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SELF-ASSOCIATED INDISULAM IN PHOSPHOLIPID-BASED NANOMICELLES: A POTENTIAL NANOMEDICINE FOR CANCER

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Abstract

This study aims to start the development of a nanomedicine, containing indisulam solubilized in sterically stabilized micelles (SSM) composed of DSPE-PEG₂₀₀₀ or sterically stabilized mixed micelles (SSMM) composed of DSPE-PEG₂₀₀₀ plus egg phosphatidylcholine (EPC). Micelles were prepared by coprecipitation and reconstitution of drug and lipids. Particle size distributions of micellar formulations were determined by quasi-elastic light scattering (QELS). Amounts of solubilized drug were determined by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC). In vitro cytotoxicity of indisulam in nanocarrier was determined on MCF-7 cell line by NCI developed Sulforhodamine B assay. Optimal solubilized indisulam concentrations in 5mM total lipid were 10 µg/ml for SSMM, and 400 µg/ml for SSM. HPLC results demonstrated encapsulation capacity of both micelles was over 95%. In vitro studies showed indisulam in micellar system was more effective than free indisulam. Optimized formulation was successfully freeze-dried without any addition of lyo- and cryo-protectants. We conclude SSM is a promising nanocarrier for indisulam, and indisulam-SSM should be developed further as a novel targeted nanomedicine.

Keywords

DSPE-PEG₂₀₀₀ and EPC; PEGylated Phospholipids; water-insoluble anticancer drug, indisulam; Sterically stabilized simple micelles; Sterically stabilized mixed micelles

INTRODUCTION

Recently, our laboratory have reported on the use of PEGylated phospholipids such as poly (ethylene glycol 2000) grafted 1,2-Distearoyl phosphoethanolamine, (DSPE-PEG₂₀₀₀), to form sterically stabilized micelles (SSM) or DSPE-PEG₂₀₀₀ plus egg phosphatidylcholine (EPC) to form sterically stabilized mixed micelles (SSMM) as targeted nanocarriers for the

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delivery of poorly water soluble drugs [1,2]. Components of these micelles are FDA approved, safe, biocompatible, and relatively nontoxic [2,3]. The PEG on the surface of the micelles renders them to be sterically stabilized, preventing opsonization and reticular endothelial system uptake [4].

These sterically stabilized micelles, because of their low critical micellar concentration (CMC) values and most likely because of strong interactions between the acyl chains in the core region, are also relatively stable on dilution [1,5]. In addition, the small size (~15nm) of these carrier systems can provide targeted delivery to cancer tissue or other injured tissues by selective extravasation through leaky vasculature [6]. Furthermore, preparation of SSM is simple and efficient compared with bile salt/phospholipid mixed micelles or liposomes [7–9].

Indisulam or also called E7070, N-(3-chloro-7-indolyl)-1, 4-benzenedisulfonamide is a sulfonamide anticancer agent that disrupts the cell cycle progression at the G1/S phase [10–12]. Although the exact mechanism of action remains uncertain, some identified effects include induced expression of p53 and p21, inhibition of phosphorylation of the tumor suppressor retinoblastoma protein (Rb), reduced expression of cyclins and cyclin-dependent kinases [13, 14]; and inhibition of carbonic anhydrase IX [15]. Currently, indisulam is being tested in combination with capecitabine and carboplatin for clinical trials [16,17]. Indisulam alone is shown to be effective on human breast cancer by in vitro and in vivo studies [12,18,19]. It was found to be considerably different from conventional anticancer drugs in clinical use with respect to its cell cycle effect and its tumor type selectivity [14,20]. However, its efficacy in clinical trials for breast cancer was not up to expectations due to dose-limiting toxicities [19, 21]. Aqueous indisulam formulations tested in clinical trials are not made of nanoparticles and drug molecules can extravasate in any vasculature in the body resulting in high toxicity on healthy tissues. One of the reasons for hematological toxicity in these formulations tested in clinical trials is that indisulam binds to plasma proteins (albumin) and to erythrocytes (carbonic anhydrase) in a saturable manner [22]. However, if indisulam is solubilized in micelles, then the drug will not be free to interact with blood components and the nanosize of micelles will significantly reduce drug distribution and toxicity to normal tissues. In addition, the drug will be accumulated by passive targeting at the tumor sites by extravasations due to leaky vasculature [4]. Furthermore, nanomicelles can improve the solubility of hydrophobic compounds (aqueous solubility of indisulam is less than 5µg/ml at 37 °C, provided by Eisai, Inc.) in the aqueous medium to render them suitable for parenteral administration. Previously, we have shown improved solubility of other anticancer drugs such as camptothecin and paclitaxel using simple and mixed micelles [2,7]. The purpose of this study was to investigate the solubility potential of SSM and SSMM for indisulam, and determine an optimized formulation to initiate the development of a novel nanomedicine for targeted treatment of breast cancer.

MATERIALS AND METHODS

Chemicals

1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy- poly(ethylene glycol 2000) (DSPE-PEG₂₀₀₀) and Egg-phosphatidylcholine (EPC) was obtained from LIPOID GmbH (Ludwigshafen, Germany). Indisulam was a gift from Eisai Inc. (Tokyo, Japan). All lipid samples were high performance liquid chromatography purified. HPLC-grade methanol, acetonitrile and phosphoric acid were purchased from Fisher Scientific (Ithaca, IL, USA). Eagle's minimal essential medium (EMEM) was purchased from ATCC (Manassas, VA, USA) All other reagents were of analytical grade.

Preparation of Aqueous Dispersions of Indisulam

Simple and mixed micelles containing indisulam were prepared by coprecipitation method [1,2]. Briefly, for simple micelles, indisulam and poly-(ethylene glycol-2000)-grafted distearoyl phosphatidylethanolamine (DSPE-PEG₂₀₀₀) were dissolved in methanol. For mixed micelles, DSPE-PEG₂₀₀₀ and egg-phosphatidylcholine at optimal molar ratio (90:10) were coprecipitated along with indisulam [1]. The solvent was then removed by vacuum rotary evaporation under a stream of argon. Obtained dry film was further dried under vacuum overnight to remove any traces of remaining solvent. The dried film was rehydrated with isotonic 0.01 M Phosphate buffer, pH 6.0, at which indisulam is in the most stable form. The solution was then flushed with argon, sealed, and equilibrated for 2 hours for simple micelles and 12 hours for mixed micelles at room temperature which was approximately 25 °. The excess of unsolubilized indisulam was removed by centrifugation at 13,000 g for 5 min to obtain a clear dispersion. The maximum solubility of indisulam was determined in simple micelles of DSPE-PEG₂₀₀₀ by keeping the phospholipid concentration fixed and changing the drug concentration (drug:phospholipid molar ratios ranged from 0.005 to 1.04 for simple micelles, and 0.001 to 0.52 for mixed micelles) until a homogenous system was determined by a quasi-elastic light scattering as a single size peak population. Each formulation was prepared in triplicate. The prepared dispersions were then characterized for their particle size and assayed for the drug content. The optimal formulations of SSM or SSMM were chosen based on their formation of a homogenous system with maximum solubilization potential for indisulam.

Particle Size Determination

Particle size distribution and mean diameter of the prepared aqueous dispersions of indisulam were determined by quasi-elastic light scattering using a NICOMP 380 submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA) equipped with a 35mW helium-neon laser at 632.8 nm. Mean hydrodynamic particle diameters (\bar{d}_h) in the aqueous dispersions were obtained from the Stokes-Einstein relation using the measured diffusion of particles in solution ($\eta = 0.933$, $T = 23^\circ\text{C}$, $n = 1.33$). Data were analyzed in terms of volume- and intensity-weighted distributions. Each reported experimental result is the average of at least three \bar{d}_h values obtained from analysis of the autocorrelation function accumulated for at least 20 minutes.

Assay of Solubilized Indisulam

The amounts of indisulam solubilized both in the SSM and the SSMM were determined by RP-HPLC. The clear aqueous dispersion was diluted with methanol and 20 μl of each sample were injected into YMC-Pack-Pro-C18 column (5 μm , 250 \times 4.6 mm, 120 Å pore size). The column was eluted with acetonitrile/water/phosphoric acid (430:570:1, v/v/v) at flow rate of 1.2 ml/min. UV absorbance was measured at 223 nm. The drug concentration was calculated from standard curves. Each solution was analyzed in triplicates.

In Vitro Cytotoxicity

MCF-7 breast cancer cell line was used to evaluate the in vitro activity of the formulations. The cell line was maintained in Eagle's minimal essential medium (EMEM) containing 2mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1mM nonessential amino acids, 1mM sodium pyruvate and supplemented with 0.01 mg/ml fetal bovine insulin at 37 °C in a humidified, 5% CO₂ atmosphere.

Optimum solutions of indisulam-SSM (composed of 5mM DSPE-PEG₂₀₀₀, 400 $\mu\text{g}/\text{ml}$ indisulam) chosen from the solubilization studies were used as the test solutions. Drug-free SSM in 0.01M phosphate buffer (pH 6.0) were prepared at the same concentrations as the test solution and used as a negative control. Indisulam in 4% DMSO was used as a positive control. In vitro indisulam cytotoxicity was assessed using the sulforhodamine B assay adopted for a

routine antitumor screening test in the National Cancer Institute [23,24]. Briefly, serial dilutions with either phosphate buffer containing lipid or 4% DMSO were made to obtain indisulam concentrations from 0.1 – 50 mg/ml. MCF-7 cells (190 μ l) were trypsinized and plated at a density of 1000 cells per well in a 96-well plate (final DMSO concentration was less than 0.5%). A total of 27 μ l/well of the test solutions and controls groups of solvents were added to the microtiter plates. Each sample was evaluated in triplicate. The plates were then incubated for 72 hours in a humidified, 5% CO₂ atmosphere.

After the incubation period, the cells were fixed to the plates by adding 100 μ l/well of cold 20% trichloroacetic acid and incubated for 1 hour at 4°C. The plates were then washed, air-dried, and stained with 100 μ l/well of 0.4% sulforhodamine B in 1% acetic acid for 30 minutes. Then the plates were washed with 1% acetic acid, and 200 μ l/well of 10mM Tris ([trihydroxymethyl]-aminomethane) buffer was added. The optical density was then read at 515 nm, and the readings obtained for the solvent controls were used to define 100% growth after normalizing for the value obtained for the zero-day control. These data were then expressed as survival percentage versus drug concentration, and ED50 values were calculated using nonlinear regression analysis.

Freeze-drying of Indisulam-SSM

The optimal solubilization ratio of indisulam in DSPE-PEG₂₀₀₀ micelles was determined as drug:lipid molar ratio 0.2:1. In our lyophilization studies, final lipid concentration of indisulam-SSM was 10 mM in order to form a good visually appealing cake [25]. The optimal indisulam-SSM formulation (composed of 10 mM DSPE-PEG₂₀₀₀, 800 μ g/mL indisulam) based on indisulam solubilization experiments, without any modification or any addition of a cryoprotectant and lyoprotectant, was placed into 1.5ml glass vials at fill volumes of 1ml and subjected to a lyophilization cycle by FreeZone® 6 (Labconco, Kansas City, MO, USA) [26]. Briefly, prepared samples were stored at –20°C overnight, followed by freezing in liquid nitrogen for 3 minutes. Then frozen samples and controls were lyophilized. Micellar size and indisulam concentration were compared for stability before and after lyophilization.

Data and Statistical Analysis

All the data are expressed as means \pm SD. Formulations and characterization data are averages of at least triplicate. In vitro cytotoxicity data are average of triplicates. ED50 values were calculated for each formulation and compared statistically using one-way analysis of variance. A p value < 0.05 was considered statistically significant.

RESULTS

Particle Size

A given phospholipid concentration can solubilize poorly water soluble drug only up to a certain concentration of the drug [26]. Once this maximal threshold is attained, populations of other species are observed by quasi-elastic light scattering. These particles that do not settle down after centrifugation are named as sterically stabilized particles (SSP), since most likely they are stabilized by DSPE-PEG₂₀₀₀ on their surface. Mean hydrodynamic diameters of SSM and SSMM without any presence of SSP were 12.6 \pm 2.9 nm (n=20) and 13.0 \pm 2.5 nm (n=20) respectively (Table 1).

Solubilization of Indisulam

With 5mM fixed total phospholipid concentration of SSMM, we performed experiments at different indisulam concentrations ranging from 2 μ g/ml to 1000 μ g/ml. For concentrations at and below 10 μ g/ml, one uniform size distribution was observed by Nicomp analysis (Fig. 1A).

Above this concentration, there were other populations of lipid coated particles coexisting with indisulam-SSMM as indicated by size distribution (Fig. 1B). Therefore, solubilization potential of 5mM SSM for indisulam was low and only 10 μ g/ml (drug: lipid molar ratio 0.005: 1).

Next, we tested SSM for its potential to solubilize indisulam. Sixteen different indisulam concentrations ranging from 2 to 2000 μ g/ml were tested with 5mM SSM composed of just DSPE-PEG₂₀₀₀ alone. Surprisingly, all samples at and below 400 μ g/ml of indisulam had single uniform size distribution, and above this concentration SSP were observed (Fig. 2A and 2B).

The amount of solubilized drug in the system was calculated by standard curves of RP-HPLC. All standard curves were linear over the tested indisulam concentration range of 20–100 μ g/ml. RP-HPLC results showed that at least 95% of the drug was incorporated in the system for all optimum drug concentrations.

In Vitro Cytotoxicity

We investigated whether the interaction between indisulam and the lipids in the formulation affected an anticancer activity of the drug. Since the solubility of indisulam in SSM was 40 fold higher than the solubility in SSMM, we performed in vitro cytotoxicity studies only with indisulam-SSM. The formulations of free indisulam in DMSO and indisulam-SSM were tested against MCF-7, a human breast cancer cell line.

As shown on Figure 3, indisulam in simple micelles was readily available to interact with cancer cells and retained its anticancer activity. As shown on Figure 4, ED₅₀ of indisulam in DMSO was 36.3 \pm 3.6 μ M, which is similar to the value reported by Nakatsu [18]. Indisulam in SSM proved to be more effective with ED₅₀ value of 16.2 \pm 2.7 μ M.

Freeze Drying

PEGylated lipids are not stable in aqueous media and subject to degradation by hydrolysis and oxidation [27]. Therefore, we tested the option to keep the optimal indisulam-SSM formulation in dry form after lyophilization. Visual appearance of lyophilized cakes of indisulam-SSM was similar to SSM controls: white, cotton like cakes elegant in appearance. Particle sizes of both SSM alone and indisulam-SSM were not significantly different before and after lyophilization (Table 1). Based on HPLC results, all the drug molecules were incorporated in the micelles after lyophilization.

DISCUSSION

Drug incorporation did not change the particle size of simple and mixed micelles (Table 1), most probably because of the dominating effect of the PEG molecules to the overall size of micelles. Similarly addition of small sized drug molecules into the core did not make a significant change in size of SSM and SSMM.

Indisulam was solubilized 40 folds higher in SSM than in SSMM. The big difference in the solubilization potential of SSM and SSMM can be explained with the fact that indisulam is located at the interface between the hydrophobic core and relatively hydrophilic palisade region. For SSMM, most of this area may be occupied by EPC molecules, allowing only very few molecules of indisulam to reside, causing lower drug solubilization in SSMM.

Based on in vitro cytotoxicity experiments on MCF-7 cell line, indisulam in SSM proved to be more effective than in DMSO (Fig. 4). The reason for increased activity is yet to be investigated. However, it was not due to the additive effect of PEGylated lipids, since PEGylated lipids alone did not show any significant toxicity upto 150 μ M.

Micellar formulations were successfully lyophilized. We believe that slightly smaller size for indisulam-SSM prepared for freeze drying (Table 1) is due to the higher concentration of lipids (5mM vs. 10mM), which may affect the extension of flexible PEG chains. However, particle size of samples in each case was not significantly different ($p>0.05$ 5mM micellar samples compared to 10mM micellar samples).

In conclusion, molecular dispersion of indisulam in aqueous media was increased at least 80 folds when solubilized in SSM (drug concentration in aqueous media $\leq 5\mu\text{g/ml}$ verses in SSM 400 $\mu\text{g/ml}$). SSMM were not as efficient solubilizer as SSM, and only improved aqueous solubility by 2 folds. This was probably due to competition of indisulam molecules with EPC for the same location in SSMM micelles. Drug incorporation did not change the mean size of the micelles. Based on HPLC results more than 95% of the drug was incorporated in the optimized micellar system with no precipitation and SSP formation. In vitro studies using MCF-7 breast cancer cell line showed that indisulam-SSM is more effective than indisulam in DMSO. Optimized indisulam-SSM formulation was successfully lyophilized without any addition of lyo- and cryo-protectants indicating that acceptable shelf life can be achieved for this optimized indisulam formulation. We propose that indisulam-SSM should be further developed as a novel nanomedicine for targeted treatment of breast cancer.

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Abbreviations

E7070, N-(3-chloro-7-indolyl)-1, 4-benzenedisulfonamide
 BSS, balanced salt solution
 CMC, Critical Micellar Concentration
 DSPE-PEG₂₀₀₀, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy- poly (ethylene glycol 2000)
 EPC, egg phosphatidylcholine
 ED50, median effective dose
 EMEM, Eagle's minimal essential medium
 FDA, Food and Drug Administration
 NCI, National Cancer Institute
 PEG, polyethylene glycol
 QELS, quasi-elastic light scattering
 RP-HPLC, Reverse Phase-High Performance Liquid Chromatography
 SRB, Sulforhodamine B
 SSM, Sterically Stabilized Micelles
 SSMM, Sterically Stabilized Mixed Micelles
 SSP, Sterically Stabilized Particles

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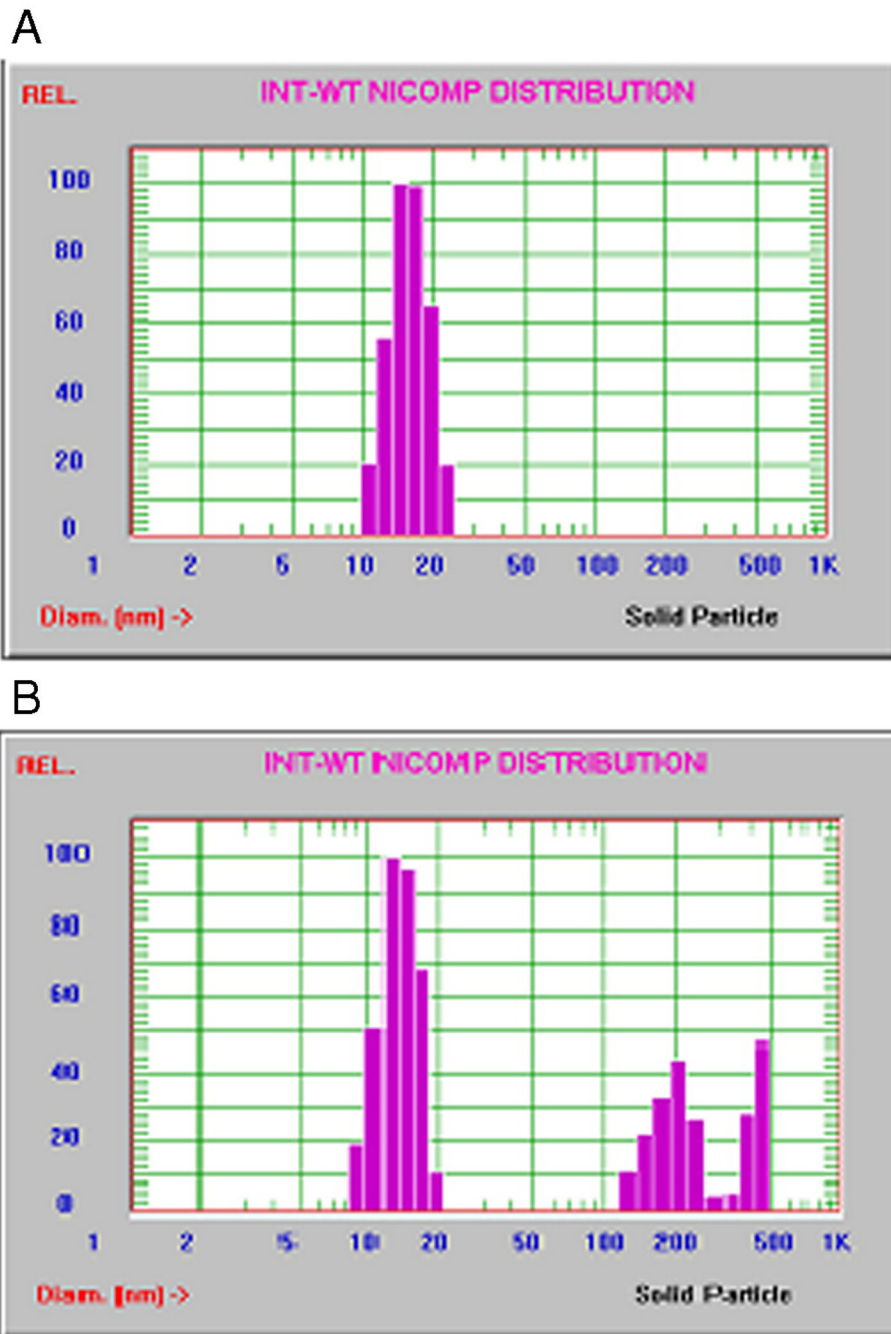


Figure 1. Representative Nicomp size distribution of indisulam with SSMM; a) Indisulam-SSMM at optimum drug concentration of $10\mu\text{g/ml}$, b) Indisulam-SSMM at excessive drug concentration of $25\mu\text{g/ml}$.

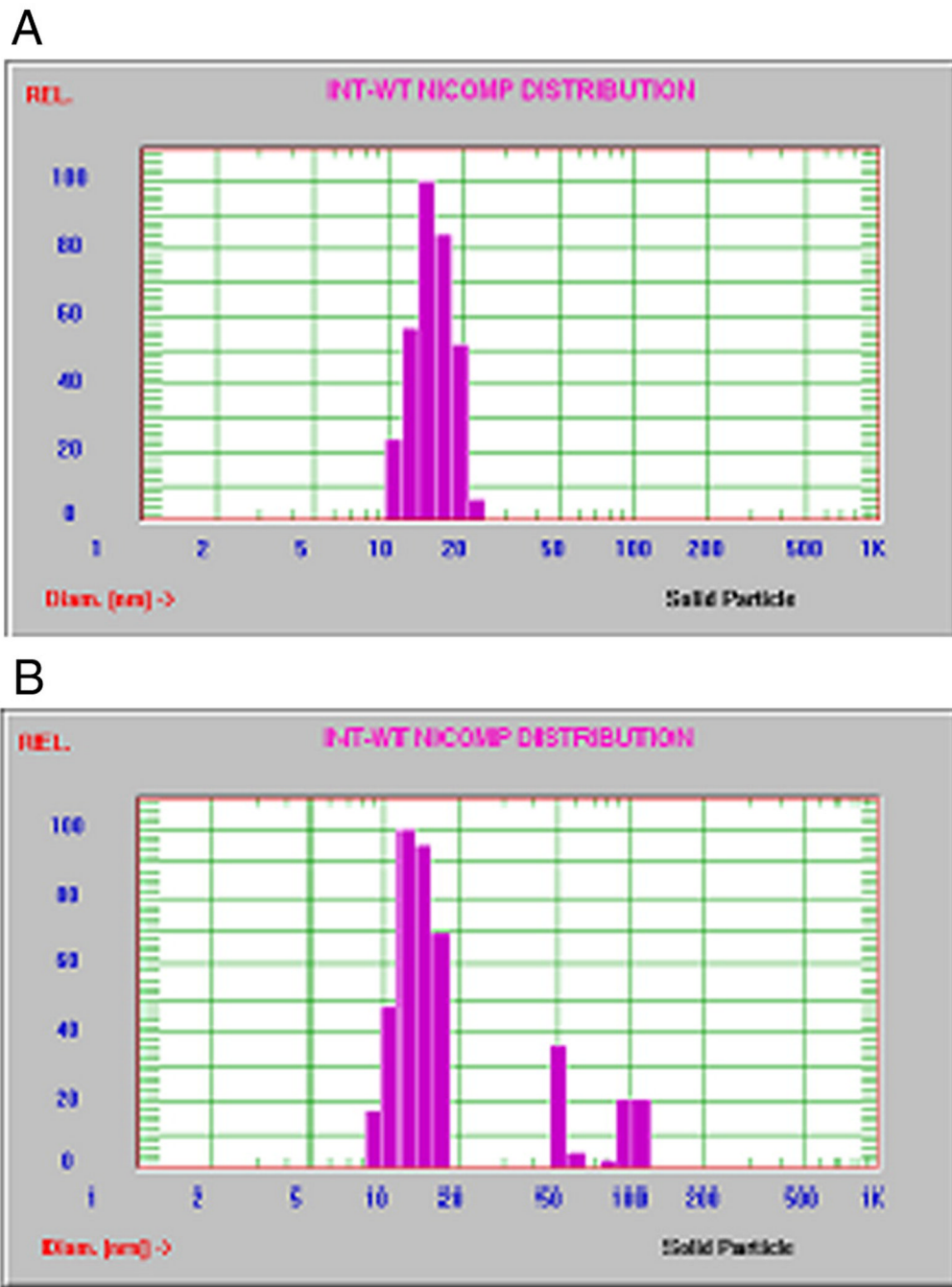


Figure 2. Representative Nicomp size distribution of indisulam with SSM; a) Indisulam-SSM at optimum drug concentration of 400µg/ml, b) Indisulam-SSM at excessive drug concentration of 500µg/ml.

In vitro Cytotoxicity of Indisulam

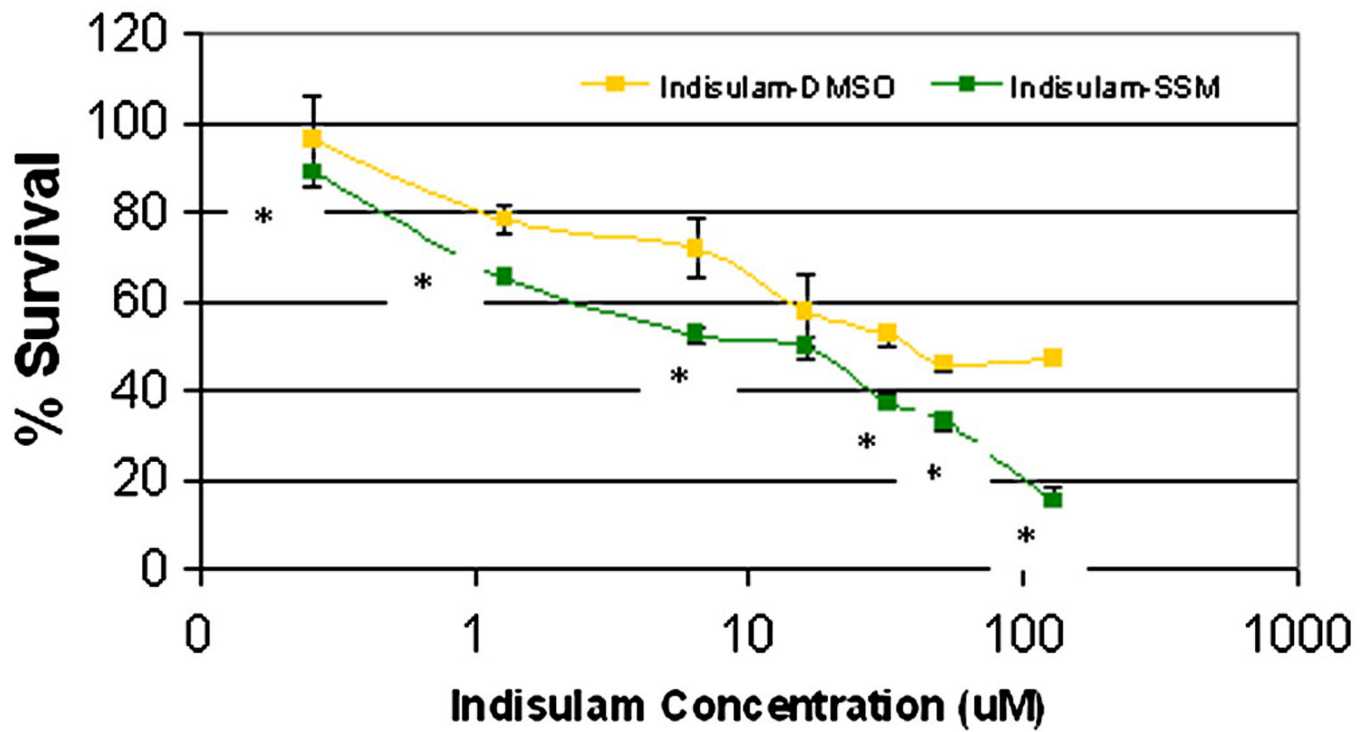


Figure 3.

In vitro cytotoxicity of indisulam-SSM after 72 hours by Sulforhodamine B assay (n=3, mean \pm SD, * p<0.05 compared to respective samples of indisulam in DMSO).

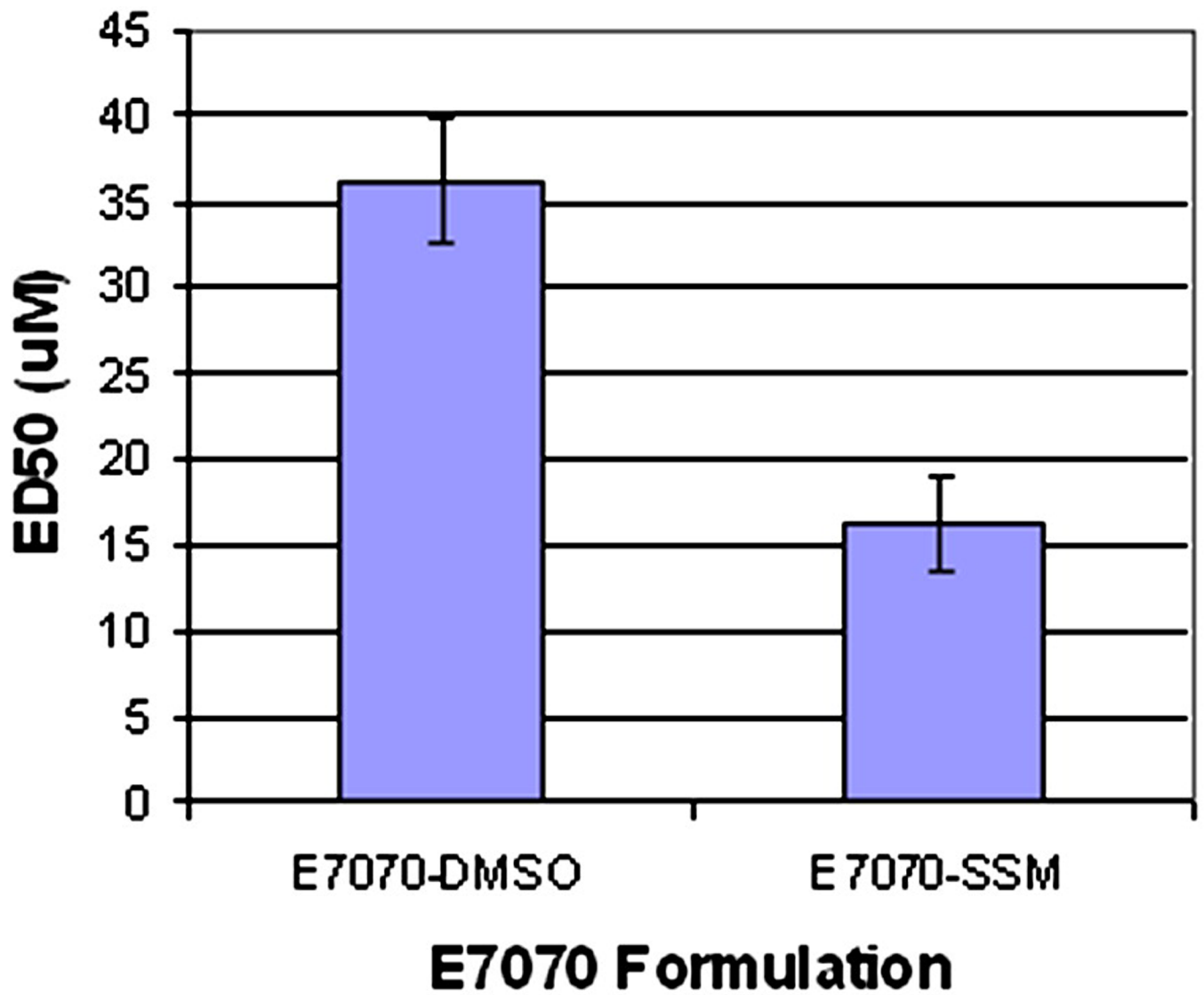


Figure 4. ED50 values of indissulam in DMSO and indissulam-SSM ($3.63 \pm 3.6 \mu\text{M}$ and $16.2 \pm 2.7 \mu\text{M}$ respectively).

Table 1

Solubility of indisulam in 5mM SSM and SSMM; particle sizes of SSM and indisulam-SSM before and after lyophilization

Formulation	Mean Particle Diameter		Optimum Drug Concentration
Indisulam-SSMM (5mM lipids)	12.6 ± 2.9 nm (n=20)		10 µg/ml
Indisulam-SSM (5mM lipids)	13.0 ± 2.5 nm (n=20)		400 µg/ml
	Before Lyophilization	After Lyophilization	
SSM (10mM lipids)	10.4 ± 1.8 nm (n=3)	10.7 ± 2.2 nm (n=3)	0
Indisulam-SSM (10mM lipids)	10.8 ± 3.1 nm (n=3)	10.3 ± 2.2 nm (n=3)	800 µg/ml

More (40×) drug solubilization is achieved in SSM than in SSMM. (n=20, mean ± SD, p>0.05, SSM mean particle diameter compared to SSMM). Particle sizes of SSM and Indisulam-SSM before and after lyophilization did not significantly change (p>0.05).