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Biodistribution and Pharmacokinetic Evaluations of a Novel Taxoid DHA-SBT-1214 in an Oil-in-Water Nanoemulsion Formulation in Naïve and Tumor-Bearing Mice

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Abstract

Purpose—The main purpose of this study was to formulate an oil-in-water nanoemulsion of a next generation taxoid DHA-SBT-1214 and evaluate its biodistribution and pharmacokinetics.

Methods—DHA-SBT-1214 was encapsulated in a fish oil containing nanoemulsion using a high pressure homogenization method. Following morphological characterization of the nanoemulsions, qualitative and quantitative biodistribution was evaluated in naïve and cancer stem cell-enriched PPT-2 human prostate tumor bearing mice.

Results—DHA-SBT-1214 was successfully encapsulated up to 20 mg/ml in the nanoemulsion formulation and had an average oil droplet size of 200 nm. Using a DiR near infra-red dye encapsulated nanoemulsion, we have shown the delivery of nanoemulsion to mouse tumor region. By quantitative analysis, DHA-SBT-1214 encapsulated nanoemulsion demonstrated improved pharmacokinetic properties in plasma and different tissues as compared to its solution form. Furthermore, the nanoemulsions were stable and had slower *in vitro* drug release compared to its solution form.

Conclusions—The results from this study demonstrated effective encapsulation of the drug in a nanoemulsion and this nanoemulsion showed sustained plasma levels and enhanced tumor delivery relative to the solution form.

Keywords

biodistribution and pharmacokinetic; nanoemulsion formulation; prostate tumor; taxoid

INTRODUCTION

In the United States, cancer remains one of the leading cause of morbidity and mortality despite many advances in the molecular understanding of this disease (1). Prostate cancer (PrC) is one of the main causes of mortality in men in United States (2). One of the primary reasons for failure of therapy in PrC is that almost 70% of patients have recurrence after the initial treatment by surgery, radiation, and chemotherapy. Upon recurrence, the tumor becomes much more aggressive, metastatic, and resistant to chemotherapy that leads to poor prognosis (3).

Recently, it has been reported that recurrence of PrC and other tumors might be due to the presence of small residual population of cells termed cancer stem cells (CSCs) or tumor-initiating cells (TICs) (4–6). These CSCs have the potential to self-renew and initiate tumor. Moreover, these cells can produce an entire spectrum of differentiated progeny, which not only form the tumor mass, but are also able to cause metastasis and acquire resistance to current therapies (4–6). One of the flaws of current therapeutic drugs is that they only kill rapidly proliferating prostate cancer cells and do not target quiescent CSCs (7,8). This inefficiency of current therapies explains the lower success rate of anticancer drug development for PrC and other solid tumors (9,10). Along with the presence of CSCs that are resistant to current therapies, traditional models of preclinical evaluation of anticancer agents are also limited in their ability to predict clinical response. Indeed, these preclinical evaluations are traditionally done in unselected high-passage commercial cancer cell lines which over the period of time accumulate additional genomic and epigenomic changes and become a subpopulation of dominant cell. As a result, these cancer cell lines have little or poor correlation with the original clinical samples and hence their use for the study of genomic alterations, discovery of clinically relevant molecular targets, and anticancer drug development is compromised (11). All of the above issues, place a great emphasis on studying CSC-targeted therapies in physiologically and clinically more relevant *in vitro* and *in vivo* models to recapitulate the human disease. Therefore, in this study we have used a patient-derived ultra-low passage PrC cell line (PPT2 cell line), which has retained the features of immature and stem-like cells (12).

The current standard of care in PrC includes surgery, radiation and chemotherapy (7,8). In advanced stage PrC, especially when the tumor has metastasized to other parts of the body, chemotherapy with taxanes, such as paclitaxel and docetaxel, is considered to be critical for treatment. However, these taxanes lack tumor specificity and are highly prone to development of multi-drug resistance (MDR) (13). To address these chemotherapy challenges, our medicinal chemistry laboratory has developed a series of highly potent next-generation taxanes (toxoids) (13–16). Several of these novel taxoids exhibited better potency than those of paclitaxel and docetaxel against drug-resistant cell lines expressing MDR phenotypes (13–16). In particular, one of the new-generation taxoids, SBT-1214, showed

excellent efficacy against a highly drug-resistant (Pgp+) colon tumor xenograft in SCID mice (16). In another study, SBT-1214 totally suppressed the tumor recurrence (17). These results facilitated our decision to use this taxoid for current study. To further improve the tumor specificity and decrease systemic toxicity, we have conjugated SBT-1214 with docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid (18,19). DHA-conjugation to paclitaxel has been shown to reduce toxicity and to increase drug retention in the tumor and DHA-paclitaxel conjugate Taxoprexin® have shown improved efficacy in Phase II clinical trials against prostate, breast, gastric, NSCL cancers and metastatic melanoma (20–22). Previously, we have studied the efficacy of DHA-SBT-1214 in colon and ovarian cancer in mouse models and it has proven more effective than other drugs studied (23).

To further improve the efficacy and targeted delivery of the drug to the tumor mass, we have formulated an omega-3 rich fish oil containing oil-in-water nanoemulsion formulation of DHA-SBT-1214. Nanoemulsions are heterogeneous dispersions of liquid which usually range in size from 100 to 250 nm scale. Nanoemulsion formulations are commonly used carriers for hydrophobic drug delivery. Many anticancer drug encapsulated nanoemulsions have shown enhanced efficacy due to systemic delivery to the tumor site (24). The nanoemulsions formulations contain Tween® 80 as an emulsifying agent to stabilize hydrophobic oil droplet particles (25). The surface of the oil droplets is decorated with amphiphilic molecules to lower the interfacial tension and increase its stability in aqueous medium. Poly(ethylene glycol) (PEG) surface modified oil droplet allows its long circulation upon systemic administration and passive targeting to solid tumors by the enhanced permeability and retention effect (26–30).

In the present study, we have evaluated the qualitative and quantitative biodistribution and pharmacokinetics of DHA-SBT-1214 in solution and nanoemulsion formulations upon intravenous administration in naïve and cancer stem cell-enriched PPT2 human prostate tumor-bearing mice.

MATERIALS AND METHODS

Materials

Docosahexaenoic acid conjugate of SBT-1214 (i.e., DHA-SBT-1214) was synthesized by ChemMaster International, Inc. (Stony Brook, NY) following the previously reported method (14–16). Extra pure grade omega-3 rich fish oil was purchased from Jedwards International (Quincy, MA), Lipoid E80 from Lipoid GMBH (Ludwigshafen, Germany), DSPE PEG2000 from Avanti Polar Lipids, Inc. (Alabaster, AL), Tween 80 from Sigma Chemicals, Inc. (St. Louis, MO), Mesenchymal stem cell growth media (MSCGM) from Lonza (Portsmouth, NH), LAL chromogenic endotoxin quantitation kit from Thermo Scientific (Rockford, IL), Rat collagen type I from Sigma-Aldrich. Penicillin, streptomycin and Trypsin were obtained from Invitrogen (Grand Island, NY, USA). Male CD-1® mice (4–6 weeks old) for naïve and NOD.SCID/NCr mice for tumor bearing studies were purchased from Charles River Laboratories (Frederick Research Model Facility-NCI) (Cambridge, MA, USA). Dialysis membranes (molecular weight cutoff, 3000) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Amicon Ultra-0.5 ml,

Centrifugal filters from Millipore (Cork, Ireland). All other analytical grade reagents were purchased through Fisher Scientific.

Preparation of Nanoemulsion Formulations

Preparation of nanoemulsion formulations was carried out with a well-established protocol as reported recently with some modifications (31–33). Instead of a sonication method, oil-in-water nanoemulsions were prepared by high pressure homogenization method. Briefly, pre-warmed oil phase (10 ml) consisting of fish oil alone (for placebo) or 25 mg/ml of DHA-SBT-1214 dissolved in ethanol, was gradually added after ethanol evaporation from the oil mixture with compressed nitrogen gas to the pre-warmed water phase (40 ml) containing egg phosphatidylcholine (Lipoid E80®) (1200 mg), polysorbate 80 (Tween80®) (0.5 ml), DSPE-PEG2000 (1, 2-distearoyl-Sn- glycerol- 3- phosphoethanolamine- N-[amino (polyethylene glycol)-2000]) (75 mg). The resultant mixture was homogenized and the oil-water suspension was passed through a zirconia plunger of a M-110EH-30 (Microfluidics, Inc., Westwood, MA) high shear fluid processor at 10,000 psi for 3 cycles to achieve a uniform nanoemulsion formulation.

Characterization of the Nanoemulsion Formulations

The oil-in-water nanoemulsion formulation was characterized by well-established protocols in our laboratory (31–33). Particle size and surface charge of water diluted nanoemulsion were measured by using the Malvern Zetasizer (Worcestershire, WR14 1XZ, UK), morphology of oil droplets in the nanoemulsion formulations was visualized with transmission electron microscopy (TEM). Drug loading, encapsulation efficiency and stability were evaluated using HPLC as described previously (31). In short, for drug loading, the nanoemulsion was sufficiently diluted with organic (acetonitrile), and 80 µL aliquot was injected into the High Performance Liquid Chromatography (HPLC). For encapsulation efficiency, ultra-filtration method using centrifugal filter device (molecular weight cut-off 3000 Da; Millipore, Bedford, MA) was used. All batches of nanoemulsions were tested for endotoxin level through Limulus Amebocyte Lysate (LAL) assay according to manufacturer's instructions before using the nanoemulsion for both *in vivo* and *in vitro* studies.

High Performance Liquid Chromatography Analysis

The Waters LC (model 2487, Waters Corporation, Milford) comprising two pumps, an autosampler and a UV-detector was used for the analysis. The LC system was interfaced with Empower software for instrument control, data acquisition, and processing. The mobile phase consisting of (A) 0.1% trifluoroacetic acid (TFA) in water, and (B) 0.1% TFA in acetonitrile was pumped through the Grace Vydac 218TP54 column (C18, particle size 5 µm, 4.6 mm × 150 mm) at a flow rate of 1 ml/min. The gradient was 60%B to 95%B in 15 min and drug elution was monitored at a wavelength of 230 nm. The HPLC method followed was a modified version of a reported study (33). The retention time for the DHA-SBT-1214 was obtained at 10.24 min. The calibration curve was prepared with R² values of 0.997. Limit of detection (LOD) and limit of quantification (LOQ) was calculated through Excel according to the following formulae:

$$\text{LOD} = (\text{STEYX} / \text{Slope})^{*3.3}$$

$$\text{LOQ} = (\text{STEYX} / \text{Slope})^{*10}$$

Limit of detection (LOD) for our HPLC chromatographic method was 2.13 µg/ml and limit of quantitation (LOQ) was 6.45 µg/ml.

Storage Stability of the Nanoemulsion Formulations

DHA-SBT-1214 in solution form is light sensitive, therefore to minimize the effect of undersized factors, we carried out stability studies in an air-tight amber-colored glass container. Blank and DHA-SBT-1214 nanoemulsions were stored for up to 6 months at -20°C, 4°C, and at room temperature (~25°C). Samples were obtained at predetermined time intervals for analysis of oil droplet particle size and drug encapsulation.

In Vitro Drug Release Studies

In vitro drug release kinetics from aqueous solution and nanoemulsion formulations were determined using an equilibrium dialysis method (34). Briefly, 5 mg of DHA-SBT-1214 in aqueous solution and in nanoemulsion in 0.5 ml total volume were placed in the dialysis bags (MW cutoff 3000). Then, dialysis bags were immersed in 100 ml phosphate buffer (pH 7.4) with 1% (w/v) sodium lauryl sulfate (SLS) for different time points at room temperature. At predetermined time intervals, 1 ml of the released medium was withdrawn and replaced with an equivalent volume of fresh buffer to maintain the sink condition. The content of released DHA-SBT-1214 from both aqueous and nanoemulsion formulations was measured using HPLC analysis.

Cell Culture

The human prostate adenocarcinoma cancer stem cells (CSC)-enriched cell line PPT-2 used in this study was established from the stage pT2c pNX pMX prostate cancer patient (12). Briefly, PPT2 cells were cultured on rat collagen type I-coated cell culture dishes or flasks (Corning) in serum-free Mesenchymal Stem Cell Growth medium (MSCGM; Lonza) under 5% CO₂ atmosphere at 37°C.

In Vivo Tumor Xenografts in NOD/SCID Mice

All experiments involving the use of naïve and tumor-bearing mice were carried out in strict accordance with the recommendations in the guide for the care and use of laboratory animals of the National Institutes of Health. The experimental protocol was evaluated and approved by Northeastern University's Institutional Animal Care and Use Committee (IACUC). Briefly, after sufficient propagation, clonogenic human primary prostate cancer stem cells (PPT2) cells were resuspended in 1:1 MSCGM/Matrigel and one million cells injected subcutaneously to the right flanks of a 6 weeks old NOD/SCID mice. Tumor development was monitored weekly. The tumor size was measured with a caliper on a weekly basis and approximate tumor volumes determined using the formula $0.5ab^2$, where b is the smaller of the two perpendicular diameters.

Qualitative Biodistribution Studies with Near-IR Fluorescent Dye-Encapsulated Nanoemulsions

For qualitative evaluation of nanoemulsion formulation in naïve and tumor-bearing mice, DiR near-infrared fluorescent dye was encapsulated in the oil phase of the nanoemulsion. The droplet size and surface charge of nanoemulsion with near-IR dye were similar to those containing DHA-SBT-1214.

Male CD-1 naïve and NOD/SCID PPT-2 tumor bearing mice were dosed intravenously with encapsulated 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 0, 2, 4, 8, 12 and 24 h in the Caliper IVIS equipped with high lamp power with excitation filter centered at 745 nm and emission filter at 820 nm for exposure of 1 s. Image analysis was performed using Living Image 4 software (Caliper Life Sciences).

Plasma Pharmacokinetic Analysis of DHA-SBT-1214 in Solution and Nanoemulsion Formulations

DHA-SBT-1214 was given as a single dose intravenously as solution or nanoemulsion formulation to naïve and tumor-bearing mice, via tail vein injection at 120 mg/kg in injection volume of 150 µl. This administrated drug dose is based on our previous unpublished work. The solution of DHA-SBT-1214 was prepared by dissolving the drug in ethanol and diluted with Cremophor EL at 50:50 ratio and then the desired concentration was achieved in a sterile saline solution. Whereas, the nanoemulsion was directly diluted in sterile saline solution to get the required concentration of the drug. Blood was collected in EDTA-treated tubes at various times: 0.5, 4, 10, 24 and 48 h 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. Plasma (100 µl) was mixed with 300 µl of acetonitrile, and following vortexing, centrifuged at 10,000 rpm for 10 min to extract DHA-SBT-1214. Plasma (400 µl sample was mixed with 20 µl of paclitaxel (PTX) (25 µg/ml, as the internal standard) and vortexed. The extracted DHA-SBT-1214 was analyzed using an HPLC assay and the area under the peak of the drug relative to internal standard PTX was obtained from the chromatogram for determining the plasma concentration *versus* time profile.

Tissue Biodistribution of DHA-SBT-1214 in Solution and Nanoemulsion Formulations

DHA-SBT-1214 was dosed intravenously as solution or nanoemulsion formulation to mice via tail vein injection at 120 mg/kg. Following intravenous dosing of DHA-SBT-1214, the mice were anesthetized and at pre-determined time points of 0.5, 4, 10, 24 and 48 h, the blood was completely withdrawn by cardiac puncture. Animals were perfused with PBS and then sacrificed by cervical dislocation. Various tissues including heart, prostate, pancreas, brain, colon, lungs, spleen, kidneys, liver and tumor tissues were harvested, weighed, snap frozen in liquid nitrogen and stored at -80°C. For analysis, the frozen tissues were thawed, washed with PBS and homogenized using a tissue homogenizer at 5000 rpm for 2 min in 400 µl mixture of Ethyl acetate:methanol:acetonitrile (50:25:25) twice and following vortexing, the sample was centrifuged at 10,000 rpm for 10 min at 4°C to extract DHA-SBT-1214. Supernatant was evaporated with nitrogen gas and sample reconstituted in 400 µl

of acetonitrile which contains 20 μ l of paclitaxel (PTX) (25 μ g/ml, as the internal standard). Samples were analyzed by HPLC and the concentration of the drug in each of the tissues at different points was calculated from the areas under the peak of the HPLC chromatogram.

Pharmacokinetic Data Analysis

DHA-SBT-1214 pharmacokinetic parameters were determined using non-compartmental analysis for sparse data with Phoenix® WinNonlin® v. 1.3 software (Certara, Princeton, USA). Area under the plasma concentration-time curve (AUC) from zero to the last sampling time (AUC_{0-tlast}) was calculated using the linear trapezoidal method. The slope of the log-linear terminal part of the curve (Lambda z) was estimated using the best-fit method. The AUC was extrapolated to infinity (AUC_{0-∞}) using the following formula: AUC_{0-∞} = AUC_{0-tlast} + C_{last}/Lambda z. PK parameters including volume of distribution at steady state (V_{ss}), clearance (CL), terminal half-life (t_{1/2}), and mean residence time (MRT), were calculated using the non-compartmental formula. The maximal measured concentration C_{max} and the corresponding time T_{max} were obtained from the mean plasma concentration vs time curve. The targeting efficiency was evaluated by the ratio of the AUC_{last} in the tumor mass divided by the sum of the AUC_{last} of the other tissues. For statistical analysis, the AUC_{last} (or targeting efficiency) of the nanoemulsion were compared to the AUC_{last} (or targeting efficiency) of the solution using the Student's unpaired t-test with Welch correction for unequal variances.

RESULTS

Characterization of DHA-SBT-1214 Nanoemulsion Formulation

Nanoemulsion delivery approach has shown enhanced therapeutic potential in our previous studies (33). In this study, we have formulated an oil-in-water nanoemulsion of DHA-SBT-1214, a new-generation taxoid using fish oil which is rich in PUFAs such as omega-3 and omega-6 fatty acids. The non-ionic surfactant, Tween® 80, in nanoemulsions, reduced their aggregation by saturating the hydrophobic interfaces and providing steric repulsion (25). This taxoid encapsulated nanoemulsion was used to study its comparative biodistribution and pharmacokinetics in naïve and prostate tumor bearing mouse models. We used a microfluidic technique to formulate this uniform, milky-white nanoemulsion (35). Nanoemulsions were near spherical in structure with a maximum size of 220 nm, as observed with transmission electron microscopy (TEM) (Fig. 1a). The average particle size of the blank nanoemulsion (without any drug) was 209 ± 7 nm. The incorporation of DHA-SBT-1214 in nanoemulsions did not significantly change the particle size and it was measured 213.2 ± 6 nm. Along with particle size, uniformity of the nanoemulsions also predicts their bioavailability and is represented by polydispersity index (PDI). The lower value of PDI indicates the uniform and stable form of the nanoemulsions generated. PDI values of both placebo and drug encapsulated nanoemulsions was less than 0.11. Along with particle size and PDI, charge of the nanoemulsion also dictates their uniformity and stability. The average surface charge of the oil droplets in the nanoemulsions was -28.9 mV which shows that the nanoemulsions are stable. The negative charge of the nanoemulsion could be due to the presence of free fatty acids of the fish oil used in the preparation of these nanoemulsions. The surface charge of both formulations was not significantly different

employing the maximum encapsulation of the drug inside oil droplets. Table I shows the average particle sizes, PDI and zeta potentials of all formulations used in the present study. Representative graphs of size and zeta potential of DHA-SBT-1214 nanoemulsion formulations are shown in Fig. 1(b) and (c), respectively. The hydrodynamic diameter of the oil droplets and surface charge of our nanoemulsions are favorable for their stability on the basis of the DLVO theory (36). An HPLC assay was used to determine the drug concentrations in the nanoemulsion formulations. DHA-SBT-1214 nanoemulsion at 20 mg/ml represents drug loading efficiency of 97%. This high drug encapsulation efficiency of nanoemulsions was attributed to the relative lipophilicity of the drug, as this drug was retained in the oil core of the nanoemulsion. All the formulations were filtered through 0.2- μ m filter and had a minimum level of endotoxin as confirmed through Limulus Amebocyte Lysate (LAL) assay during the storage period.

Stability of Nanoemulsion Formulation

The stability of the nanoemulsion formulations is very important for their therapeutic efficacy. Usually nanoemulsions become unstable due to different phenomena including creaming, cracking and phase inversion. The stability of NE-DHA-SBT-1214 was assessed at three different temperature i.e. room temperature, at 4°C and at -20°C for a time period of 6 months. During this period droplet size (Fig. 2b), PDI (Fig. 2c), zeta potential (Fig. 2d) and drug encapsulation (Fig. 2e) were periodically measured. Our results showed better storage stability of the prepared nanoemulsion at 4°C compared to other two storage conditions. At 4°C, the droplet size was 233 nm and PDI was 0.052 at the end of 6 months storage. Similarly, there was no significant change in zeta potential and encapsulation efficiency and the nanoemulsion did not show any signs of phase separation. Overall room temperature is not suitable for storage of the nanoemulsion formulation and at -20°C nanoemulsion showed slightly increased particle size but did not affect their encapsulation level. This slight increase in particle size at -20°C might be due to the aggregation of nanoemulsion droplets that is evident from their increased poly dispersity index. Most likely this aggregation happens due to the formation of freeze-concentrate at this freezing temperature that leads to decreased inter-particle distance and subsequent coagulation of particles based on DLVO theory. The higher negative zeta-potential, smaller size and well-constructed shell of nanoemulsion in the light of the DLVO theory (36), could be the reason of their better stability at 4°C which prevents their breakdown or fusion.

Drug Release Studies

The *in vitro* drug release kinetics of nanoemulsion were investigated in a solution of PBS at pH 7.4 and 1% Sodium Lauryl sulfate (SLS). Compared to drug solution with 32% DHA-SBT-1214 release after 48 h, nanoemulsion had a significantly lower DHA-SBT-1214 release of 8%. This decrease in drug release may be due to complete distribution of the co-surfactant, PEG and Lipoid 80 on the surface of the nanoemulsion (37,38). Another reason for slow release of drug from nanoemulsion might be the longer diffusion pathway that the internal drug molecules had to travel to reach dissolution medium (39,40), compared to drug solution which has higher content of DHA-SBT-1214 in the solution (Fig. 2a). Slow release of nanoemulsion formulation at this pH indicates its enhances retention in blood circulation.

Qualitative Biodistribution Studies with NIR-Encapsulated Nanoemulsions

The DiR near infra-red dye-encapsulated nanoemulsion in tumor-bearing animals induced a massive signal in the periphery of tumor region, which became more concentrated in the tumor region over the period of time and persisted over 24 h, as shown in Fig. 3. Contrarily, in naïve animals, the nanoemulsions showed diffused pattern that gradually decreased over the period of time and almost disappeared within 24 h.

Plasma, Tumor, and Tissue Pharmacokinetic Analyses

DHA-SBT-1214 in both solution and nanoemulsion formulation was administered intravenously in this study. The plasma concentration of DHA-SBT-1214 was measured by HPLC through a method explained in Table II and Fig. 1d, e and f. The mean plasma concentrations *versus* time curves are plotted in Fig. 4 for better comparison of solution and nanoemulsion in naïve and tumor bearing animals. The shape of the log-linear concentrations *versus* time curve shows a multi-phasic decrease, which strongly suggests the presence of differential distribution in a central and at least one deep tissue compartment. Detailed pharmacokinetic parameters are described in Table III. The data were analyzed using a non-compartmental method. The maximum observed plasma concentration of DHA-SBT-1214 was at 0.5 h for both solution and nanoemulsion formulation which decreased gradually. The AUC_{0-∞} was 4539 h.μg/ml for the solution and 7858 h.μg/ml for nanoemulsion in naïve animals and 1086 h.μg/ml and 6685 h.μg/ml tumor-bearing mice, respectively.

For both formulations, the presence of a tumor mass is associated with a decreased plasma exposure (AUC_{last}, AUC_∞). This decreased exposure is also associated with a higher clearance and higher volume of distribution. The higher volume might be associated with the presence of a new distribution compartment, the tumor. The nanoemulsion formulation significantly increases the plasma exposure (AUC_{last}, AUC_∞) in both naïve and tumor-bearing animals but this effect is more intense in tumor-bearing animals with an almost 6-fold increase compared to less than 2-fold in naïve animals. This increased exposure time of the drug is associated with lower clearance. The lower clearance is mainly linked to a lower volume of distribution. This lower volume might indicate a restrained diffusion to tissues, which could be associated with a better targeting efficiency due to less unspecific exposure in tumor-bearing mice. Accordingly, tumor exposure (Figs. 5 and 6) was higher with the nanoemulsion than with the solution and the targeting efficiency was almost doubled which is consistent with the enhanced permeability and retention (EPR) effect (26–29).

We also studied the distribution of the DHA-SBT-1214 solution and nanoemulsion in naïve and tumor-bearing CD-1 mice. Overall, the pattern of biodistribution was similar in naïve and tumor-bearing mice. Spleen and liver were the most exposed organs with both solution and nanoemulsion formulations. Biodistribution of drug was lower in kidney, lung and heart and negligible in other tissues, including tumor (Fig. 6). Except prostate and brain, the exposure (AUC_{last}) was lower in all organs in tumor-bearing mice compared to naïve mice. This appears contradictory to the lower volume of distribution calculated from plasma data. However, since V_{ss} is a dilution factor between the plasma quantity and concentration at any time, it can be explained by higher elimination rate (as reflected by Lambda z) which will

lead to lower quantities and concentrations in plasma and in any organ of the body. The strongest effect of nanoemulsion formulation on biodistribution was a significant increase of spleen exposure which can be expected from a nanoemulsion due to the elimination route involving the mononuclear phagocytic system. Exposure was also significantly increased in heart and kidney in tumor-bearing mice.

DISCUSSION

The intravenous (IV) delivery of hydrophobic drugs usually can lead to higher toxicity and the high plasma concentration peak value. Current commercially available taxane formulations have issues of toxicity due to the vehicle used and lack of tumor specific delivery. To overcome these delivery issues, we have successfully formulated and studied the nanoemulsion carrier system containing DHA-SBT-1214 in fish oil droplets, which favorably acted as a drug reservoir and can be injected through IV. Through IV administration of the nanoemulsion, higher and accurate dose of encapsulated drug can be administrated. Also, higher affinity of nanoemulsion component towards serum protein leads to longer blood circular and ultimately higher therapeutic efficiency. Furthermore, this mode of nanoemulsion delivery is non-toxic and no-irritant. For any colloidal system, the particle size and zeta potential are two primary parameters which significantly affect the stability of the formulation *in vitro* and performance *in vivo*. The non-ionic emulsifying agent Tween 80 in our nanoemulsion facilitated desired oil droplet size and charge by inhibiting the aggregation through competitive adsorption to interfaces (25). The size of the nanoemulsions also facilitate their bioavailability to the target site. To ascertain that the prepared nanoemulsions meet the desired quality attributes, we used the processing pressure of 10,000 psi of the microfluidizer with 3 cycles. These parameters were found to be adequate to produce the disruptive forces to overcome the strong force which resists the disruption and deformation of oil droplets. Along with a smaller particle size, a negative zeta potential is desirable for long term stability of the NE-DHA-SBT-1214. The surface charge of the nanoemulsions is represented by zeta potential and is measure of its electrostatic attraction or repulsion between nanodroplets of the nanoemulsion. As DLVO theory predicts, particles with low surface charges has no electrostatic activation energy barrier for coagulation but at higher surface charge, an electrostatic energy barrier slows their coagulation (36). With these results, the lower zeta potential value represents higher attraction between droplets and vice versa. These parameters ensures that the Brownian motion forces are in the same order as the gravitational forces and there is a certain degree of change in the relative magnitude of attractive and repulsive forces (41). Our formulated nanoemulsions have desired stability without any signs of coalescence or flocculation when stored at 4°C for 6 months and retained its particle size, PDI and zeta potential without significant change. The surface morphology of both blank and DHA-SBT-1214 nanoemulsion formulation was studied using transmission electron microscope and was spherical in morphology with no visible drug crystals. This implies that the drug remains inside the droplet reservoir and does not leak out and form crystals. The *in vitro* release of drug from nanoemulsion is comparatively slower than its solution form. This slow release, increases their residence time in the blood circulation and ultimately its bioavailability in tumor microenvironment. The biodistribution of DiR near infra-red dye loaded

nanoemulsion showed that nanoemulsion allows delivery of the dye to the tumor microenvironment. Our plasma pharmacokinetic data showed a significantly higher exposure with the administration of DHA-SBT-1214 nanoemulsion in both naïve and tumor-bearing mice compared to solution. Moreover, the nanoemulsion delivery system led to higher exposure of the drug in the tumor and almost doubled the tumor targeting efficiency *versus* the solution demonstrating the improved therapeutic potential of the nanoemulsion encapsulated drug.

Overall, our formulated nanoemulsions not only resulted in increased drug payload but also lead to better stability and increased bioavailability.

CONCLUSIONS

In conclusion, DHA-SBT-1214 nanoemulsion was successfully formulated with the desired quality attributes and performance. The fish oil nanoemulsion performed better in terms of biodistribution and pharmacokinetics. Thus, this nanoemulsion formulation could be a promising approach for intravenous delivery of DHA-SBT-1214 and other chemotherapeutic drugs with similar properties.

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ABBREVIATIONS

AUC	Area under curve
CL	Clearance
CSCs	Cancer Stem Cells
DHA	Docosahexaenoic acid
DSPE-PEG2000	1, 2-distearoyl-Sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000
HPLC	High Performance Liquid Chromatography
IACUC	Institutional Animal Care and Use Committee
LAL	Limulus Amebocyte Lysate
LOD	Limit of Detection
LOQ	Limit of Quantification
MDR	Multi Drug Resistance
MRT	Mean residence time

MSCGM	Mesenchymal Stem Cell Growth Media
PEG	Poly Ethylene Glycol
PDI	Polydispersity index
PrC	Prostate cancer
PTX	Paclitaxel
SLS	Sodium Lauryl Sulfate
t_{1/2}	Terminal half-life
TEM	Transmission Electron Microscopy
TFA	Trifluoroacetic acid
TICs	Tumor Initiating Cells
Vss	Volume of distribution at steady state

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Fig. 1.

(a) Transmission electron microscopy (TEM) of nanoemulsion. (b) The oil droplet particle size determination in nm, (c) the measurement of zeta potential or surface charge on the oil droplets in mV, (d & e) HPLC Chromatogram of DHA-SBT-1214 and Fdclitaxel, and (f) Standard curve for calculating concentration of drug.

Fig. 2.

(a) *In vitro* dialysis-based release profile. (b-e) Stability of DHA-SBT-1214 from solution and nanoemulsions (NE) formulations.

Fig. 3.
Qualitative biodistribution of near infra-red dye encapsulated nanoemulsion formulation following intravenous administration in naïve and PPT-2 tumor bearing mice.

Fig. 4. Plasma concentration ($\pm$ standard deviation) in $\mu\text{g/ml}$ *versus* time in hours for DHA-SBT-1214 solution and nanoemulsion (NE) following intravenous dosing at 120 mg/kg in naïve and PPT-2 tumor bearing mice.

Fig. 5. (a) Tumor concentration ($\pm$ standard deviation) in $\mu\text{g/g}$ versus time hours for DHA-SBT-1214 solution and nanoemulsion (NE) following intravenous dosing at 120 mg/kg to PPT2 tumor-bearing mice. (b) – The targeting efficiency ($\pm$ standard deviation) of solution and NE-DHA-SBT-1214 in PPT2 tumor-bearing mice as evaluated by the ratio of tumor exposure to other organs/tissue exposure.

Fig. 6. Exposure of DHA-SBT-1214 in different organs upon administration of the solution and nanoemulsion (NE) formulations as evaluated by AUClast ($\pm$ standard error) following intravenous dosing at 120 mg/kg in naïve and PPT-2 tumor bearing male mice.

Table I

Characterization of Placebo and DHA-SBT-1214 Nanoemulsion Formulation

Nanoemulsion	Hydrodynamic diameter of the oil droplet, nm ($\pm$ S.D.)	Polydispersity index	Surface charge, mV ($\pm$ S.D.)
NE Placebo	209.0 $\pm$ 7.0	0.09	-26.7 $\pm$ 3.7
NE DHA-SBT-1214	213.2 $\pm$ 6.0	0.11	-28.9 $\pm$ 4.3

S.D. standard deviation

Table II**HPLC Method for DHA-SBT-1214 Analysis**

Condition	Description
Mobile phase	A: water B: acetonitrile
Column	CI8, 5 μm , 4.6 mm $\times$ 150 mm
Flow rate	1 ml/min
Gradient	60% B to 95% B in 15 min
Injection volume	80 μl
Wavelength of detection	230 nm

Table III

Plasma Pharmacokinetic Parameters in Plasma Following Intravenous Administration of 120 mg/kg DHA-SBT-1214 Solution or Nanoemulsion in Naïve and Tumor Bearing Mice

	Units	DHA-SBT-1214 solution in naïve mice	NE-DHA-SBT-1214 in Naïve mice	DHA-SBT-1214 solution in tumor bearing mice	NE-DHA-SBT-1214 in tumor bearing mice
Rs _q		0.605	0.609	0.416	0.860
Lambda _z	1/h	0.130	0.146	0.0595	0.0751
HL _{Lambda_z}	h	5.32	4.75	11.6	9.23
C ₀	µg/ml	1163	2767	208	943
C _{last}	µg/ml	1.54	1.07	9.06	19.7
AUC _{last}	h*µg/ml	4539	7858	1086	6685
SE _{AUC_{last}}	h*µg/ml	2047	345	96.3	597
AUC _{INF}	h*µg/ml	4551	7865	1238	6947
CL	ml/h	0.659	0.381	2.42	0.432
MRT	h	2.63	1.85	15.3	10.5
V _{ss}	ml	1.73	0.704	37.1	4.52

Rs_q: coefficient of determination of the line used to calculate Lambda_z. Lambda_z: slope of the log-linear terminal part of the curve. HL_{Lambda_z}: corresponding half-life. C: concentration extrapolated at time zero. C_{last}: concentration at the last time point. AUC_{last}: area under the curve between time zero and the last time point. SE_{AUC_{last}}: standard error of the AUC_{last}. AUC_{INF}: area under the curve between time zero and infinity. CL: clearance. MRT: mean residence time. V_{ss}: steady-state volume of distribution