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RESEARCH PAPER

Analgesic Efficacy and Safety of DALDA Peptide Analog Delivery to the Brain Using Oil-in-Water Nanoemulsion Formulation

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ABSTRACT

Purpose The main objective of this study was to develop and evaluate therapeutic efficacy and safety following systemic delivery of a peptide analgesic into the CNS using an oil-in-water nanoemulsion system.

Methods We have formulated a safe and effective, omega-3 rich polyunsaturated fatty acid containing oil-in-water nanoemulsion formulation, for encapsulating and delivering chemically-modified DALDA, a potent mu-opioid peptide analogue, to the CNS. One of the challenges with CNS delivery is the lack of a non-invasive bioanalytical technique to confirm CNS uptake and therapeutic efficacy. Using blood oxygen-level dependent (BOLD) functional magenetic resonance imaging (fMRI), we provide quantitative evidence of nanoemulsion-based delivery and analgesic activity of DALDA analogue in capsaicin-induced awake rat model of pain.

Results Nanoemulsion formulation effectively encapsulated the modified analgesic peptide and demonstrated efficacy in the capsaicin- pain induced functional magnetic resonance imaging model in rodents. Preliminary safety evaluations show that the nanoemulsion system was well tolerated and did not cause any acute negative effects.

Conclusions Overall, these results show tremendous opportunity for the development of modified peptide analgesic-encapsulated nanoemulsion formulations for CNS delivery and therapeutic efficacy.

KEY WORDS Blood-brain barrier · DALDA analgesic peptide · functional magnetic resonance imaging · oil-in-water nanoemulsion

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INTRODUCTION

Peptide and protein therapeutics for diseases of the central nervous system (CNS) possess several important advantages; however, their systemic delivery across the blood-brain barrier (BBB) is limited (1). The BBB is a highly regulated structure that protects brain from toxic solutes present in the systemic circulation, but as such also presents a major limitation for access of therapeutic compounds to the central nervous system (CNS). Complex tight junctions between the endothelial cells of the brain seal off all paracellular permeability and, together with the multiple transporters present on the luminal membrane of the EC, impart a formidable obstacle to drug entry (2), especially for proteins, peptides, and nucleic acids. Various invasive and non-invasive approaches have been explored pre-clinically as well as in clinic, to effectively deliver peptides to the CNS (3).

Peptide analgesics are regarded as good alternatives to morphine, as their use can reduce the side effects of morphine-based therapy (4). Peptides are highly potent as therapeutic agents when they are able to cross the BBB and able to gain access to the sites of action of centrally mediated pain processes, making the activity of these peptides within CNS of particular interest (1,5). However, poor metabolic stability of peptides and their low lipid solubility limit their ability to cross the BBB. [D-Arg^2, Lys^4]-dermorphin analogue (DALDA) is a potent, centrally acting μ-opioid peptide analgesic with limited permeability across the BBB, when administered by clinically relevant routes of administration, such as intravenous or subcutaneous injection (6).

Omega-3 fatty acids (FA), also known as essential n-3 polyunsaturated fatty acids (PUFA), are important for brain growth and development, but cannot be produced by the body (7). For this reason, omega-3 FA must be obtained from a food source, like flax seed or fish oil. Omega-3 FA are known to be critical modulators of neuronal function and play a key

role in regulation of neuroinflammatory and oxidative stress-mediated mechanisms in the normal CNS during aging and chronic neurological diseases (8). One of the models for fatty acid diffusion across the BBB rules out the involvement of transporters; i.e., it proposes that fatty acids cross the luminal and the transluminal leaflets of brain endothelium and plasma membrane by reversible flip-flop (9). Once fatty acids reach the neurons, acetyl-CoA synthetase traps them by forming acetyl-CoA, which no longer can diffuse out of the cell. Nanoemulsions are oil-in-water (O/W) or water-in-oil (W/O) formulations made with edible oils like the ones rich in omega-3 FA, surface-active agents (surfactants), and water, where the diameter of inner phase is reduced to nanometer length scale (10). The versatility of nanoemulsions is based on the different types of oils and surface modifiers that can be used (11). Multifunctional nanosystems with different types of payloads and targeting capabilities in a single platform are gaining more attention and focus recently, mainly due to their wider capabilities in being amenable to therapeutic, diagnostic and imaging applications. Hydrophobic payloads and imaging agents can be readily incorporated in the oil phase of the nanoemulsions (12,13). These systems can be designed to achieve active targeting, efflux transporter inhibition or their delivery using alternate routes/techniques to overcome the BBB (12,13).

Another challenge with CNS delivery is the lack of an analytical assay to measure the concentration in the brain, without involving surgical procedures. Hence, recently, different imaging techniques have been utilized in the diagnosis of brain disorders. Magnetic resonance imaging (MRI) has been one the most widely used clinical imaging modality for the assessment of CNS diseases. The newer field of functional MRI (fMRI) can provide topographic plots of brain activity using blood flow and regional oxygen consumption in the brain (14). Possibility of whole imaging in awake animals has led to growing interest in employing such pharmacological MRI systems, also called as blood oxygen- level- dependent (BOLD) functional MRI (fMRI), to study brain activities and analgesic effects upon stimulus in animal models of pain (15,16). Capsaicin- induced awake pain generates a persistent, highly painful stimuli without causing any permanant harm to the subjects. Besides, such testing is also used in a clinical setting to study analgesic responses in human volunteers.

Our study involved development of an omega-3 rich poly-unsaturated fatty acid (PUFA)-based nanoemulsion formulation, for effective encapsulation and delivery of peptide analogs of DALDA to the CNS. Testing of the analgesic potential of peptide nanoemulsion was performed with a capsaicin-induced awake animal pain model using fMRI. In addition to evaluating the systemic delivery and efficacy of the peptide nanoemulsion, preliminary safety and tolerability studies were also performed in animals.

MATERIALS AND METHODS

Materials

Extra pure omega-3 rich fish oil and Pluronic F68® were provided as a gift by Jedwards International (Quincy, MA) and BASF Corporation (Florham Park, NJ), respectively. Lipoid E80® and DSPE-PEG2000 were purchased from Lipoid GMBH (Ludwigshafen, Germany). Tween 80® was purchased from Sigma Chemicals, Inc. (St. Louis, MO). [D-Arg^2, Lys4]-dermorphin-(1-4)-amide (DALDA) was purchased from Bachem, Inc. (Torrance, CA). Pointe Scientific AST (SGOT) and ALT (SGPT) kits were purchased from Fisher Scientific, Inc. (Fair Lawn, NJ). Enzyme immunoassay kits were purchased from RayBiotech, Inc. (Norcross, GA). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).

Animals

Male CD-1® mice (4–6 weeks old) and male Sprague Dawley® (SD) rats (4–6 weeks old) were purchased from Charles River Laboratories (Cambridge, MA). All the animal procedures were approved by the Northeastern University's Institutional Animal Care and Use Committee.

DALDA Peptide Chemical Modification

As illustrated in the previous study (17), to improve the encapsulation of hydrophilic DALDA in oil phase of nanoemulsion, the epsilon-amino group of the lysine residue was structurally modified using 9-fluorenylmethyloxycarbonyl (Fmoc/tBu) solid phase peptide synthesis, followed by side chain acylation with the appropriate fatty acid (butanoic, hexanoic or octanoic acid). The product obtained by this modification were referred as DALDA-C4, DALDA-C6 or DALDA-C8. Reactions during peptide synthesis of modified DALDA analogues were monitored with the use of ninhydrin test. The identity of modified peptides was confirmed by mass spectrometry (MS) with Shimadzu Biotech Axima CFRplus utlizing Matrix-assisted laser desorption/ionization (MALDI) technique and m/z was reported. The purity was confirmed by reverse phase high performance liquid chromatography (HPLC).

Preparation and Characterization of the Nanoemulsion Formulations

Oil-in-water nanoemulsions were prepared by sonication method as described in previous study (17). The formulations were characterized for particle size and surface charge using the Brookhaven Instrument's 90Plus ZetaPALS analyzer as illustrated in previous study (17). Drug loading, encapsulation

efficiency and stability were evaluated using HPLC as described before (17). .

Analgesic Efficacy Evaluations with Hot-Water Tail Withdrawal Assay

Male CD-1® mice were randomized into 3 groups of 6 animals per group. These mice were acclimatized to the test conditions prior to dosing the formulations. Temperature controlled, Isotemp® water bath (Fisher Scientific, Fair Lawn, NJ) was set at 52°C. CD-1 mice were given an intravenous dose of 3 mg/kg DALDA solution in phosphate buffered saline (PBS) or DALDA-C8 solution in PBS or DALDA-C8 nanoemulsion (prepared as described in DALDA Peptide Chemical Modification). The animal's tail tip (1 in.) was gently lowered in the water bath at set time points of 0 min (before injection) and at every 30 min interval up to 180 min post-dosing. Time taken for the mouse to withdraw its tail out of the hot water (i.e. the latency period) was recorded. A cut off time of 8 s was set to avoid burn to the tail due to prolonged exposure to hot water.

Analgesic Efficacy Evaluations with Functional MRI in Capsaicin-Induced Pain Model

Capsaicin- Induced Awake Animal Model

Capsaicin, a major component of chili peppers, is an irritant compound and upon injection in the skin, causes stimulation of unmyelinated and poorly myelinated Aδ primary sensory afferent fibers (18). This causes release of bradykinin and prostaglandin E_2, thereby causing inflammation via modulation of a heat-sensitive ion channel on sensory neurons, which is opened in response to thermosensitive stimulus (19). Being a receptor-mediated event, there is no permanent damage to tissues (18).

Capsaicin stimulation was performed by placing a PE20 (polyethylene) tubing (Braintree Scientific Inc., Braintree, MA) fitted with a small needle (26-gauge), that would be introduced subcutaneously in the right dorsal hindpaw of the test rat after the rat is set up in the magnet. The tubing was hooked to a syringe filled with capsaicin (Sigma-Aldrich, St. Louis, MO) solution (1 mg/mL in 25% w/w captisol® in pH 7.4 phosphate buffered saline). In the experiments involving DALDA-C8 nanoemulsion formulation dosing, the SD® rats were dosed via tail vein injection at 3 mg/kg dose, prior to setting them up in the magnet and prior to capsaicin injection as described in the protocol in Fig. 1.

The Pain Neuronal Circuit

Pain phenomenon is quite complex and no single pre-clinical pain model can address the wide range of modulatory factors that are associated with pain response mechanisms in humans (20) (21) . The dorsal horn neurons for spinal cord perceive the noxious stimulus (like capsaicin) from the peripheral nociceptors. The sensory component (location and intensity) of pain is conveyed via spinal neurons to the higher centers in brain namely, the thalamus and somatosensory cortex (21). The affective component (duration and emotion) is processed by amygdala, anterior cingulate cortex and the bed nucleus of stria terminalis.

Functional Magnetic Resonance Imaging (fMRI) (22,23)

The rats were lightly anesthetized and placed into a head coil and restrainer developed by the Animal Imaging Research (Holden, MA) (22,23). When fully conscious, the animals were placed in a dark mock scanner tube with a recording of a standard MRI pulse sequence playing in the background. This procedure when repeated every other day for four days has been shown to significantly reduce plasma corticosterone levels, respiration, heart rate, and motor movements when compared to the first day of acclimation (24). The reduction in autonomic and somatic response measures of arousal and stress improve the signal resolution and MR image quality (24).

Experiments were conducted using a Bruker Biospec 7.0 T/20-cm USR horizontal magnet and a 20-G/cm magnetic field gradient insert (ID = 12 cm) capable of a 120-μs rise time. Radiofrequency signals were sent and received with the quad-coil electronics built into the animal restrainer. Functional images were acquired using a multi-slice, half-fourier acquisition, single shot, turbo-spin echo sequence (RARE-st). A single scanning session acquired 250 time points of 22 slices, 1.0 mm thick, every 6.0 s (FOV 3 cm, matrix size 96×96, ETL 36, NEX 1). First 50 time points were baseline and approximately 180 time points were collected after injection. At the end of each imaging session, a high-resolution anatomical data set was collected using the RARE pulse sequence (22 slice; 1.0 mm; field of vision [FOV] 3 cm; 256×256; repetition time [TR] 2.5 s; echo time [TE] 12.4 msec; NEX 6; 6.5-minute acquisition time).

fMRI Data Acquisition and Analyses (22,23)

Functional images were acquired using a T_2 weighted RARE-st (multi-slice, half-fourier acquisition, single shot, turbo-spin echo sequence) (22,23). A single data acquisition has 22; 1.0 mm slices in 6 s (FOV 3 cm). This sequence was repeated as per protocol design. Functional imaging studies were bracketed by a single-slice, arterial-spin labeling scan of the dorsal striatum to assess blood flow before and after administration of the test article.

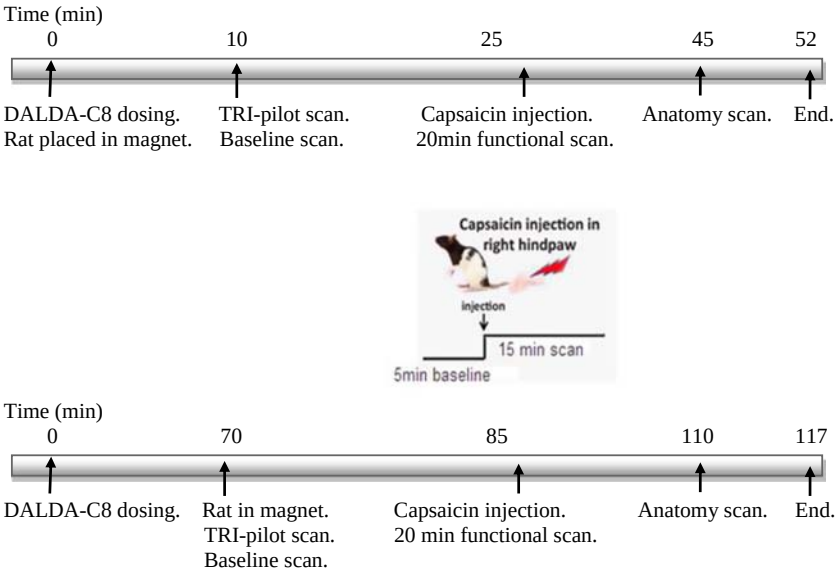

Fig. 1 Functional magnetic resonance imaging (fMRI) protocol for DALDA-C8 dosing in a capsaicin-induced pain model. (**a**) Capsaicin shock at 30 min after dosing, followed by functional scan. (**b**) Capsaicin shock at 90 min after dosing, followed by functional scan. Cartoon representation of capsaicin injection and scan time for the experiments. Time axis not to scale.

Functional data was pre-processed using SPM8 and in-house Matlab® software. SPM8 was used for motion estimation/correction followed by trend correction. Data was registered to brain atlas using MIVA software and processed using in-house Matlab® code. Statistical *t* tests were performed on each voxel of each subject within their original coordinate system. The baseline threshold was set at 2%. *T*-test statistics using a 95% confidence level, 2-tailed distributions, and heteroscedastic variance assumptions were performed.

A statistical composite was created for each treatment group of rats. The composite statistics was built using the inverse transformation matrices. Each composite pixel location (i.e. row, column, and slice), premultiplied by $[T_i]^{-1}$, mapped it within a voxel of subject (i). A trilinear interpolation of the subject's voxel values (percentage change) determined the statistical contribution of subject (i) to the composite (row, column, and slice) location. The use of $[T_i]^{-1}$ ensured that the full volume set of the composite was populated with subject contributions. The average value from all subjects within the group was used to determine the composite value. The average number of activated pixels that had the highest composite percent change values in a particular ROI was displayed in a composite map. Activated composite pixels were calculated as follows:

$$\textit{Activated Composite Pixels ROI}(j) = \frac{\sum_{i=1}^{N} \textit{Activated Pixels Subject}(i)\ \textit{ROI}(j)}{N}$$

The composite percent changes for the time history graphs for each region were based on the weighted average of each subject, as follows:

$$\textit{Composite Percent Change} = \frac{\sum_{i=1}^{N} \textit{Activated Pixel Subject}(i) \times \textit{Percent Change}(i)}{\textit{Activated Composite Pixels}}$$

Formulation Safety Evaluations

Changes in Animal Body Weight

Mice were weighed before dosing (day 0), followed by dosing a 125 µl of saline or DALDA-C8 solution or DALDA-C8 nanoemulsion (3 mg/kg dose) and then weighed continuously at day 1 (24 h), day 4 and day 7. The change in body weight was determined and compared to the initial body weight (day 0) of all animals in the three groups.

Changes in Liver Enzyme and Natriuretic Peptide Levels

Aspartate aminotransferase (AST) is found in a variety of tissues including liver, heart, kidney and brain. It is released into the serum when any of these tissues is damaged. It is therefore not a highly specific indicator of liver injury (25). Whereas, the alanine aminotransferase (ALT) is exclusively found in liver and it is released in plasma in case of liver injury (25). Thus, it serves as a fairly specific indicator of any liver damage. To measure the liver enzyme levels namely ALT and AST, plasma from various treatment groups: DALDA-C8 solution or nanoemulsion

at 20 min and 180 min was compared to untreated (control) group, using the manufacturer's instructions.

Atrial natriuretic peptide (ANP) is a peptide hormone secreted by cardiac myocytes of the atrium and it plays an important role in homeostatic regulation of body water, sodium, potassium and fat. It has been previously shown that μ-opioid agonists like morphine, stimulate diuresis and natriuresis, and these effects are mediated by increased ANP release (26). Brain natriuretic peptide (BNP), similar to ANP, is secreted by ventricles of heart in response to excessive stretching of myocytes and is been used as an approved marker for congestive heart failure. To measure the ANP and BNP levels in mice, plasma from various treatment groups: DALDA-C8 solution or nanoemulsion at 20 min and 180 min, was compared to untreated (control) group, following manufacturer's instructions of enzyme immunoassay.

Tissue Histopathology Analyses

Liver, heart and kidney samples were collected for histopathological analysis from mice at early time point, 20 min and at the end of the study for DALDA-C8 solution or nanoemulsion. These tissue samples were preserved in formalin before analysis. Paraffin embedded tissues were cut into 5 μm sections and mounted on a glass slides. Tissue sections were dried and deparaffinized using xylene substitute followed by decreasing concentrations of ethanol to finally running purified water. Sections were incubated in hematoxylin, rinsed with water, and incubated with 1% acid alcohol (clearing reagent). Sections were rinsed and incubated with 4% ammonia solution (bluing reagent). Sections were then incubated with Eosin followed by dehydration by two changes each in 95% ethanol and 100% ethanol followed by final change of xylene substitute. Tissues were cover slipped and digital image was captured using a light microscopy. Blinded analysis of tissue toxicological profile and tissue damage, if any, was carried out by Dr. Jerry Lyon, a certified veterinary pathologist, at the Tufts University Veterinary School in Grafton, MA.

Statistical Data Analysis

The two-tailed unpaired homoscedastic *t*-test was used to compare mean values ± standard deviation obtained for the treated and untreated samples and $p<0.05$ was considered statistically significant.

RESULTS

DALDA Peptide Chemical Modification

DALDA analogues with modified side- chain fatty acid (Table I) could be successfully prepared by 9-fluorenylmethyloxycarbonyl (Fmoc/tBu) solid phase peptide synthesis.

Modified DALDA analogues were characterized using MALDI mass spectrometry to confirm the molecular weight. Figure 2 shows the chromatograms of the various modified chemistry of DALDA analogues analyzed by MALDI mass spectrometry.

HPLC analysis of modified DALDA analogues was performed using 0.1% trifluoroacetic acid in water as mobile phase A and 0.1% trifluoroacetic acid in acetonitrile:water:: 60:40, v/v mobile phase B and flow rate of 1 ml/min. Simple gradient was run from 0% to 100% B in 40 min on Grace Vydac 218TP54 C18, 5 μm, 4.6 mm × 250 mm column. An injection volume of 50 μl and a wavelength of 220 nm was used for DALDA-C8 analysis.. The HPLC showed a linear profile with R^2 of 0.9996 for the concentration range of 2–20 μg/ml. The chromatograms indicated high purity for all the peptides analogues made using solid phase synthesis. Figure 3 illustrates the HPLC chromatogram of DALDA-C8. The peak purity was 98.85%.

Preparation and Characterization of the Nanoemulsion Formulations

The sonication technique yielded a uniform, milky-white emulsion formulation.

Particle size and polydispersity index (PDI) were determined for both placebo and peptide loaded nanoemulsion formulations. All the formulations made by sonication had small size (<200 nm) and narrow PDI (<0.3) and were negatively charged as described in Table II. Surface charge was significantly different (less negative) for the peptide-loaded nanoemulsion *versus* the placebo formulation, owing to positively charged nature on the peptide, except for DALDA-C8 analogue. DALDA-C8 nanoemulsion at 0.7 mg/ml had the drug loading of 87%. Nanoemulsion formulations retained their particle size and surface charge during storage, and peptide loaded nanoemulsion formulations were chemically stable for at least up to 3 months upon storage at 4°C.

Analgesic Efficacy Evaluations in Hot-Water Tail Withdrawal Assay

Unmodified DALDA solution, DALDA-C8 solution and DALDA-C8 nanoemulsion, dosed at 3 mg/kg, via tail vein injection produced analgesia, as shown in (17). The latency time increased 2–3.5- fold over baseline at 60–120 min post-dose for DALDA and DALDA-C8 solution. The response returned to baseline for solution formulations after 120 min.

Table I Molecular Properties of DALDA Analogues

Modified peptide	Substitution on the epsilon-amino group of lysine	Molecular weight (g/mol)	Chemical name
DALDA-C4	Butanoic acid	682.46	H-Tyr-D-Arg-Phe-Lys(Butanoyl)-NH_2
DALDA-C6	Hexanoic acid	710.69	H-Tyr-D-Arg-Phe-Lys(Hexanoyl)-NH_2
DALDA-C8	Octanoic acid	738.82	H-Tyr-D-Arg-Phe-Lys(Octanoyl)-NH_2

For the DALDA-C8 nanoemulsion, a 2–3.5- fold increase in latency time at 60–90 min and an extended duration of analgesia, for up to 180 min was observed. This highlights the potential for improved duration of action with the nanoemulsion formulation for the modified peptide. The experiment also suggests that the C8-linker i.e. modification introduced on the DALDA peptide did not adversely impact its efficacy.

Analgesic Efficacy Evaluations by fMRI

Capsaicin-Induced Awake Pain Model

A 3-D representative map of the different brain areas comprising the putative pain neural circuit of the rat (coronal view) is shown in Fig. 4a. The pain neural circuit is comprised of 17 areas based on tract tracing studies in rats showing connectivity to and from the parabrachial nucleus of the brainstem (27) and critical nodes identified from meta-analysis from human pain imaging studies (28). Since the rats were acclimatized for 4 days prior to testing, there was minimal motion of the head in all planes (X, Y or Z) during the experiment, despite capsaicin shock. This helped to improve the image analysis and reduce any background/noise arising due to motion. Activation of key nodes in the putative pain neural circuit, namely the parabrachial nucleus, gigantocellular reticularis, prefrontal cortex and anterior cingulate was seen in the current capsaicin-induced pain model in awake rats during fMRI scans.

fMRI Data Acquisition and DALDA-C8 Nanoemulsion Analgesia Effect

A significant ($p<0.05$) difference was observed in the positive BOLD signal in various brain regions involved in pain circuit, as represented by the decreased volume of activation, in rats treated with DALDA-C8 nanoemulsion 30 or 90 min prior to capsaicin shock (Fig. 4b–c). A significantly higher volume of activation (positive BOLD signal) is seen for untreated (capsaicin alone) rats. Vehicle corresponds to saline dosing only, where no capsaicin or peptide was administered. As seen in Fig. 4b, the posterior thalamus, nucleus brachium and infralimbic cortex regions showed a small to none positive BOLD signal, equivalent to vehicle arm, indicating the peptide nanoemulsion caused a complete recovery from capsaicin shock. Other regions, namely the central amygdala, ventral medial hypothalamus and entorhinal cortex showed a significant reduced positive BOLD response when compared to untreated rats. While, the temporal cortex data does not look to be different visually due to the error bars, comparing the drug treated and untreated rats, the statistical significance was seen during the composite map analysis.

As seen in Fig. 4c, the central amygdala, temporal cortex, parietal cortex, infralimbic cortex and anterior hypothalamus showed a small positive BOLD signal compared to untreated rats, indicating the peptide nanoemulsion caused recovery from capsaicin shock. While, the medial amygdala and entorhinal cortex data does not seem to be different visually due to the error

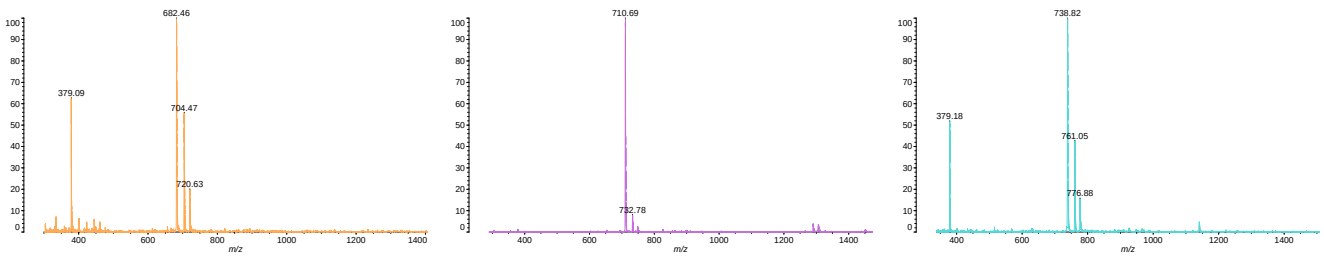

Fig. 2 MALDA mass spectrometry of modified DALDA analogues. Confirmation of identity of modified DALDA peptides with DALDA-C4 = 682.46 g/mol, DALDA-C6 = 710.69 g/mol and DALDA-C8 = 738.82 g/mol.

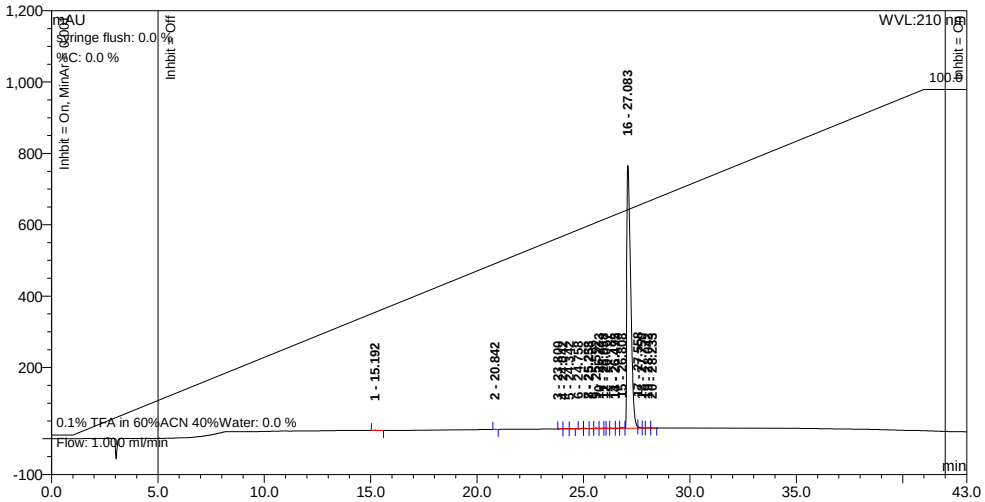

Fig. 3 HPLC chromatogram of modified DALDA-C8 peptide. HPLC method was used to determine the purity of DALDA-C8 and the purity was 98.85%.

bars, comparing the drug treated and untreated rats, the statistical significance was seen during the composite map analysis.

Formulation Safety Evaluations

Changes in Animal Body Weight

For the three groups, saline treated (control), DALDA-C8 solution or DALDA-C8 nanoemulsion, no difference in body weight was seen at day 1, 4 or 7 post-dosing. Mice in the three groups showed a steady gain in weight (Fig. 5). This shows that the modified peptide and nanoemulsion formulation are well tolerated in mice.

Changes in Liver Enzyme and Natriuretic Peptide Levels

There was no statistically significant ($p > 0.05$) elevation in liver enzyme levels observed in DALDA-C8 solution or nanoemulsion treated mice and the levels were comparable to control mice and reported range for normal enzyme level values in literature (29). AST and ALT levels for various time points and treatment groups is shown in Table III. This highlights that the modified peptide and the nanoemulsion formulation are well tolerated in mice. There was no statistically significant ($p > 0.05$) change in ANP or BNP levels in the treated mice, compared to untreated mice as determined by the enzyme immunoassay as shown in Table IV.

Table II Particle Size, Polydispersity Index (PDI) and Surface Charge of Placebo, Unmodified DALDA and Modified DALDA Nanoemulsion

Formulation	Hydrodynamic diameter of the oil droplet, nm (± S.D.)	PDI	Surface charge, mV (± S.D.)
Placebo NE	181 ± 3	0.30	−43.4 ± 0.4
DALDA	182 ± 2	0.30	−27.2 ± 1.8
DALDA-C4	175 ± 3	0.30	−28.9 ± 0.5
DALDA-C6	175 ± 4	0.20	−26.3 ± 0.7
DALDA-C8	177 ± 3	0.25	−40.6 ± 0.4

Tissue Histopathology Analysis

Excised liver, heart and kidney tissues were cut into 5 μm sections and stained with hematoxylin/eosin followed by histo-pathological characterization to investigate any tissue damage due to administration of DALDA-C8 solution or nanoemulsion in mice. Digital images of the tissue sections from four treatment groups: (1) DALDA-C8 solution- 20 min, (2) DALDA-C8 nanoemulsion- 20 min, (3) DALDA-C8 solution- 180 min, and (4) DALDA-C8 nanoemulsion- 180 min were captured and analyzed.

Liver Biopsy

As shown in Fig. 6a, there was moderate periacinar and diffuse hepatocellular vacuolation. Occasional multifocal aggregates of cells were present in portal areas consistent with extramedullary hematopoiesis (EMH). Mild extramedullary hematopoiesis and lipid vacuolation are considered as common incidental findings in the liver and are not thought to be related to the treatment. Hence, the

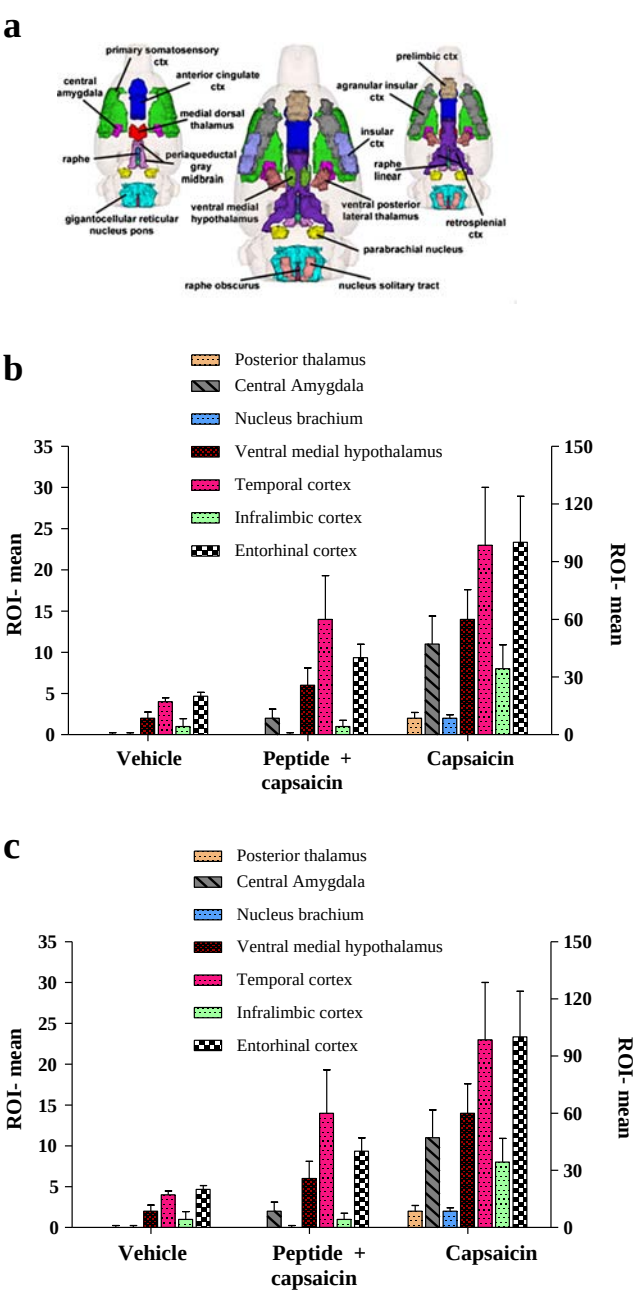

Fig. 4 (**a**) 3-D color representation of the 17 brain areas comprising the putative pain neural circuit of the rat (coronal view). (**b**) Changes in volume of activation (ROI) of 7 brain regions 30 min post- dosing. fMRI data collected for capsaicin shock given 30 min after DALDA-C8 nanoemulsion (30 min peptide-capsaicin data) intravenous dosing (3 mg/kg dose) showed a reduced volume of activation when compared to untreated (capsaicin alone). Vehicle corresponds to saline dosing only- no capsaicin or peptide dosed. $P < 0.05$, $n = 5$ rats. (**c**) Changes in volume of activation of 7 brain regions 90 min post-dosing. fMRI data collected for capsaicin shock given 90 min after DALDA-C8 nanoemulsion (90 min peptide-capsaicin data) intravenous dosing (3 mg/kg dose) showed a reduced volume of activation when compared to untreated (capsaicin alone). $P < 0.05$, $n = 5$ rats.

liver tissues histo-pathological findings for treated groups are consistent with what is regarded as within normal limits.

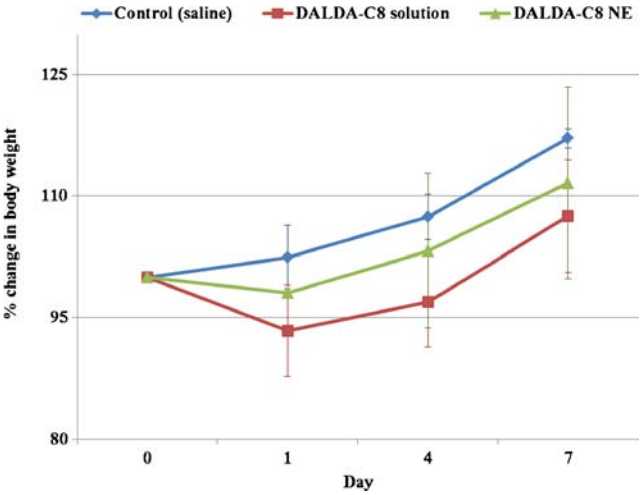

Fig. 5 Body weight change in mice. Saline-treated (control) or DALDA-C8 solution or DALDA-C8 nanoemulsion for up to 1 week.

Kidney Biopsy

No significant lesions were observed in kidney tissues for any of the treatment groups and the tissues could be classified as normal as shown in Fig. 6b.

Heart Biopsy

No significant lesions were observed in heart tissues for any of the treatment groups and the tissues could be classified as normal as shown in Fig. 6c.

DISCUSSION

Biotherapeutics are attractive owing to their potency, specificity and safety profile. However, their delivery to the CNS has been challenging due to presence of the blood brain barrier, blood-CSF barrier, presence of efflux transporters and the systemic dilution effect and clearance. Various invasive and non-invasive approaches have been explored preclinically as well as in clinic, to effectively deliver peptide

Table III AST and ALT Levels in Untreated (Control) and Treated Mice

Treatment group	AST level IU/L ± S.D.* (p value)	ALT level IU/L ± S.D.* (p value)
Untreated	46 ± 6	36 ± 13
DALDA-C8 solution, 20 min	78 ± 16 (0.06)	73 ± 14 (0.06)
DALDA-C8 nanoemulsion, 20 min	52 ± 5 (0.27)	56 ± 12 (0.18)
DALDA-C8 solution, 180 min	51 ± 10 (0.50)	28 ± 6 (0.06)
DALDA-C8 nanoemulsion, 180 min	71 ± 15 (0.06)	31 ± 7 (0.54)

*Mean ± S.D. ($n = 3$), p value (two-tailed) >0.05 indicates not statistically significant

Table IV ANP and BNP Levels in Untreated (Control) and Treated Mice

Treatment group	Concentration of ANP (pg/ml) ± S.D.* (*p* value)	Concentration of BNP (pg/ml) ± S.D.* (*p* value)
Untreated	7.5 ± 2.4	35.8 ± 3.5
DALDA-C8 solution, 20 min	3.7 ± 0.6 (0.18)	32.9 ± 4.1 (0.62)
DALDA-C8 nanoemulsion, 20 min	7.9 ± 0.5 (0.86)	34.7 ± 1.3 (0.81)
DALDA-C8 solution, 180 min	5.9 ± 2.6 (0.56)	26.2 ± 7.2 (0.28)
DALDA-C8 nanoemulsion, 180 min	2.9 ± 2.0 (0.14)	20.8 ± 8.4 (0.17)

*Mean ± S.D. ($n = 3$), p value (two-tailed) >0.05 indicates not statistically significant

analgesics to the CNS. Several examples of these peptide chemistries are highlighted by (30). While chronic pain therapy has been responsive to traditional opioids, it suffers from severe side effects like respiratory depression, constipation, hallucinations, and diuresis. Besides, the success rate for treating pain in clinic is only 30%. Nanoemulsions containing oil, that are rich in omega-3 polyunsaturated fatty acids (PUFA) can play a very important role in overcoming biological barriers, including the BBB (3). Hydrophobically modified peptide, when appropriately encapsulated in a poly (ethylene glycol) (PEG)-modified nanoemulsion along with an efflux modulator, can prove to be a clinically relevant therapy for chronic pain.

The oil-in-water nanoemulsion could be reproducibly made by ultrasonication method. In addition, the characterization of formulations demonstrated that the nanoemulsions were <200 nm in size, spherical and about −40 mV surface charge. More importantly, the morphology, size and surface charge remained unchanged for up to 6 months when stored at 4°C, thus signifying the stability of nanoemulsion formulations. Modifications applied to DALDA peptide helped to improve the drug loading and encapsulation efficiency of this peptide in the nanoemulsion formulation. The higher encapsulation efficiency for DALDA-C8 modified peptide compared to other analogues also translated to, very similar surface charge between the peptide encapsulated and placebo nanoemulsion, hence, indicating that the octanoic acid modification is optimal for the DALDA peptide for the nanoemulsion formulation. This provides an exciting new formulation approach combined with chemistry effort, to improve the delivery of soluble peptides using lipid-based drug delivery system.

Imaging in awake animals using BOLD fMRI has enabled the study of brain activities and analgesic effects upon painful stimulus. Utilizing capsaicin-induced persistent pain model coupled with imaging, we demonstrate that the DALDA-C8 chemical modification did not alter its analgesic effect. We hereby demonstrate this model to correlate to the findings in traditional hot water- tail withdrawal model (17) and provide additional details related to particular regions in the brain that respond to pain, which is not evident in traditional non-surgical pain models. The template used to define the brain areas comprising the pain neural circuit of the rat came primarily from the work of Gauriau and Bernard (31) and meta-analysis data from various neuroimaging modalities used to study acute pain in humans (28). Using the parabrachial nucleus as a central node Gauriau and Bernard described its many efferent connections to brainstem, hypothalamus and forebrain areas as the sites comprising the distributed neural circuit of pain (31). They and others have provided clear anatomical and electrophysiological evidence showing two major pathways of nociceptive fibers emanating

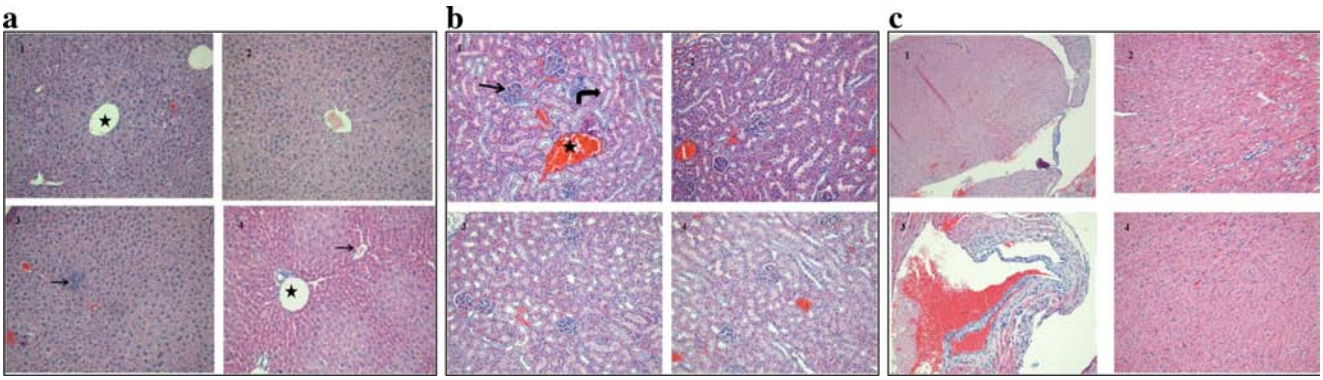

Fig. 6 (**a**) Histology of liver tissues. 1: DALDA-C8 NE, 20 min: Normal hepatic architecture with central vein (star) 2: DALDA-C8 NE, 180 min: Normal hepatic architecture with mild to moderate vacuolation 3: DALDA-C8 S, 20 min: Single focus of cells corresponding to extramedullary hematopoiesis (*arrow*). 4: DALDA-C8 S, 180 min: Liver with normal architecture, portal area (*star*) and periacinar area (*arrow*). (**b**) Histology of kidney tissues. 1: DALDA-C8 NE, 20 min: - Normal kidney with glomeruli (*arrow*), tubules (*bent-arrow*) and blood vessel (*star*) 2: DALDA-C8 NE, 180 min: - Normal kidney architecture 3: DALDA-C8 S, 20 min: - Normal kidney architecture 4: DALDA-C8 S, 180 min: - Normal kidney architecture (**c**) Histology of heart tissues. 1: DALDA-C8 NE, 20 min: - Normal heart with A-V valve (*arrow*) 2: DALDA-C8 NE, 180 min: - Normal heart architecture 3: DALDA-C8 S, 20 min: - Normal heart and aortic valve (*arrows*). 4: DALDA-C8 S, 180 min: - Normal well-organized myocardial cells of the heart. *S* solution, *NE* nanoemulsion.

from lamina 1 of the dorsal horn of the spinal cord. One pathway projects to the somatosensory cortex through the lateral thalamus (e.g. ventral posterior lateral thalamus) shown in our results and is involved in sensory discrimination of nociceptive processing. A second, linking the brainstem with hypothalamus (shown in our results), midline thalamic areas and limbic cortex (shown in our results) is thought to deal with the attentional and motivational aspects of pain. Similar lateral and medial pain systems attending to sensory discrimination and emotion, respectively, have been described in the human imaging literature (32). Nanoemulsion provides an alternate improved formulation to prolong the analgesia of the peptide, thereby providing an exciting platform technology for exploiting other peptides and analgesics which are often restricted to acute pain therapy and need more frequent dosing regimen. This successful demonstration of a functional MRI setting to study the pain neural circuit in awake animals can be translated to a clinical setting in humans thereby, providing an effective means to titrate the dosing regimen and provide a customized therapy option to patients.

No body weight change or liver enzyme elevation or change in ANP or BNP levels were observed in any of the treatment groups analyzed. Tissue histology did not show any abnormal findings in liver, heart or kidney in any of the treatment groups. These tests suggest that the modified DALDA-C8 peptide, when administered as solution or nanoemulsion is well tolerated in mice.

CONCLUSIONS

In the current study, a polyunsaturated fatty acid based nanoemulsion system effectively encapsulated the analgesic peptide and demonstrated efficacy in the capsaicin- pain induced functional magnetic resonance imaging model in rodents. The modified peptide and the formulation were also well tolerated as tested in mice.

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