

CNS Delivery and Pharmacokinetic Evaluations of DALDA Analgesic Peptide Analog Administered in Nano-Sized Oil-in-Water Emulsion Formulation

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Received: 16 August 2013 / Accepted: 10 November 2013
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ABSTRACT

Purpose Although neuro-active peptides are highly potent as central nervous system (CNS) therapeutics, their systemic delivery across the blood-brain barrier (BBB) is limited due to lack of permeability in the brain and rapid systemic metabolism. In this study, we aimed at enhancing the brain delivery and stability of chemically modified [D-Arg², Lys⁴]-dermorphin-(1–4)-amide]] (DALDA) peptide to achieve prolonged analgesic effects.

Methods The C8-DALDA peptide analog was encapsulated in an oil-in-water nanoemulsion formulation made specifically with oils rich in omega-3 rich polyunsaturated fatty acid (PUFA) to enhance CNS availability. The nanoemulsion formulation was administered systemically in CD-1 mice and qualitative and quantitative biodistribution was evaluated. We have also examined the effect of curcumin, which is known to down-regulate efflux transporters and inhibit systemic metabolism, on the pharmacokinetic properties of the peptide.

Results Qualitative and quantitative biodistribution and pharmacokinetic studies in mice clearly demonstrated improved plasma and brain exposure of modified DALDA when administered in nanoemulsion, thereby providing an exciting opportunity towards improved efficacy and/or lowered dose of the peptide. The various dosing regimens tested for modified DALDA solution and curcumin nanoemulsion directed towards a novel combination strategy for improved systemic delivery of peptides across the BBB.

Conclusions Encapsulation of the drug in PUFA nanoemulsion is an effective strategy for delivery of peptides. This work provides a novel combination strategy for improved delivery of peptides to the brain.

KEY WORDS biodistribution · CNS delivery · curcumin · DALDA · nanoemulsion · pharmacokinetics

INTRODUCTION

Biological molecules, like peptides and proteins, are highly potent as central nervous system (CNS) therapeutic agents based on their specificity to the target site and potential for disease-modifying effects (1). However, due to their hydrophilicity, ionization properties, and large molecular weight, peptides and proteins do not cross the blood-brain barrier (BBB) upon systemic administration. Additionally, these molecules are susceptible to rapid metabolism and clearance in the systemic circulation. While there are some invasive approaches, such as convection-enhanced delivery, can be used for systemic delivery of peptides and proteins across the BBB, the potential for serious CNS toxicity, especially upon chronic administration is a concern (2). As such, there is a significant medical need for safe and efficient delivery of bio-molecules, like opioid peptide analgesics across the BBB, for effective pain therapy.

DALDA ([D-Arg², Lys⁴]-dermorphin-(1–4)-amide), a potent and selective μ opioid receptor agonist, is an analogue of the N-terminal tetrapeptide segment of dermorphin (3). It is relatively metabolically stable: the placement of a D-amino acid at the 2-position makes the peptide resistant to aminopeptidases, and the carboxyl terminal amidation reduces susceptibility of the peptide to carboxypeptidase (4). DALDA is eliminated from plasma presumably by glomerular filtration. The metabolic stability of DALDA limits the systemic clearance of this peptide, and accordingly the plasma exposure is relatively high. Since DALDA slowly traverses the BBB via lipid-mediated free diffusion, the brain exposure is directly proportional to the plasma exposure. High plasma exposure for DALDA allows the brain delivery to reach a value of 0.02% injected dose/g in Sprague-Dawley rats, which was

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allowed to induce analgesia only after a high 5 mg/kg dose being administered intravenously (5).

In this study, we aimed at enhancing systemic stability and brain delivery of chemically modified DALDA, with the aim to achieve better and prolonged analgesic effect with decreased dose and dose-related side-effects. Two strategies were considered to reach this goal. First, an omega-3 rich polyunsaturated fatty acid (PUFA)-based nanoemulsion formulation was developed for effective encapsulation of this peptide. PUFA 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 (6). 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 (7). Once fatty acids reach the neurons, acetyl-CoA synthetase traps them by forming acetyl-CoA, which no longer can diffuse out of the cell. Another theory indicates that transport of fatty acids across the BBB is derived from fatty acid-albumin complexes, where the fatty acid undergoes translocation from extracellular to intracellular leaflet of the membrane via transporters (7). Also, a recent article describes the role of PUFA as effective absorption enhancers at the BBB where they cause membrane fluidification and impact the tight junctions thereby increasing the paracellular and transcellular transport (8).

Second, curcumin was incorporated in the nanoemulsion and tested *in vivo*, as there is evidence that curcumin has the ability to down-regulate drug efflux transporters such as P-glycoprotein (9). Using the nanoemulsion strategy, we carried out whole body imaging in anesthetized mice which were administered modified peptide tagged to a near infra-red (NIR) probe to study the qualitative biodistribution of the peptide. We also performed the quantitative biodistribution and pharmacokinetic studies of modified peptide when administered as a solution or nanoemulsion formulation with and without curcumin.

MATERIALS AND METHODS

Materials

Extra pure omega-3 rich fish oil and Pluronic F68[®] were kindly 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[®] and Curcumin were purchased from Sigma Chemicals, Inc. (St. Louis, MO). [D-Arg², Lys⁴]-dermorphin-(1–4)-amide (DALDA) and modified DALDA were purchased from Bachem, Inc. (Torrance, CA). Strata[®]-X-CW SPE columns

were purchased from Phenomenex Inc. (Torrance, CA). All other chemicals and solvents were purchased from Fisher Scientific (Fair Lawn, NJ). LC/MS/MS analyses were carried out at using API5000 AB Sciex system at PhenoLogix, division of Phenomenex Inc. (Torrance, CA). Imaging experiments were conducted using a Lumina II *In Vivo* Imaging System (IVIS) (Caliper Life Sciences, Hopkinton, MA).

Male CD-1[®] mice (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.

Synthesis of Lipid-Modified DALDA Analog

To improve the encapsulation of hydrophilic DALDA peptide in the oil phase of the nanoemulsion, the epsilon-amino group of the lysine residue was structurally modified with octanoic acid to increase its lipophilicity. Briefly, the side-chain fatty acid modified DALDA was prepared by 9-fluorenylmethyloxycarbonyl (Fmoc/tBu) solid phase peptide synthesis (SPPS) at Bachem Inc. (Torrance, CA). At the conclusion of chain assembly, epsilon-amino group of the lysine residue (Dde) group was deprotected, to allow for side chain acylation with octanoic acid. This was followed by global deprotection and cleavage from the resin, with trifluoroacetic acid (TFA). Crude products from the above chemistry were precipitated and purified by reversed-phase HPLC (RP-HPLC), and finally lyophilized. Several different fatty acids with varying carbon chain lengths were used for chemical modification and the C8 modified peptide showed high encapsulation efficiency in the nanoemulsion formulations. The peptide analog is referred as C8-DALDA in this paper.

For qualitative biodistribution study of C8-DALDA, NIR dye 800CW (Li-Cor, Lincoln, NE) was coupled via N-hydroxysuccinimide (NHS) ester chemistry to C8-DALDA conjugate at Bachem Inc. Briefly, the DALDA sequence was prepared by Fmoc/tBu SPPS as above, except for the 8-aminooctanoic acid residue was coupled to the lysine side chain. The product was then cleaved from the peptide resin with the N-terminal amine protected. IRDye800-NHS was coupled to the terminal amine of the 8-aminooctanoic acid residue under standard neutral pH buffered conditions. The N-terminal amine protecting group was then removed. The product, IRDye800-DALDA-C8, was purified by RP-HPLC and lyophilized.

Preparation and Characterization of Multifunctional Nanoemulsion Formulations

Preparation of the Nanoemulsion Formulations

Oil-in-water nanoemulsion was prepared by the sonication method as described previously (10). Briefly, pre-warmed oil

phase (1 ml) consisting of fish oil and the peptide alone (or with curcumin) was gradually added to the pre-warmed water phase (4 ml) containing egg phosphatidylcholine (Lipoid E80[®]) (120 mg), polysorbate 80 (Tween80[®]) (0.05 ml), DSPE-PEG2000 (1, 2-distearoyl-*Sn*-glycero-3-phosphoethanolamine-*N*-[amino (polyethylene glycol)-2000]) (15 mg). The resultant mixture was stirred for 2 min using a silverson homogenizer (Model: L4RT-A, Silverson Machines, MA) at 6,000 rpm and ultrasonicated for 10 min using the Vibra Cell VC 505 probe sonicator (Sonics and Material Inc., Newtown, CT) at 22% amplitude and 50% duty cycle.

Characterization of the Nanoemulsion Formulations

The nanoemulsions were characterized for particle size and surface charge using the Brookhaven Instrument's 90Plus ZetaPALS particle size analyzer (Holtsville, NY), at a 90° fixed angle, and at 25°C up on greater than 10,000-fold dilution with deionized distilled water. The morphology of oil droplets in the nanoemulsion formulations was visualized with transmission electron microscopy (TEM). Nanoemulsion sample was placed on formvar-coated copper grids (EM Sciences, Hatfield, PA), and negatively-stained for 10 min with 50 µL of 1.5% phosphotungstic dye at room temperature. Excess liquid was drained off with a filter paper, and the grid containing dry film of nanoemulsion sample was observed with a JEOL 100-X transmission electron microscope (Peabody, MA). The formulation was analyzed for extent of drug loading (i.e., amount of peptide incorporated in the nanoemulsion) and encapsulation efficiency (i.e. amount of peptide present in the internal or oil phase of the nanoemulsion) by using HPLC. For drug loading, the nanoemulsion was sufficiently diluted with organic (ethanol: acetonitrile), and a 50 µL aliquot was injected into the HPLC. For encapsulation efficiency, ultra-filtration method was used. The nanoemulsion sample (0.5 mL) was placed in the upper donor chamber of a centrifugal filter device (molecular weight cut-off 3,000 Daltons; Millipore, Bedford, MA), and the unit was centrifuged at 10,000 rpm for 30 min at 4°C. The sample, along with encapsulated drug, remains in the donor chamber and the aqueous phase moves through the filter into the sample recovery chamber. The concentration of the peptide in the aqueous phase was estimated with use of HPLC and the encapsulation efficiency was calculated by mass balance and the drug loading measurements.

Quantitation of C8-DALDA Peptide in Plasma and Tissue Samples

Sample Preparation

Owing to the complexity of *in vivo* sample matrix and possibility of interference of plasma proteins with analytical

techniques, sample preparation was performed using solid phase extraction (SPE). This is a well-known technique which is based on the principle of selective partitioning of analyte between a solid (sorbent) phase and a liquid (solvent) phase (11, 12). SPE for C8-DALDA was developed based on the ion exchange principle. Due to the presence of predominantly basic groups on the peptide, cationic polymer-based columns, Strata®-X-CW, with affinity for basic groups were used for purification and extraction of peptide from *in vivo* sample matrix. Table I describes the SPE process utilized. C8-DALDA from *in vivo* samples was extracted using methanol. Briefly, 75 µl of plasma was mixed with 225 µl of methanol and centrifuged at 4,500 rpm for 20 min. Frozen tissue samples, including brain, were weighed and an equal amount of saline was added and the tissue homogenized at 6,000 rpm for 2 min. An excess of 4-fold by weight of methanol was added to the tissue homogenate and following vortexing, it was centrifuged at 4,500 rpm for 20 min. The clear supernatant was collected and loaded to the SPE columns. The % recovery of C8-DALDA after SPE was also determined by spiking plasma and tissue samples with known amounts of peptide and subjecting the spiked samples to the same procedure of SPE as the *in vivo* samples.

LC/MS/MS Analysis

LC/MS/MS is a widely recognized technique for sensitive and accurate detection of nanogram levels of peptide analytes biological matrix. API5000 AB Sciex system at PhenoLogix was used for quantification of the SPE elute of the *in vivo* samples. Described in Table II is the LC/MS/MS method and conditions used for assaying the samples. A standard curve was obtained on LC/MS/MS using this method by running known amounts of C8-DALDA (solution in 0.1% formic acid in methanol) spiked in blank plasma sample matrix and eluted through SPE. Appropriate QC solutions with known

Table I Solid Phase Extraction for C8-DALDA

	Description
Sorbent (column)	Strata®-X-CW- cationic (33 µm polymeric weak cation, 30 mg/1 mL)
Condition step	Methanol -1 ml
Equilibration step	Water-1 ml
Sample load step	Methanol extract of tissue or plasma sample-300 µl
Wash 1	Water-1 ml
Wash 2	Methanol-1 ml
Elute	5% formic acid in methanol (freshly prepared) (2 × 150 µl)

Table II LC/MS/MS Method for C8-DALDA Analysis

Condition	Description	
Mobile phase	A: 0.1% formic acid in water B: 0.1% formic acid in methanol	
Column	1) Kinetix, C18, 2.6 μm , 2 \times 50 mm, 100 Å pore size 2) Mixing column, 5 μm , Silica, 30 \times 3 mm	
Flow rate	0.5 ml/min	
Injection volume	10 μl	
Detector	API5000	
Temperature	Ambient	
Pressure	312 bars, 5,500 psi	
Gradient run		
Time (min)	A (%)	B (%)
0.00	80	20
3.00	10	90
4.50	10	90
4.51	80	20
6.00	80	20
Mass transitions	Q1, Da	Q3, Da
	369.9	484.5
	369.9	612.4

amounts of C8-DALDA spiked plasma or tissue samples were run with every sample set.

Qualitative Biodistribution Studies with NIR-Conjugated C8-DALDA Peptide

Male CD-1[®] mice were dosed intravenously with IRDye800-C8-DALDA solution or nanoemulsion via tail-vein injection at a dose of 30 nmol equivalent of dye. Animals were imaged under anesthesia at pre-determined time points of 15, 60 and 180 min in the Caliper IVIS with high lamp power, excitation filter centered at 745 nm, emission filter at 820 nm, and a 1 s exposure. Image analysis was performed using Living Image[®] 4 software (Caliper Life Sciences).

In Vivo Biodistribution and Pharmacokinetic Analyses of C8-DALDA Peptide

C8-DALDA was dosed intravenously as solution or nanoemulsion formulation to male CD-1[®] mice, via tail vein injection at 3 mg/kg dose (i.e., 75 μg for 25 g weighing mice). Blood was collected in EDTA-treated tubes at various time intervals: 10, 20, 30, 60, 120 and 180 min post dosing and kept on ice. The samples were centrifuged at 10,000 rpm for 20 min at 4°C to separate plasma. The plasma was stored at -20°C or used immediately for analyses. For biodistribution study, the blood was completely withdrawn by cardiac puncture. Mice were sacrificed via cervical dislocation and the whole brain, liver, lungs, kidneys, spleen and heart were

collected, weighed, and frozen at -80°C until the time of analyses.

Effects of Curcumin on In Vivo Biodistribution and Pharmacokinetics

To compare and contrast the *in vivo* pharmacokinetics of C8-DALDA solution and nanoemulsion when administered with curcumin, different dosing options (co-dosing or pre-dosing) for curcumin were evaluated. Curcumin nanoemulsion at 2 mg/mL drug loading was prepared by ultrasonication, using the procedure described above. Curcumin nanoemulsion was administered intravenously to male CD-1[®] mice at 10 mg/kg dose 24 h or 3 h prior to C8-DALDA injection, or simultaneously. C8-DALDA was injected in solution or nanoemulsion at 3 mg/kg, except when curcumin nanoemulsion was co-administered with C8-DALDA nanoemulsion. In that case, the C8-DALDA nanoemulsion dose had to be reduced to half (1.5 mg/kg dose) due to viscosity and volume restriction. For the 3 h and 24 h curcumin pre-treatment groups and C8-DALDA solution dosing, blood was collected in EDTA-treated tubes at 10, 20 and 60 min and brain was collected as mentioned in the previous section. All samples were stored at -80°C until the time of analyses.

Analgesic Efficacy Evaluations in 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. The animal's tail tip (1 inch) 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.

Data Analysis

Pharmacokinetic parameters were calculated taking into account individual data using Phoenix[®] WinNonlin[®] v. 1.3 software (Pharsight, Cary, NC). A non-compartmental analysis (NCA) was performed for plasma and brain, using an intravenous bolus administration model for sparse data. The intravenous bolus model was chosen for brain based on the shape of the concentrations versus time curve, which suggested that distribution to brain could be assumed to be

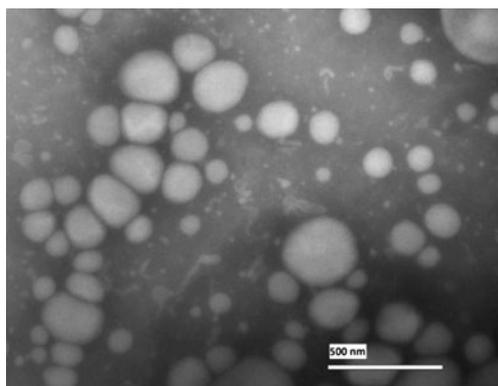


Fig. 1 Transmission electron microscopy (TEM) of the nanoemulsion: The oil droplets of the nanoemulsion sample were spherical and their size was in the range of 180–220 nm. Scale bar is 500 nm.

instantaneous. The slope of the terminal log-linear part of the concentration versus time curve (λ_z) was calculated using the best-fit method, with uniform weighting. Graph Pad Prism[®] software (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. The two-tailed unpaired homoscedastic t-test was used to compare mean values \pm standard errors obtained for the solution and the nanoemulsion: $p < 0.05$ was considered statistically significant.

RESULTS

Lipid Modification of DALDA for Encapsulation in Nanoemulsions

C8-DALDA and IRDye800-C8-DALDA analogues could be successfully prepared by 9-fluorenylmethyloxycarbonyl

(Fmoc/tBu) solid phase peptide synthesis and purified by RP-HPLC. The purity was greater than 90% by RP-HPLC and the identity was confirmed by MALDI mass spectrometry.

Preparation and Characterization of Multifunctional Nanoemulsion Formulations

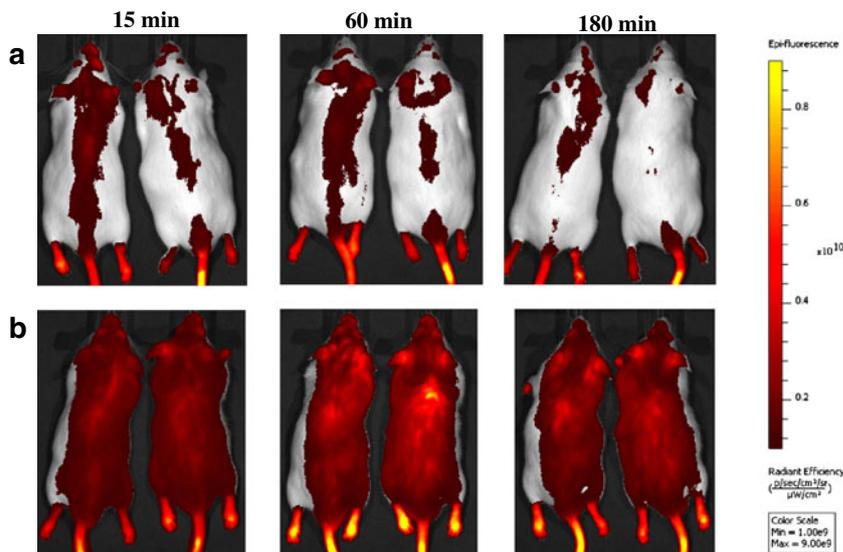
Ultrasonication led to uniform nanoemulsion formulation. Particle size and polydispersity index (PDI) were determined for peptide loaded nanoemulsion formulation. The formulation size was 177 ± 3 nm and PDI was 0.25. The charge was -40.6 ± 0.4 mV. TEM of nanoemulsion formulation confirmed its spherical shape and the size range of 180–220 nm as shown in Fig. 1.

C8-DALDA had a drug loading of 0.7 mg/ml. C8-DALDA achieved encapsulation efficiency of 87% (versus unmodified DALDA which had less than 10% encapsulation), suggesting that C8 chain length modification applied to the DALDA peptide provided sufficient solubility in the internal oil phase.

Quantitation of C8-DALDA Peptide in Plasma and Tissue Samples

For solid phase extraction, the working hypothesis was that C8-DALDA peptide will form relatively strong ionic bonds with the carboxylic acid moieties on the SPE sorbent under neutral pH conditions, allowing the use of a strong wash with organic solvent to eliminate hydrophobic matrix components. An initial wash with water removes polar matrix components and salts. Adding 5% formic acid to the organic elution solvent (methanol) neutralizes the carboxylic acid groups on the SPE sorbent, allowing the plasma C8-DALDA to elute. A

Fig. 2 Qualitative biodistribution of near infra-red labeled C8-DALDA as solution and nanoemulsion following intravenous dosing at 30 nmol equivalent of dye in CD-1 male mice under anesthesia, $n = 2$. **(a)** *In vivo* image of mice at 15, 60 and 180 min post-administration of solution. **(b)** *In vivo* image of mice at 15, 60 and 180 min post-administration of nanoemulsion formulation. The bar to the right is the epi-fluorescence scale with a range of 0.1×10^{10} – 0.9×10^{10} radiant frequency.



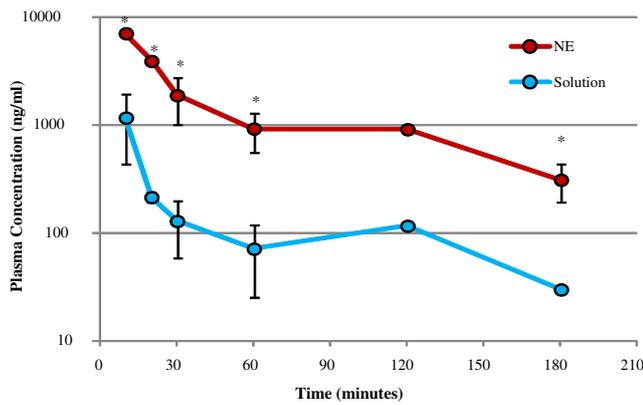


Fig. 3 Plasma concentration (ng/ml) versus time (minutes) for C8-DALDA solution and nanoemulsion (NE) following intravenous dosing at 3 mg/kg to CD-1 male mice. $n = 3$ for 10, 20, 30, 60 and 180 min, $n = 1$. NE = nanoemulsion; S = solution (* $p < 0.05$, two-tailed unpaired homoscedastic t-test).

recovery of >98% was obtained using this method for the *in vivo* plasma and tissue samples. The LC/MS/MS method gave a standard curve with $R^2 = 0.9977$ and concentration range of 1–2,000 ng/ml.

Qualitative Biodistribution Studies with NIR-Conjugated C8-DALDA Peptide

The IRDye800-C8-DALDA nanoemulsion induced a massive and diffuse signal, which persisted over 180 min, as shown in Fig. 2. Contrarily, the solution formulation induced a low signal, which was concentrated on the backbone axis, and almost disappeared in 180 min.

Quantitative Pharmacokinetics and Biodistribution of C8-DALDA Peptide in Solution and Nanoemulsion

Figure 3 displays the plasma concentration (C_p) - time data for C8-DALDA solution and nanoemulsion on a log-linear scale, after intravenous administration at a 3 mg/kg dose. A biphasic decrease was observed for both formulations. Pharmacokinetic parameters calculated using Phoenix[®] WinNonlin[®] NCA are shown in Table III. Nanoemulsion induced

Table III Pharmacokinetic Parameters in Plasma Following Intravenous Administration of 3 mg/kg C8-DALDA Solution or Nanoemulsion in Mice

PK parameter	Solution dosing*	Nanoemulsion dosing*	Units
AUC _{0-∞}	62,803	347,244	ng.min/ml
Cl	1.194	0.216	ml/min
V _{ss}	45.2	13.4	ml
λ_z	0.00915	0.0104	l/min
$t_{1/2} \lambda_z$	75.7	66.4	min
MRT	37.8	62.1	min

* $n = 3$, two-tailed unpaired homoscedastic t-test, $p < 0.05$

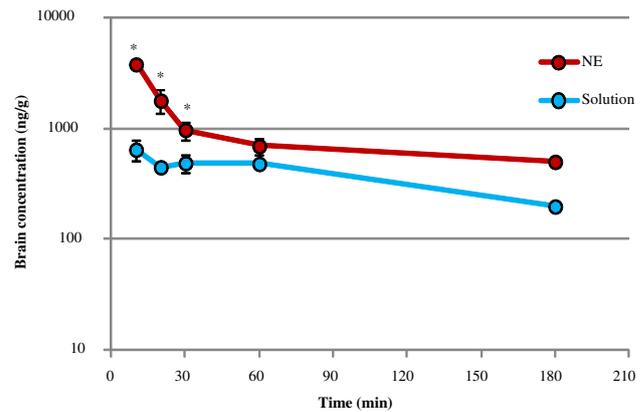


Fig. 4 Brain concentration (ng/g) versus time (minutes) for C8-DALDA solution and nanoemulsion (NE) following intravenous dosing at 3 mg/kg to CD-1 male mice. $n = 3$ for 10, 20, 30, 60 min. NE = nanoemulsion; S = solution (* $p < 0.05$, two-tailed unpaired homoscedastic t-test).

significantly higher peptide levels in plasma when compared to the solution formulation, leading to an increased exposure, with a 5.5 fold higher AUC_{0-∞}. This increased exposure was associated to an accordingly 5.5 fold decreased clearance. Steady-state volume of distribution (V_{ss}) was also decreased (3.3 fold) by nanoemulsion formulation, while the slope of the log-linear terminal part of the curve (λ_z) and corresponding half-lives ($t_{1/2}$) were similar for both formulations, consistently with the parallel decrease observed in Fig. 3. MRT was 1.6 fold higher for nanoemulsion, presumably due to a lower distribution rate in the first phase, as can be seen in Fig. 3. This distribution rate could not be calculated because there were not enough data to perform a bi-compartmental analysis with sparse data.

Brain concentration-time data (Fig. 4) shows a similar trend. A biphasic decay was observed in brain, and nanoemulsion allowed significantly higher brain concentration of peptide when compared to the solution formulation, leading to an increased exposure, with a 3.3 fold higher AUC_{0-∞} (Table IV). MRT was also higher in brain for nanoemulsion, suggesting that the nanoemulsion would allow a longer residence of the peptide in the brain. Accordingly, λ_z was lower in brain for nanoemulsion.

Tables V and VI and Fig. 5 shows the biodistribution of C8-DALDA in highly perfused tissues (i.e., heart, lungs,

Table IV Pharmacokinetic Parameters in Brain Following Intravenous Administration of 3 mg/kg C8-DALDA Solution or Nanoemulsion in Mice

PK parameter	Solution dosing*	Nanoemulsion dosing*	Units
AUC _{0-∞}	104,634	325,851	ng.min/g
MRT	145.4	198.4	min
λ_z	0.00640	0.00396	l/min
$t_{1/2} \lambda_z$	108.3	174.9	min

* $n = 3$, two-tailed unpaired homoscedastic t-test, $p < 0.05$

Table V Biodistribution of DALDA-C8 Solution or Nanoemulsion in Tissues at 20 min Following Intravenous Dosing at 3 mg/kg

Tissue	Concentration from solution (ng/g) ± S.D.*	Concentration from Nanoemulsion (ng/g) ± S.D.*	p value
Brain	449 ± 31	1,804 ± 434	0.006
Heart	567 ± 30	7,144 ± 865	0.0002
Spleen	590 ± 70	1,804 ± 279	0.002
Lungs	1,166 ± 230	4,002 ± 1,080	0.01
Liver	3,517 ± 568	1,754 ± 423	0.012
Kidney	633 ± 107	1,148 ± 84	0.003

*n = 3, two-tailed unpaired homoscedastic t-test, p < 0.05

spleen, kidneys and liver) at 20 min (Fig. 5a) and 180 min (Fig. 5b) following dosing of solution or nanoemulsion formulation. At 20 min, C8-DALDA concentrations are significantly twice lower in liver after nanoemulsion administration when compared to solution, but significantly higher in all other organs. The most striking difference is observed in heart, where concentration is 12.6 fold higher after nanoemulsion administration, reaching a level even higher than in plasma, while for other kidney, lungs and spleen, nanoemulsion induces a more modest 1.81 to 3.43 fold increase of concentration. At 180 min, concentrations have decreased in liver and lungs, but increased in spleen for both formulations. In heart, concentrations have increased for the solution and decreased for the nanoemulsion, leading to an only 3.54 fold higher concentration for nanoemulsion. In kidney, concentrations appear stable in time for both formulations. Comparing nanoemulsion to solution at 180 min, all organs display 1.70 to 4.11 fold higher concentrations after nanoemulsion administration.

Quantitative Pharmacokinetics of C8-DALDA Peptide in Solution and Nanoemulsion in the Presence of Curcumin

Figure 6 shows the plasma and brain concentrations for C8-DALDA after solution and nanoemulsion administration

Table VI Biodistribution of DALDA-C8 Solution or Nanoemulsion in Tissues at 180 min Following Intravenous Dosing at 3 mg/kg

Tissue	Concentration from solution (ng/g) ± S.D.*	Concentration from nanoemulsion (ng/g) ± S.D.*	p value
Brain	199 ± 8	502 ± 16	0.001
Heart	675 ± 72	2,387 ± 587	0.007
Spleen	1,309 ± 385	5,375 ± 960	0.002
Lungs	683 ± 90	1,721 ± 591	0.04
Liver	563 ± 107	955 ± 115	0.012
Kidney	631 ± 258	1,297 ± 370	0.06

*n = 3, two-tailed unpaired homoscedastic t-test, p < 0.05

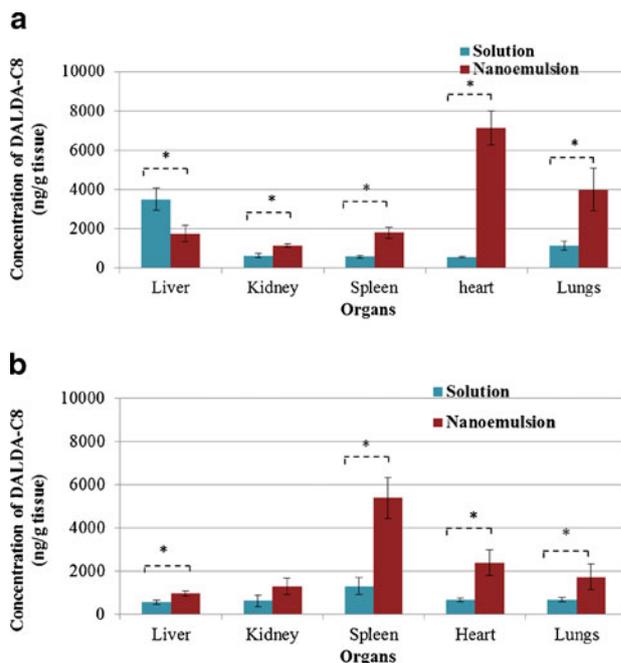


Fig. 5 C8-DALDA concentration in various organs for solution and nanoemulsion (NE) following intravenous dosing at 3 mg/kg to CD-1 male mice (*p < 0.05, two-tailed unpaired homoscedastic t-test). (a) Biodistribution at 20 min post-dosing, n = 3. (b) Biodistribution at 180 min post-dosing, n = 3.

when curcumin was added following various schedules. For solution formulation of C8-DALDA peptide, addition of

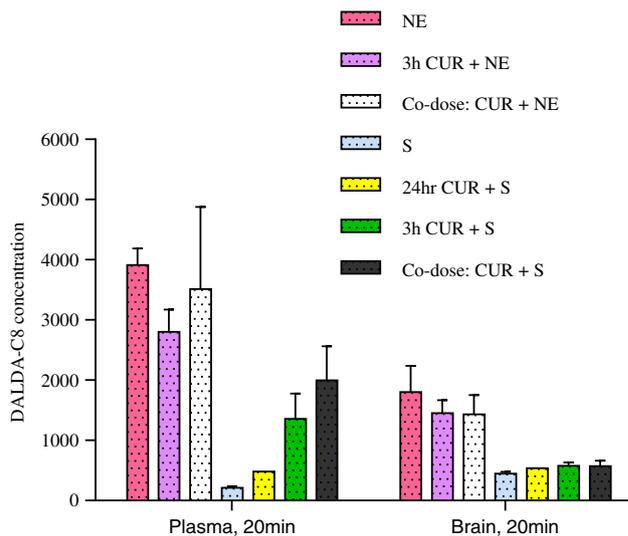


Fig. 6 Mean plasma concentration (ng/ml) or brain concentration (ng/g) at 20 min after intravenous injection of C8-DALDA solution (S) or nanoemulsion (NE) dosing at 3 mg/kg (except for co-dose (corrected arm) of 1.5 mg/kg) with or without curcumin (CUR) nanoemulsion administered intravenously at 10 mg/kg. Nanoemulsion formulation for C8-DALDA dosed alone (NE) or dosed after 3-hour of CUR NE dosing (3 h CUR + NE) or upon co-dosing with CUR NE (Co-dose: CUR + NE) shows higher plasma and brain concentration when compared to solution formulation of C8-DALDA(S) dosed alone or after 3-hour of CUR NE dosing (3 h CUR + S) or upon co-dosing with CUR NE (Co-dose: CUR + S). **p < 0.05, two-tailed unpaired homoscedastic t-test, n = 3.

curcumin was able to induce an increase in plasma concentration at 20 min for all schedules tested, the co-dosing option being the most efficient with a 9.30-fold increase ($p=0.005$). However, this increase in plasma concentrations did not translate into an increase of brain concentration. For nanoemulsion formulation, addition of curcumin led to a slightly lowered (0.7-fold) plasma concentration of C8-DALDA peptide upon 3 h curcumin pre-treatment, but no significant difference when co-dosing curcumin, when compared to nanoemulsion alone at 20 min. For co-dosing of curcumin with C8-DALDA nanoemulsion, a half-dose has been given (see methods) and the concentrations have been dose-normalized.

Analgesic Efficacy Evaluation Using Hot-Water Tail Withdrawal Assay

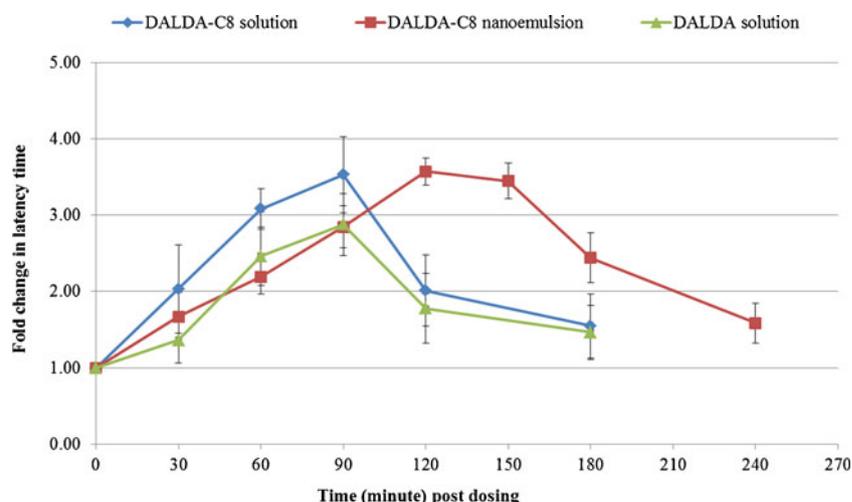
Unmodified DALDA solution, DALDA-C8 solution and DALDA-C8 nanoemulsion, dosed at 3 mg/kg, via tail vein injection produced analgesia, as shown in Fig. 7. The latency time increased 2–3.5-fold over baseline at 60–120 min post-dose for DALDA and DALDA-C8 solution. The response returned to baseline for solution formulations after 120 min. 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.

DISCUSSION

Qualitative and quantitative data consistently showed that nanoemulsion induced an increase in C8-DALDA levels

throughout the body, maintaining high levels 180 min after administration, when compared to solution. A biphasic decay profile was observed in plasma after C8-DALDA solution administration, in line with results obtained with DALDA in Sprague-Dawley rats by Samii *et al.* (5), with some minor differences in the values of PK parameters, likely due to difference in animal species (mouse versus rat) and modification of the lysine residue. Brain delivery of C8-DALDA in solution was comprised between 648 ng/g (or 0.864%ID/g) at 10 min and 199 ng/g (or 0.265%ID/g) at 180 min, which meant that brain delivery was higher than reported by Samii *et al.* for DALDA in rat (0.019%ID/g at 30 min). A biphasic decay profile was also observed in plasma and brain with nanoemulsion of DALDA-C8. The two phases could not be characterized due to the sparseness of the data, which allowed only for non-compartmental analysis. Nanoemulsion formulation of C8-DALDA induced a higher brain exposure than solution, with values between 3,831 ng/g (ie 5.11%ID/g) at 10 min and 502 ng/g (ie 0.669%ID/g) at 180 min post-administration. This level of brain exposure constitutes a major improvement not only over the solution formulation of DALDA-C8, but also over DALDA peptide (5) and even DALDA-[Dmt1] peptide, which display similar pharmacokinetic properties as DALDA (13) and does not allow for high brain delivery in male CD-1 mice (14). Nanoemulsion seems to increase brain exposure through an increase in plasma level, in line with the observation by Samii *et al.*, that the brain delivery is directly proportional to the plasma exposure. Since DALDA clearance is supposed to be mainly due to glomerular filtration (5), nanoemulsion might protect C8-DALDA from kidney elimination. With particle size of ~200 nm, nanoemulsion entrapping C8-DALDA is indeed too big to be filtrated by the glomerule (15), and will likely be eliminated by other routes, such as reticulo-endothelial system. For both solution and nanoemulsion formulations, exposure in brain (as quantitated by AUC) was similar to exposure in plasma,

Fig. 7 Hot-water tail withdrawal analgesia assay. Fold change in latency time for CD-1® male mice dosed with DALDA solution (green curve) or DALDA-C8 solution (blue curve) or DALDA-C8 nanoemulsion (red curve) at 3 mg/kg dose. The latency time does not change for DALDA-C8 when compared to unmodified DALDA peptide in solution. The DALDA-C8 nanoemulsion showed an extended duration of analgesic action as depicted by the prolonged latency time compared to solution, $n = 6$ mice.



suggesting a free diffusion process, which is in line with published results for DALDA (5). Interestingly, concentrations in brain got higher at later time points when compared to plasma, causing a higher MRT in brain than in plasma. This can be due to the affinity of the peptide to the target receptors, leading to a slight accumulation in the brain.

Curcumin had no effect on peptide kinetics, when peptide analog was administered as nanoemulsion. When C8-DALDA was administered as solution, curcumin enhanced plasma concentrations, but had no significant effect on brain concentration, despite down-regulation of BCRP, MDR1 and MRP1, and enhanced permeability and reduced efflux ratio, as demonstrated in Caco-2 cells (*in vitro* studies, data not shown here). This result suggests that the effect of curcumin on peptide pharmacokinetics might be associated to a decreased clearance of the peptide, without any effect on BBB permeability. The low brain exposure of the peptide might accordingly not be due to efflux associated to BCRP, MDR1 or MRP. Since DALDA is eliminated mainly by glomerular filtration, the increase of DALDA plasma concentration induced by curcumin should be associated to an effect of curcumin on glomerular filtration. To our knowledge, the effect of curcumin on glomerular filtration in physiological conditions has never been studied. Another hypothesis would be that, though considered metabolically stable, C8-DALDA could still be metabolized to some extent. Curcumin could decrease peptide degradation rate by reducing proteolysis, resulting in lower clearance of the peptide. Although never assessed in a pharmacokinetics context, a few studies suggest that curcumin could display such antiproteolytic activity. Siddiqui *et al.* (16), showed that curcumin had a dose-dependent effect on protein degradation as assayed by tyrosine release during serum starvation. Proteasome activity was inhibited in HeLa cells (17) and less increased in the muscle tissues of tumour-bearing mice (18) when treated with curcumin. Interestingly, in a transcriptomic study of nuclear factor E2-related factor 2 (Nrf2)-dependent genes, curcumin decreased expression of peptidases, such as kallikrein 26 and carboxypeptidase A4 (19). The effect of curcumin on drug glomerular filtration and on protein drugs proteolysis deserves further investigation.

CONCLUSIONS

The pharmacokinetics and biodistribution studies clearly demonstrated the improved plasma and brain exposure of C8-DALDA peptide from omega-3 rich fish oil-based nanoemulsion formulation, thereby providing an exciting opportunity towards improved efficacy and/or lowered dose of peptide. The various dosing regimen options tested for C8-DALDA solution and curcumin nanoemulsion formulations directly recommending the co-dosing scenario based on the

plasma and brain concentrations observed for C8-DALDA at the 20 min time point. This provides a novel combination strategy for improved delivery of such peptide drugs from solution. The precedence of much higher brain exposures from C8-DALDA nanoemulsion formulation as compared to any of the curcumin-based options and the negligible impact of curcumin on this nanoemulsion formulation, shows that encapsulation of the drug in polyunsaturated fatty acid (PUFA) based nanoemulsions should be retained as an effective delivery strategy for the DALDA peptide.

ACKNOWLEDGMENTS AND DISCLOSURES

This study was partially supported by a grant (R21-NS066984) from the National Institute of Neurological Disorders and Stroke of the National Institutes of Health. We would like to thank Dr. Kevin Cash and Dr. Heather Clark for their assistance with the IVIS imaging studies. We appreciate the help from Seyed Sadjadi and Jenny Wei at Phenomenex with LC/MS/MS analyses. We also thank Ms. Purva Pandya for her assistance with the *in vivo* studies.

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