp1a prevented uptake of Hsp1a-FL in sciatic nerves, suggesting that the probe was specific and its target saturable (Figure S4).

Pharmacokinetics.

Under mesoscopic imaging conditions, we observed rapid and selective accumulation of Hsp1a-FL (Figure 4a and Figure S5). Mice were injected intravenously with Hsp1a-FL alone (7 nmol, 68 μ M in 100 μ L of PBS) or in combination with an excess of unmodified peptide (21 nmol, 204 μ M in 100 μ L of PBS) (block), and then they were sacrificed 30 min after injection. The right and left sciatic nerves (RSN and LSN) were exposed (Figure 4a) and epifluorescence imaging performed using an IVIS Spectrum in vivo imaging system (excitation 465/30 nm; emission 520–580 nm). In mice receiving just the imaging agent, the sciatic nerves were clearly visible, whereas uptake was significantly reduced in mice that received the imaging agent in combination with excess unmodified peptide, (radiant efficiency: $(6.3 \pm 3.2) \times 10^7$ and $(0.03 \pm 0.01) \times 10^7$ for Hsp1a-FL and coinjection (blocking), respectively; Student's unpaired *t*-test, $P < 0.001$, Figure 4a).

Ex vivo, the differences were equally pronounced (Figure 4b,c). In mice receiving the imaging agent only, the sciatic nerves are clearly visible, with a mean radiant efficiency of $(26 \pm 0.13) \times 10^7$ (Figure 4a), whereas mice receiving the imaging agent in combination with the unmodified peptide (21 nmol, 204 μ M in 100 μ L of PBS) had a statistically significant 200-fold reduction in radiant efficiency to $(0.13 \pm 0.08) \times 10^7$ (Student's unpaired *t*-test, $P < 0.05$). While it should be noted that quantitative assessment with fluorophores across organ systems is not feasible, particularly in the visible range, we have observed a trend toward higher fluorescence signals from the kidneys (radiant efficiency: $(0.1 \pm 0.01) \times 10^7$ and $(0.71 \pm 0.06) \times 10^7$ for kidneys with and without injection, respectively; Student's unpaired *t* test, $P = 0.14$, Figure S6). We have not observed significantly higher fluorescent signals in other organs, including muscle and liver when

comparing animals injected with Hsp1a-FL and PBS (Figure S6). When examining fresh tissue under a confocal microscope, we observed nerve patterns similar to what was seen histologically (Figure S7).

Surgical Microscope Imaging.

To confirm the feasibility of imaging in a more clinically relevant setting, we chose to test Hsp1a-FL with a Lumar surgical fluorescence stereoscope (SteREO Lumar v12, Zeiss, Jena, Germany). Analogous to our previous work, mice were injected with Hsp1a-FL (7 nmol, 68 μ M in 100 μ L of PBS) or 100 μ L of PBS. After 30 min, mice were sacrificed and their sciatic nerves exposed for imaging (Figure 1d and Figure 5a). Figure 5b shows images of sciatic nerves obtained under white light imaging conditions (top row) and Hsp1a-FL fluorescence (bottom row) for both mice injected with Hsp1a-FL and PBS. Unlike the IVIS Spectrum, the Lumar Fluorescence imaging system does not support autofluorescence suppression. Irrespectively, we detected strong specific fluorescence in nerves of animals injected with Hsp1a-FL (fluorescence intensity: $(2.6 \pm 1.7) \times 10^4$), which was significantly lower for PBS-injected mice ($(0.8 \pm 0.2) \times 10^4$, Mann-Whitney's test, $P < 0.05$, Figure 5c,d). At higher magnification, the Lumar fluorescence stereoscope revealed structures within the nerves that resemble the same tubular and axonal features that were observed on nerve sections during confocal imaging, providing additional support for Hsp1a-FL being highly specific for Na_v1.7. The combination of the Lumar stereoscope and Hsp1a-FL could thus potentially be used clinically for intraoperative, contemporaneous mapping of peripheral nerves.

DISCUSSION

This study investigated the use of the recently discovered peptide, Hsp1a, as a Na_v1.7-targeting vector for imaging the peripheral nervous system. Specifically, we corroborated the expression of Na_v1.7 in human vagus nerves and confirmed that expression in mouse sciatic nerves is comparable, allowing mice to serve as models in the development of the imaging agent (Figure 1, Figure S1). We labeled the spider-venom peptide Hsp1a with BODIPY-FL ($\text{ex}_{\text{max}} = 503$ nm, $\text{em}_{\text{max}} = 509$ nm) to yield a fluorescent peptide, Hsp1a-FL, with high affinity for Na_v1.7. When injected into mice, Hsp1a-FL yielded uptake patterns resembling Na_v1.7 expression, irrespective of whether the tissue was imaged as a histological section or a freshly resected nerve. Further corroborating that Hsp1a-FL is a highly specific Na_v1.7-targeted agent, uptake in sciatic nerves was elevated 200-fold compared to sciatic nerves from mice coinjected with unlabeled Hsp1a when using an epifluorescence imaging system. Similarly, we found that mouse sciatic nerves were clearly visible when using a clinically relevant Lumar fluorescence stereomicroscope. This is notable because the Lumar stereomicroscope does not provide image deconvolution (and therefore autofluorescence correction), and yet Hsp1a-FL accumulation was still clearly resolvable. Ultimately, this data suggests that it is possible to delineate peripheral nerves noninvasively and with high contrast when using Hsp1a-based imaging.

Recent decades have seen an impressive leap in our ability to identify and treat malignant growths, including the operating room, where surgeons and their teams are now often

surrounded by arrays of elaborate technology. These technologies have not only made it easier to monitor and maintain a patient's condition, but also improve the overall effectiveness and success rate of surgical interventions. However, it is often overlooked that the assignment and identification of tumor and its surrounding tissues, while changed,³⁰ still heavily rely on visual cues and palpation.³¹ The advent of minimally invasive laparoscopic and endoscopic procedures eliminates a surgeon's direct access to the tissue within the surgical bed, increasing the need for alternative solutions. One such solution is fluorescence-assisted intraoperative imaging. Two well-known fluorescence dyes that find regular use in the operating room are fluorescein/fluorescein isothiocyanate (FITC) and indocyanine green (ICG).^{32–35} However, it is well understood that while these dyes are incredibly valuable in some applications, they are not specific, and they cannot be used to target and highlight specific biomarkers.

In 2006, it was reported that nonsense-mutations in *SCN9A* (the gene encoding the α -subunit of Na_v1.7) lead to congenital inability to sense pain.³⁶ This discovery was precipitated by a 10-year-old child in Pakistan who was well-known to the local medical staff due to his self-inflicted injuries, and his inability to sense noxious stimuli. Significantly, aside from impaired olfaction, all other sensory functions were normal, including sensitivity to heat, touch, and pressure. This genetic study therefore suggested that Na_v1.7 might be a potent analgesic target. Particularly in light of the opioid crisis, the possibility of establishing a new class of painkillers created substantial interest in both industry and academia-but while the loss of function of Na_v1.7 undoubtedly leads to the inability to sense pain, reproducing the painless state associated with loss of Na_v1.7 with an administered Na_v1.7 inhibitor has proven difficult.

For one, humans express eight other Na_v channels that are highly homologous to Na_v1.7, and some of these channels have vital roles in respiratory, neuronal, or cardiovascular function. A clinically successful Na_v1.7 inhibitor should therefore be exceptionally selective. Second, while Na_v1.7 is predominantly expressed in peripheral sensory neurons, particularly nociceptive small-diameter dorsal root ganglia,^{25–27} any inhibition within the central nervous system has to be avoided. Inhibitors of Na_v1.7 should therefore be impermeable to the blood brain barrier in order to avoid interacting with channels in the central nervous system. Third, it is unclear to what degree Na_v1.7 must be inhibited, or what population of Na_v1.7 channels must be inhibited, to induce an analgesic effect.^{24,37} Lastly, it remains to be seen if a lifelong absence and the temporary deactivation of Na_v1.7 result in comparable phenotypes.³⁸

For an imaging agent, some of the above-mentioned translational hurdles do not apply, because imaging agents should not elicit a pharmacological response. It is rather the absence of pharmacodynamic effects (both immediate and long-term) which are desired. This is achieved by injecting sufficient imaging agent for visualization, but not enough to perturb the patient's metabolism or neuronal function. Thus, in fundamental contrast to Na_v1.7 therapeutics, the clinical utility of a Na_v1.7 imaging agent is therefore defined by its ability to generate contrast while simultaneously not inhibiting large fractions of the channel, thereby allowing surgeons to inject tracer quantities of the agent. Such a regimen would

resemble that of other fluorescent agents that have been injected intravenously before.^{23,31,39} The generated contrast, however, is key-and is one of the prime measures of success.

Critically, we did not observe any visible signs of toxicity (for example, respiratory or motor dysfunction) when Hsp1a-FL was injected intravenously into mice. Moreover, our data suggests that Hsp1a-FL does not penetrate the central nervous system (Figures S6 and S7). This suggests that a more comprehensive toxicity study should be performed to determine the maximum tolerated dose of Hsp1a-FL. Perhaps most importantly, though, the dye we used for modifying Hsp1a emits in the visible range, which limits its tissue penetration range. While this is not a problem for every type of intraoperative application, the clinical impact of a nerve imaging agent is directly correlated to the maximum depth below the tissue surface from which it can provide contrast.

We believe that Hsp1a has the potential to stimulate a paradigm shift in intraoperative imaging of the peripheral nervous system. For instance, it is very convenient to image after 30 min of injection, which is ideal for surgical applications in the operating room, instead of injecting and waiting for longer periods of time. The next logical step toward a second-generation, clinical candidate for Hsp1a-based nerve imaging is incorporation of a longer-wavelength fluorophore. This could be achieved using a click-chemistry-based approach, analogous to our previous work.^{28,29} Similarly, such a defined synthetic strategy would allow for rapid testing of a library of different linker types, attachment sites, and appended fluorophores,⁴⁰ potentially retaining the Na_v1.7 binding affinity of the parental Hsp1a vector.

EXPERIMENTAL SECTION

General.

Unless otherwise stated, all solvents and reagents were obtained from Sigma-Aldrich or Fisher Scientific and were used without further purification. BODIPY-FL was purchased from Invitrogen (Carlsbad, CA). Anti-Na_v1.7 antibody [N68/6] was purchased from Abcam (ab85015). Water (>18.2 MΩ cm⁻¹ at 25 °C) was obtained from an Alpha-Q Ultrapure water system (Millipore). Acetonitrile (AcN) and ethanol (EtOH) were of high-performance liquid chromatography (HPLC) grade. PBS without Ca²⁺ or Mg²⁺ was obtained from the Media Preparation Facility at Memorial Sloan Kettering Cancer Center and used for all in vivo injections. Reverse-phase (RP) HPLC purifications were performed on a Shimadzu HPLC system equipped with a DGU-20A degasser, SPD-M20A UV detector, LC-20AB pump system, and a CBM-20A communication BUS module using RP-HPLC columns (Atlantis T3 C18, 5 μm, 4.6 × 250 mm, P/N: 186003748). Epifluorescence imaging was performed on an IVIS Spectrum (PerkinElmer). Fluorescence stereoscope images were obtained with a Lumar fluorescence stereoscope (SteREO Luma.V12, Zeiss, Jena, Germany). Confocal microscopy images were captured using a Zeiss-LSM880 (Oberkochen, Germany) point-scanning confocal microscope equipped with a 405 nm laser for detection of Hoechst 33342, and a 488 nm laser for detection of Hsp1a-FL.

Synthesis of Hsp1a.

Hsp1a was synthesized using standard Fmoc chemistry⁴¹ on a Symphony peptide synthesizer (Gyros Protein Technologies, Tucson, AZ, USA). C-terminal amidation was achieved using a Rink-amide resin at a scale of 0.125 mmol. Simultaneous release of peptide from the resin and removal of side chain protecting groups was achieved with trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/water (48:1:1 v/v/v) for 2.5 h. Crude Hsp1a was triturated in chilled diethyl ether, and then the precipitated peptide was dissolved in solvent A/B (45% v/v acetonitrile, 0.05% v/v TFA), lyophilized, and then prepurified using C18 RP-HPLC. The peptide was eluted using a linear gradient of 10–60% solvent B (90% v/v ACN; 0.05% v/v TFA) over 50 min using a flow rate of 50 mL·min⁻¹. Fractions were collected and analyzed using electrospray ionization mass spectroscopy (ESI-MS), then fractions of interest were pooled, lyophilized, and stored at -20 °C. Free cysteines were oxidized for 16 h at room temperature in a buffer containing 2 M urea, 0.1 M Tris pH 8, 0.15 mM reduced glutathione, and 0.3 mM oxidized glutathione. Oxidation was quenched by acidification to pH 3, before the peptide was filtered and purified using preparatory and semipreparatory RP-HPLC as previously described.⁴² LC-ESI-MS (ES+), *m/z* calculated for [C₁₄₈H₂₂₀N₄₀O₄₀S₆] 3389.52, [C₁₄₈H₂₂₀N₄₀O₄₀S₆+2H]²⁺ 1696.76, found [M+2H]²⁺ 1696.00, [C₁₄₈H₂₂₀N₄₀O₄₀S₆+3H]³⁺ 1130.84, found [M+3H]³⁺ 1131.05, [C₁₄₈H₂₂₀N₄₀O₄₀S₆+4H]⁴⁺ 848.38, found [M+4H]⁴⁺ 848.55, [C₁₄₈H₂₂₀N₄₀O₄₀S₆+5H]⁵⁺ 678.90, found [M+5H]⁵⁺ 679.05.

Synthesis of Hsp1a-FL.

Hsp1a peptide (0.37 mM, 250 µg in 200 µL of ACN) and Na₂CO₃ (1 M, 40 µL) were transferred into a 3 mL amber vial with a magnetic bar stirrer. BODIPY-NHS (4 µL of a 26 mM solution) was dissolved in AcN and added dropwise to the reaction mixture. The final volume of the reaction mixture was 350 µL. The reaction mixture was allowed to stir for 10–20 min before dilution with 100 µL of water. This reaction afforded the mono- and diadduct, which were purified and separated using RP-HPLC. Fractions containing Hsp1a-FL were concentrated and solvent removed *in vacuo* to afford a red-yellowish powder (150 µg, 55% yield from Hsp1a peptide). This purified compound was formulated in 100% Ca²⁺/Mg²⁺-free PBS or 10% dimethyl sulfoxide (DMSO) and PBS. LC-ESI-MS (ES+), *m/z* calculated for [C₁₆₂H₂₂₈BF₂N₄₃O₄₀S₆] 3663.63, [C₁₆₂H₂₂₈BF₂N₄₃O₄₀S₆+2H]²⁺ 1832.83, found [M+2H]²⁺ 1833.10, [C₁₆₂H₂₂₈BF₂N₄₃O₄₀S₆+3H]³⁺ 1222.22, found [M+3H]³⁺ 1222.40, [C₁₆₂H₂₂₈BF₂N₄₃O₄₀S₆+4H]⁴⁺ 916.91, found [M+4H]⁴⁺ 917.05.

Tryptic Digest of Hsp1a-FL and Hsp1a.

15 µL of digestion buffer and 1.5 µL of reducing buffer were added to a 0.5 mL microcentrifuge tube. 10.5 µL of a solution containing 4 µg of Hsp1a-FL was added to the same tube, and then the final volume was adjusted to 27 µL with ultrapure water. The sample was then incubated at 95 °C for 5 min. The sample was allowed to cool to room temperature before 2 µL of activated trypsin were added, followed by incubation at 37 °C overnight. The sample was analyzed with LC-ESI-MS. LC-ESI-MS (ES+), *m/z* calculated for the fragmentation of Hsp1a-FL was observed as follows, for fragment [C₁₂₆H₁₈₅N₃₃O₃₄S₅]

2864.24, m/z calculated for $[C_{126}H_{185}N_{33}O_{34}S_5+2H]^{2+}$ 1433.12, found $[M+2H]^{2+}$ 1432.95, $[C_{126}H_{185}N_{33}O_{34}S_5+3H]^{3+}$ 955.75, found $[M+3H]^{3+}$ 956.00.

Hsp1a was digested following the same tryptic digestion protocol and the sample was analyzed with LC-ESI-MS. LC-ESI-MS (ES⁻), m/z calculated for the fragmentation of Hsp1a was observed as follows, one fragment $[C_{112}H_{171}N_{31}O_{33}S_5]$ 1318.70 and second fragment $[C_{90}H_{138}N_{24}O_{27}S_4]$ 1056.45, m/z calculated for $[C_{112}H_{171}N_{31}O_{33}S_5-2H]^{2-}$ 1318.07, found $[M-2H]^{2-}$ 1318.70, m/z calculated for $[C_{90}H_{138}N_{24}O_{27}S_4]$ 1057.45, found $[M-2H]^{2-}$ 1057.10. In addition, for Hsp1a, the following fragmentation was observed in LCESI-MS (ES⁺), m/z calculated for $[C_{90}H_{138}N_{24}O_{27}S_4+2H]^{2+}$ 1058.45, found $[M+2H]^{2+}$ 1058.80, $[C_{90}H_{138}N_{24}O_{27}S_4+3H]^{3+}$ 705.97, found $[M+3H]^{3+}$ 706.35.

Cell Lines.

HEK293 cells stably expressing the human Na_V channel $\beta 1$ subunit (hNa_V $\beta 1$) in combination with the α subunit of hNa_V1.7 (Scottish Biomedical, Glasgow, UK) were seeded into a 175 cm² cell culture flask 2 days prior to patching and were detached at 60% confluency using 2 mL Detachin (Genlantic, San Diego, CA, USA). After pelleting, cells were centrifuged at 8000 rpm for 8 min, and then the supernatant was removed and cells were resuspended in 5 mL QPatch media containing 96.5% CD293 medium, 25 mM HEPES (Gibco), and glutamine (Gibco).

Electrophysiology.

Whole-cell patch-clamp experiments were performed at room temperature using a QPatch 16× automated electrophysiology platform (Sophion Bioscience, Denmark) using 16-channel planar patch-chip plates (QPlates) with a patch-hole diameter of 1 μ m and resistance of 2 M Ω . Whole-cell currents were filtered at 5 kHz (8-pole Bessel) and digitized at 25 kHz. A P4 online leak-subtraction protocol was used with non-leak-subtracted currents acquired in parallel. The extracellular solution was 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 4 mM KCl, 145 mM NaCl at pH 7.4, and the intracellular solution was 140 mM CsF, 1 mM/5 mM EGTA/CsOH, 10 mM HEPES, 10 mM NaCl at pH 7.3. Hsp1a and Hsp1a-FL peptides were dissolved in extracellular solution with 0.1% bovine serum albumin (BSA). Concentration-response data were obtained using five concentrations of peptide (0.2, 2, 20, 200, and 2000 nM). HEK293-hNa_V cells were clamped at a holding potential of -80 mV, and then for each concentration, 10 μ L of peptide was added for 6 s before applying the following voltage protocol: -80 mV for 10 ms, -120 mV for 200 ms, 0 mV for 20 ms, and then return to the holding potential of -80 mV. The total incubation time for each peptide concentration was 7.5 min: the voltage protocol was applied 15 times, with cells clamped at a holding potential of -80 mV for 30 s between runs. Concentration-response data were analyzed in GraphPad Prism 8 to obtain IC₅₀ values.

Human Tissue.

Human nerves (vagus nerves, $n = 3$) were a donation from the Fusion Solutions Bioskills Laboratory, Long Island, NY. The nerves were paraffin-embedded and formalin fixed and sectioned at 10 μ m thickness for H&E and immunohistochemical detection experiments.

Animal Model.

Female athymic nude mice (4–8-week-old, athymic-Nude (outbred) (Stock#:069); Envigo, USA) were allowed to acclimatize at the Memorial Sloan Kettering Cancer Center (MSK) vivarium for 1 week with ad libitum food and water prior to the experimental procedure. For imaging experiments, animals were sacrificed 30 min post tail vein injection of Hsp1a-FL, Hsp1a/Hsp1a-FL, or PBS. All animal experiments were performed in accordance with institutional guidelines and approved by the IACUC of MSK, following NIH guidelines for animal welfare.

Immunohistochemistry.

Na_v1.7 in human vagus nerves and mouse sciatic nerves was detected using immunohistochemical (IHC) staining techniques, which were performed at the Molecular Cytology Core Facility of MSK using the Discovery XT processor (Ventana Medical System, Tucson, AZ). Anti-Na_v1.7 antibody [N68/6] (Abcam ab85015) specifically bound to both human and mouse Na_v1.7 (0.5 µg/mL). Paraffin-embedded formalin-fixed 10 µm sections were deparaffinized with EZPrep buffer. For IHC detection, a 3,3'-diaminobenzidine (DAB) detection kit (Ventana Medical Systems, Tucson, AZ) was used according to the manufacturer's instructions. Sections were counterstained with H&E and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

Confocal Microscopy.

To determine the distribution and localization of Hsp1a-FL, we used 10 µm cryosections of OCT-embedded sciatic nerve tissues from mice intravenously injected with Hsp1a-FL (7 nmol, 68 µM of Hsp1a-FL in 100 µL of PBS), Hsp1a/Hsp1a-FL (Hsp1a-FL, 68 µM, 7 nmol, and Hsp1a 204 µM, 21 nmol in 100 µL PBS) or PBS. Animal organs were incubated with Hoechst 33342 (blue, 20 µM, 1 nmol in 50 µL of PBS) to counterstain nuclei before embedding in Mowiol mounting medium. Fresh tissue samples were counterstained with 33342 (blue, 20 µM, 1 nmol in 50 µL of PBS) up to 90 min post-mortem and placed directly on a microscope slide for imaging.

Epifluorescence Imaging.

Animals were intravenously injected with Hsp1a-FL (7 nmol, 68 µM of Hsp1a-FL in 100 µL of PBS, *n* = 9). To assess the specificity of the Hsp1a-FL accumulation, we injected a combination of Hsp1a and Hsp1a-FL (Hsp1a-FL, 68 µM, 7 nmol and Hsp1a 204 µM, 21 nmol in 100 µL PBS, *n* = 9) or PBS (*n* = 9). Animals were sacrificed 30 min post-injection and epifluorescence images obtained. Epifluorescence images of RSN and LSN were obtained in situ and of the excised RSN, LSN, muscle, heart, kidney, liver, and brain with an IVIS Spectrum (PerkinElmer) using a predefined GFP filterset (excitation = 465/30 nm, emission = 520–580 nm). Autofluorescence was removed through spectral unmixing. Semiquantitative analysis of the Hsp1a-FL signal was conducted by measuring the average radiant efficiency (in units of unit [p/s/cm²/sr]/[µW/cm²]) in regions of interest (ROIs) that were placed on all resected organs under white light guidance.

Fluorescence Stereoscope Imaging.

The Hsp1a-FL fluorescence signal in mouse sciatic nerves was also visualized using a fluorescence stereoscope 30 min after intravenous injection of Hsp1a-FL (7 nmol, 68 μ M of Hsp1a-FL in 100 μ L of PBS) or PBS ($n = 3$ /group). Fluorescence images were obtained using mice with exposed but otherwise intact sciatic nerves. Images were also obtained from excised sciatic nerves and muscle. Imaging was performed in bright field and fluorescence mode, with a 500/20 nm laser excitation and 535/30 emission filter and an exposure time of 200–400 ms.

Statistical Analyses.

Statistical analyses were performed using GraphPad Prism 8. Unless otherwise stated, data points represent mean values, and error bars represent standard deviations of biological replicates. *P* values were calculated using a Student's unpaired *t*-test or a Mann-Whitney test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Na_v1.7	voltage-gated sodium channel subtype 1.7
Hsp1a	<i>Homeomma</i> spec. Peru
IHC	immunohistochemical
H&E	hematoxylin and eosin stain
hNa_vβ1	human Na _v channel β1 subunit
HPLC	high performance liquid chromatography
Hsp1a-FL	fluorescence Hsp1a
DMSO	dimethyl sulfoxide

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Figure 1.

Na_v1.7 expression in human peripheral nerves. The ubiquitous expression of Na_v1.7 in nociceptive human nerves is used to develop a peripheral nerve-targeted fluorescent imaging probe. (a) Schematic showing the anatomic location of human vagus nerves within the head and neck region. (b) White light photograph of a surgically removed vagus nerve autopsy specimen ($n = 3$). (c) H&E (top row), IgG control (middle row), and anti-Na_v1.7 (bottom row) staining of a vagus nerve autopsy specimen. Immunohistochemistry shows that Na_v1.7 is expressed within the human peripheral nerve, and that the staining was specific. (d) Representative image of an exposed mouse sciatic nerve under white light conditions. (e) Schematic representation of Hsp1a transformation into a fluorescent agent, together with the workflow for imaging mouse sciatic nerves with Hsp1a-FL.

Figure 2.

Chemical synthesis, characterization, and validation of Hsp1a-FL. The figure describes synthesis, analysis, and Na_v1.7 binding affinity of Hsp1a-FL. (a) Reaction scheme for conjugation of Hsp1a peptide with BODIPY-FL-NHS ester dye. The ribbon model of Hsp1a-FL shows disulfide bridges (in yellow), highlighting lysine K4 (in red/blue) where BODIPY-FL primarily attaches. (b) Amino acid sequences, molecular weights, and observed ions for the unmodified Hsp1a peptide and the fluorescent tracer Hsp1a-FL. (c) RP-HPLC chromatograms of Hsp1a (black) and Hsp1a-FL (green) with absorbances observed at 280 nm. (d) LC-MS spectra of Hsp1a-FL tracer showing three major ion species that confirm the calculated mass of Hsp1a-FL after dye conjugation. (e) Concentration-response curves for inhibition of hNav_v1.7 by Hsp1a-FL and Hsp1a. Data were fit in prism 8 ($n = 5$; mean $\pm$ SEM). The calculated IC₅₀ values for Hsp1a-FL and Hsp1a were 62 nM and 13 nM, respectively. (f) Representative hNav_v1.7 current traces in the absence (solid) or presence of 2 μ M Hsp1a-FL or Hsp1a (dashed).

Figure 3.

Histological validation of Hsp1a-FL. Hsp1a-FL uptake was validated in mouse sciatic nerve cryosections from mice injected with Hsp1a-FL (first column, green, 68 μ M, 7 nmol Hsp1a-FL in 100 μ L of PBS). Hoechst 33342 (blue, 20 μ M, 1 nmol in 50 μ L of PBS) was used to highlight Schwann cell nuclei. Adjacent slides were stained with H&E (second column) and anti-Na_v1.7 antibody (third column). The scale bar for entire nerves (first row) represents 1000 μ m. Mouse sciatic nerves were sectioned transversely (second row, sciatic and tibial nodes), longitudinally close to the surface (third row, epineurium/perineurium), and more centrally (fourth row, endoneurium/nerve fibers).

Figure 4.

Hsp1a-FL accumulation in mouse sciatic nerves. The figure describes imaging of fresh, unprocessed mouse sciatic nerves with Hsp1a-FL. (a) Epifluorescence images of animals injected with PBS, Hsp1a-FL (7 nmol, 68 μ M of Hsp1a-FL in 100 μ L PBS) or a Hsp1a-FL/Hsp1a formulation (Hsp1a-FL, 68 μ M, 7 nmol and Hsp1a, 204 μ M, 21 nmol in 100 μ L PBS). Images were taken 30 min after tail vein injection. (b) Epifluorescence images of resected right and left mouse sciatic nerves that were injected with PBS, Hsp1a-FL or Hsp1a-FL/Hsp1a. High fluorescence intensities (due to dye accumulation) were only observed in sciatic nerves injected with Hsp1a-FL alone. Yellow arrows indicate the sciatic nerves. (c) Fluorescence intensity quantification of panel b. Parametric unpaired Student's *t*-test was used to calculate the significance. ** $P < 0.005$; $P = 0.53$. Error bars are standard deviations.

Figure 5.

Imaging of Hsp1a-FL with a Lumar fluorescence stereoscope. This figure describes imaging of sciatic nerves with Hsp1a using a surgical microscope. (a) Schematic of mouse sciatic nerve preparation. (b) Stereoscopic images of exposed mouse sciatic nerves from mice that were injected with Hsp1a-FL (7 nmol, 68 μ M of Hsp1a-FL in 100 μ L of PBS) or PBS under white light and fluorescent excitation. Images were taken in brightfield and with 488 nm laser excitation (exposure time 200 ms). (c) Stereoscopic images of resected sciatic nerves from mice injected with the fluorescent venom peptide Hsp1a-FL (exposure time 400 ms). (d) Fluorescence signal quantification of data in panel c ($n = 3$ per group). (e) Stereoscopic image at high magnification of a resected sciatic nerve from a mouse injected with Hsp1a-FL shows structural features of Hsp1a-FL accumulation. Yellow arrows point toward areas with tubular (left) and axonal (right) features. The Mann-Whitney test was used to calculate the significance. $*P < 0.01$. Error bars are standard deviations.