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A Self Assembling Nanoemulsion of Lovastatin (SANEL) Decreases Cholesterol Accumulation and Apob-100 Secretion Greater than Lovastatin alone a Hepg2 Cell Line

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Abstract

Elevated circulating low density lipoprotein-cholesterol (LDL-C) levels contribute to the development of cardiovascular disease (CVD). Lovastatin, one of the earliest statins which lowers blood LDL-C and as with the more recent statins, has been reported to reduce mortality due to CVD. However, Lovastatin requires higher doses than other newer statins for optimal efficacy and thus, has been associated with more adverse side effects. The objective of the current study was to test the hypothesis that a self assembling nanoemulsion (SANE) system which would reduce the particle size of typical Lovastatin preparations, and convert this hydrophobic entity to a stable water dispersion could consequently improve its efficacy, as it relates to cellular cholesterol accumulation and apo B (the major apoprotein of LDL) secretion, while lowering the doses needed to produce these results. A SANE Lovastatin (SANEL) and di-methyl sulfoxide (DMSO) Lovastatin (DL) (455 nM) in HepG-2 cell culture system was utilized to examine their effects on cholesterol inhibition and apo B-100 levels at a concentration of 357 nM. Previous work showed that at doses lower than 500 nM, DL had no effect on cholesterol inhibition. SANEL resulted in significantly greater reductions of cellular cholesterol accumulation (53%) and apo B-100 secretion (42%), compared to DL. Thus, a nanoemulsion delivery of Lovastatin as well as other statins may provide an additional method for drug delivery of hydrophobic pharmaceuticals with greater efficacy at lower doses, and possibly fewer side effects.

Keywords: Nanoemulsion; Lovastatin; Cholesterol synthesis; Apo B-100-Apolipoprotein B

Introduction

Oil-in-water nanoemulsion applications have been extensively studied in the areas of pharmaceutical, cosmeceutical and nutraceutical delivery [1-5]. These emulsions have advantages when compared to conventional drug delivery systems in parameters such as, but not limited to; permeability, stability and bioelimination [6-7].

Lovastatin (the only pure statin available to the researcher for investigative purposes) is a hydrophobic inactive lactone derived from a fungal strain *Aspergillus terreus* [8], which is metabolized to the corresponding β-hydroxyacid, a principle metabolite and an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the biosynthesis of cholesterol [8]. Some of the adverse side effects reported in the literature to be associated with statin therapy include myopathy/rhabdomyolysis and elevated liver enzymes, which are characteristic in varying degrees for all HMG-CoA reductase inhibitors [8-10]. While an explanation for these adverse side effects of statins are continuing to be investigated, possibilities include but are not limited to their hydrophobicity, increased dose requirements for efficacy, and it was suggested that the particle size of the typical statin preparation is an additional factor.

The laboratory has previously reported in pre-clinical studies that nano-emulsions prepared by high energy-requiring Microfluidizer® Processor technology enhances the efficacy of pharmaceuticals and nutraceuticals for indications of breast cancer [11], melanoma [12], neuroblastoma [13], and inflammation [14,15]. More recently, the laboratory has also reported in two small human clinical trials that a nanoemulsion of lutein prepared by Microfluidizer® Processor technology as stable water dispersion, and when incorporated into an orange juice beverage enhances bioavailability, compared to a supplement in pill form [15].

This communication utilizing a self assembling nanoemulsion (SANE) system [patent pending] for the preparation and delivery of a stable water dispersion of Lovastatin to HepG-2 cells, produced by a modified phase inversion temperature [PIT] process [16], will report on the significant reductions in cellular cholesterol accumulation and apo B-100 secretion, compared to the nano-emulsion blank (SANE), macro-emulsion Lovastatin (ML) and Di-methyl sulfoxide (DMSO) preparation of Lovastatin (DL).

Materials and Methods

SANE and DMSO preparations of Lovastatin

The individual components for the nanoemulsion formulations utilizing the PIT method [16] include a vegetable oil, but not limited to rice bran oil (Tsuno, Japan), Solutol HS15 (BASF, Ludwigshafen, Germany) and deionized/distilled water (Milli Q, Bedford, MA). The self-assembling nanoemulsion of Lovastatin (SANEL) was prepared in

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the following manner: 10 mg Lovastatin was added to a 50 mL beaker containing 1 gm of rice bran oil, to which was added the surfactant Solutol HS15 (5 gm) and mixed well with a magnetic stirrer until the solution became clear. Subsequently, 44 mL of water was added while mixing, at a temperature maintained (checked with a thermometer) between 60-80°C; mixing was continued until cloudiness of the mixture was observed. At this point, the temperature was cooled immediately to 37°C by keeping the beaker containing the emulsion onto ice, which yielded a clear nanoemulsion that is stable water dispersion, which is about 0.5 M. In the case of the di-methyl sulfoxide (DMSO) preparation of Lovastatin (DL), 1 mg of Lovastatin was added directly to the 5 mL DMSO (Sigma-Aldrich (St. Louis, MO)) for a final concentration of 0.5 M. The nanoemulsion blank (SANE) includes all the components in the same amounts as the nano-formulation without the drug. The macroemulsion (ML) is a combination of all the components in the same amounts as the nanoemulsion, prior to the SANE process. Mean droplet size, width, poly-dispersity index (PDI) and zeta potential were measured by a Malvern Nano-S instrument (Malvern Instruments Inc., Southborough, MA).

Determination of morphology of the nano-blank and nanoformulation of Lovastatin

Transmission electron microscope (TEM), (Philips EM400T) was used to analyze the morphology of both nano-formulations of Lovastatin (SANEL) and the SANE. The samples were diluted 200 fold with distilled-deionized water, transferred into carbon-attached Vinylec Films (Ernest F. Fullam, Inc. Latham NY), stained with 10% phosphotungstic acid (Sigma Aldrich, St Louis MO), allowed to stand 5 min for drying and placed in the vacuum chambers for 30 min prior to analysis.

Hep G-2 cell preparation, cholesterol estimation

HepG-2 cells obtained from ATCC, Manassas, VA were plated in 100 mm plates and grown to 70% confluency in minimal essential media (ATCC (Manassas, VA)), 10% fetal bovine serum (FBS) (Gemini Bio Products, West Sacramento, CA), and 5% CO_2 at 37°C. In addition, 10 µL of each of the above Lovastatin formulations and the SANE, at a concentration of 455 µM and 2 µL of insulin (Sigma-Aldrich (St. Louis, MO)) were added. Apart from this, a no treatment (NT) and only DMSO are also maintained to compare the treatments. After 72 hr of incubation, cell lysates were made by using a RIPA Lysis Buffer (Sigma St. Louis, MO, USA) and stored at -80°C. The amount of total cholesterol accumulated in the cells was determined using the Cholesterol E CHOD-DOAS method [17,18], (Wako Chemicals, Richmond, VA).

Measurement of apo B in cell-culture media

HepG-2 cells were seeded in 75 mm flasks and grown to 70-80% confluency. Each plate containing a monolayer of cells received 14 mL of medium, containing 10% lipoprotein deficient serum together with the appropriate Lovastatin formulation at a drug concentration of 357 nM. Apart from Lovastatin formulations SANE, NT is also maintained for comparisons. At 24 and 48 hr time intervals, 1 mL aliquots were removed from each well and were replaced by an equal amount of fresh medium. The amount of apo B was quantified by using a microwell enzyme immunoassay (AlerCHEK, Portland, ME), according to the manufacturer's instructions.

Bradford and MTT assays

Cell number and cellular protein measurements were performed using the Bradford assay [19] and MTT [20] (Sigma-Aldrich, St. Louis, MO), respectively; the former to ensure that the baseline cellular protein concentrations were similar per cell lysate for cholesterol accumulation values.

Statistical methods

SigmaStat software was used for all statistical evaluations (Jandel Scientific, San Rafael, CA, USA) [21]. A repeated measure one-way analysis of variance (RM ANOVA) was used to analyze the data. When statistical significance was found by ANOVA, the Student–Newman–Keuls separation of means was used to determine differences. A paired *t*-test was used to analyze cellular cholesterol and apo B secretion rate differences between the initial and final incubation time points. All values were expressed as mean+SEM, and statistical significance was set at the minimum $p<0.05$.

Results

Particle size and stability

The SANEL and the SANE were stable for more than a year at 4°C, based on insignificant particle size changes. The particle size of the DL preparations averaged 200-500 nm with polydispersity index around 0.5 Figure 2d (Supplemental data) and Macroemulsion (ML), which has every ingredient as SANEL, but not processed to nano-emulsion, has multiple peaks of sizes in micron range and PDI of 0.981 (Figure 2a (Supplementary data)), whereas SANEL and SANE remained as in a mono-disperse distribution (Figure 1a-1c, respectively and Figure 2b and 2c (supplementary data)). Dilution experiments (1:1) with water showed that the SANE was very stable above 10% (v/v) of surfactant concentration, which was decided as the optimum range. Ostwald ripening of the SANE started at 1.25% (v/v) of surfactant concentration and maintained the critical micelle concentration till 0.03%, this is same with SANEL. There were no significant changes in the physical or chemical characteristics measured (particle size, stability, zeta potential of the SANEL or SANE (Table 1). The zeta potential for SANEL approached -1.36 mv, as opposed to -13.6 mv and 0 mv for DL and SANE, respectively (Table 1). This is related to the fact that most of the Lovastatin is encapsulated in the oil part of the emulsion, and a meagre amount of Lovastatin is either unencapsulated or hanging from external surface.

Cellular cholesterol levels

SANEL inhibited cellular cholesterol accumulation (53%) compared to the DL ($p=0.007$) preparation, 72% compared to SANE ($p<0.001$), 76% compared to NT ($p<0.001$) and DMSO ($p<0.001$) treatment (Table 2). There is no significant difference between NT, SANE and DL groups.

Comparison of secreted apoB100 levels

Measurements of apo B-100 secretion into the media revealed that, although there were no significant differences between treatment groups at 24 hrs, at 48 hrs apo B-100 secreted after SANEL treatment was significantly reduced by 37% compared to DL ($p<0.001$), 42% compared to SANE ($p<0.001$), 25% compared to ML ($p<0.001$), and 48% compared to the NT group ($p<0.001$) (Table 2).

Discussion

In this communication, the potential of Lovastatin encapsulated and delivered as a nanoemulsion and used as a treatment in a HepG2 cell line was evaluated, with respect to monodispersity, physical

stability, *in vitro* stability and effectiveness on cholesterol inhibition and apo B100 concentrations. Many approaches have been investigated to increase the dissolution rate of this poor water soluble compound, but it was believed that this communication reports for the first time, the utilization of a SANE system *in vitro* that increases the efficacy of Lovastatin and produces stable water dispersion.

When compared to Lovastatin dispersion in DMSO and in the form of a macro-emulsion, where it undergoes extensive agglomeration leading to very large and heterogeneous particle sizes, the SANEL and SANE is homogeneous with a PDI<0.1, and has an average particle size of 20 nm. Upon dilution (1:1) with water, the SANEL remains stable up to a surfactant concentration of 1.25% where Ostwald ripening starts and it reaches the Critical Micelle concentration at 0.03%. The data indicate that the Lovastatin molecule following the process of encapsulation into a nano-emulsion is not affected. Although speculative, Lovastatin could be metabolized by the microsomal system more efficiently in the form of SANEL, as was suggested for the SANE formulation of the prodrug Dacarbazine (DAC), which was converted to its metabolites at least 50% more than a suspension formulation of DAC (manuscript submitted).

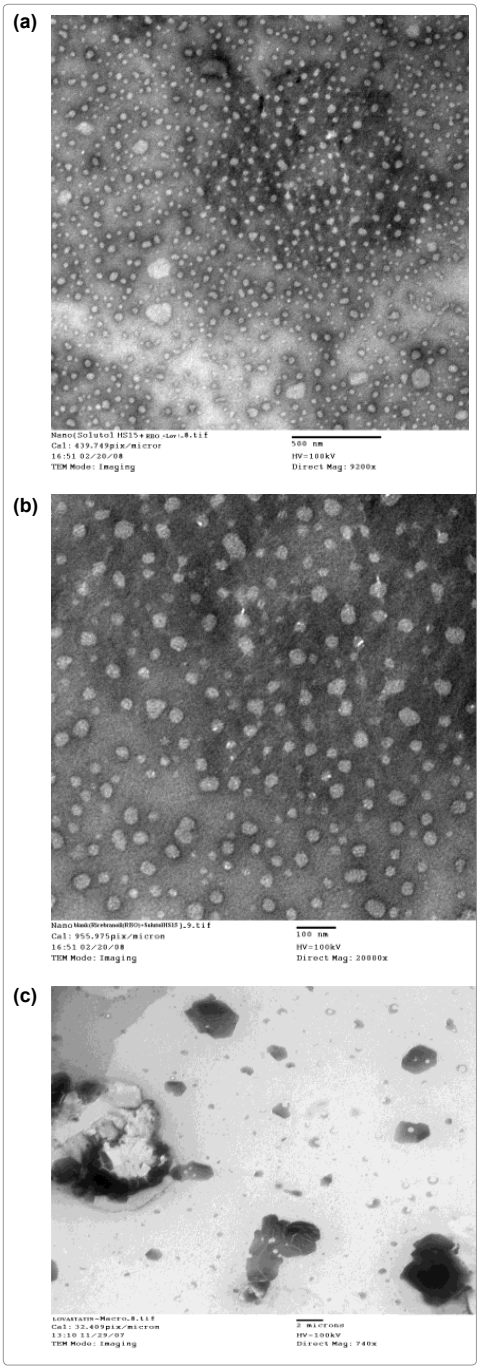

Figure 1: a, b and c TEM photographs of a Nano-lovastatin (SANEL) at 9200X, Nano-blank (SANE) at 20000X and Macro-emulsion Lovastatin (ML) at 740X, respectively.

Formulation	Particle Size	PDI	Zeta-potential
SANEL	21	0.01-0.1	0
SANE	21	0.01-0.1	0
ML	Out of range	>0.5	Out of range
DL	>2000	>0.5	-13.6

Table1: Comparison of various formulations for particle size (nm), polydispersity Index (PDI), and Zeta potential (mv).

Formulation	Cholesterol levels (µg/g protein)	apoB levels (µg/ml)
SANEL	130.0 ± 9.0[a]	5.3 ± 0.02[a]
DL	278.3 ± 13.1[b]	8.6 ± 0.04[b]
ML	……NA……	7.1 ± 0.03[c]
SANE	467.8 ± 28.1[c]	9.3 ± 0.02[b]
NT	536.5 ± 10.7[c]	10.3 ± 0.03[d]
DMSO	564.0 ± 28.2[c]	…….NA…….

Values represent Mean ± SEM; n=3. One way ANOVA analysis was done on the groups and found that values in a column not sharing common alphabet are statistically significant ($p<0.05$); NA: not available

Table 2: Comparison of the various treatments for total cellular cholesterol, HMGcoA reductase activity and apoB100 levels.

The newer statins such as Lipitor, Zocor and Crestor, as well as Lovastatin described in this communication are preferentially prescribed to lower circulating levels of atherogenic LDL [22] and in some cases, mortality associated with CVD [22-24]. However, a number of adverse side effects have been observed in individuals who use these drugs, including liver and muscle damage [22-24]. Since most of the effects occur systemically, it was suggested that direct delivery of statins encapsulated as nanoparticles could reduce the incidence and severity of these problems. In addition, there was no difference in the size of the particle in the emulsion, with and without drug (Table 1). These small particles averaging 20 nm in size within the nanoemulsion are taken up by the microsomal system and turned into β-hydroxy acid, an active inhibitor of the HMG-CoA reductase [23,24]. The thermodynamically stable formulation is compatible to cells as it is made up of biocompatible materials such as RiceBran Oil (RBO) (2%), Solutol HS15 (10%) and water (88%) [25]. Incorporation of RBO into the nano-formulation was based on the reported stability of the oil due to its high unsaponifiable levels [26,27], and the possibility that similar stability properties would be incorporated into the nanoemulsion.

HepG 2 cells are an established cell line for the study of cholesterol synthesis and apo B secretion in situations of high fatty acid influx into cells [28-30]. The finding that these cells decreased cholesterol synthesis and apo B-100 secretion into the media more efficiently when exposed to a nano-emulsion of Lovastatin supports that notion that the SANE system can play a significant role in inhibiting the apo B-100 secretion by these transformed hepatocytes, for reasons which will require further investigation.

Solutol HS15 was used as the surfactant since it is made up of amphipathic polyethylene glycols, which are known to increase the circulation time in *in vivo* models [31]. In addition, the nano-formulation generated by PIT method has a neutral zeta potential without the drug, and with the drug encapsulated it showed charge around -1.36; this value might be because of minimal amounts are left on the particles or in the dispersion phase, as they are made up of non-ionic surfactants which cover up the oil having the drug to make a nano-particle, which has been reported to avoid the mononuclear phagocytic system (MPS) [32], leading to increases in bio-circulation time of the drug [33].

Conclusion

All these factors taken together contributed to the positive findings of our nano-emulsion formulation of Lovastatin, reported here in the *in vitro* studies. Further studies will need to be conducted to evaluate the efficacy of SANEL in animals and humans.

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