Journal of Pharmaceutical Investigation
Vol. 41, No. 5, 295-300 (2011)

Preparation and Characterization of Tributyrin Sub-micron Emulsion as Carrier for Paclitaxel

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(Received August 4, 2011 · Revised September 5, 2011 · Accepted October 19, 2011)

ABSTRACT – Paclitaxel is a well known anticancer agent and has been a pharmaceutical challenge because of its extremely poor water-solubility and susceptibility to the p-glycoprotein (p-gp)-mediated efflux in multi-drug resistant (MDR) cancer cells. Tributyrin (TB), a triglyceride with relatively short fatty acid chains, was chosen as solubilizing vehicle for paclitaxel based on the solubility study (26.6 mg/mL). Tributyrin (10%) o/w emulsion containing paclitaxel (5%), egg phosphatidylcholine (5%) and pegylated phospholipid (0.5%) was prepared by high pressure homogenization to obtain sub-micron-sized emulsion. The mean particle size of the resultant TB emulsion was 395.5 nm. Paclitaxel in TB emulsion showed higher anticancer activity against human breast cancer cell line, MCF-7, than free form delivered in DMSO solution. On the other hand, its anticancer activity was significantly reduced in MCF-7/ADR, a MDR variant cancer cell line of MCF-7, and recovered by the presence of verapamil, suggesting of the susceptibility to the p-gp mediated efflux even though paclitaxel was encapsulated into emulsion. The TB emulsion showed great potential as a promising vehicle for water-insoluble anticancer agent, paclitaxel.

Key words – Paclitaxel, Tributyrin emulsion, Water-insoluble, Anticancer

Paclitaxel has been widely used as an anticancer agent and still is a challenge to the formulation scientists because of its extremely poor water-solubility and susceptibility to the efflux by p-glycoprotein (p-gp) in multi-drug resistant (MDR) cancer cells as well as in the intestinal mucosal cells (Agueros et al., 2011). The first commercially available product was intravenously injectable formulation which contains paclitaxel solubilized in a mixture of Cremophore EL and ethanol. However, Cremophore EL-based formulation have required pre-medication with antihistamins or steroids to prevent the hypersensitivity reaction caused by Cremophore EL, which has provoked extensive studies to develop Cremophore EL-free alternative formulations (Singh and Dash, 2009). Finally, a few alternative formulations have been developed and approved for clinical use after almost two decades of research, which are polymeric micelles and albumin nanoparticles (Montana et al., 2011). Even though the new formulations available, paclitaxel still needs efficient delivery systems which can make it avoid the efflux in the drug resistant cancer cells and intestinal mucosal cells because paclitaxel is a substrate of p-gp efflux protein as well as can solubilize it.

Amongst many trials to solubilize paclitaxel, lipid-based delivery systems have attracted great attentions of formulation scientists because water-insoluble paclitaxel could be solubilized or incorporated into lipid domains of the delivery systems (Li et al., 2011; Pandita et al., 2011; Sznitowska et al., 2008; Shenoy et al., 2009). However, it was reported that paclitaxel did not dissolve in vegetable oils which are comprised with triglycerides of long-chain fatty acids (Tarr et al., 1987). On the contrary, significant amount of paclitaxel dissolved in triacetin which is a triglyceride of acetic acid, leading to the development of 50% triacetin emulsion for injectable formulation of paclitaxel. Unfortunately, it showed toxicity in animal study (Tarr et al., 1987). Based on the results of triacetin emulsion, we proposed that triglycerides with moderately short or medium chain fatty acids could dissolve more paclitaxel than those with long chain fatty acids such as soybean oil and sunflower oil. Additionally, we expected the encapsulation of paclitaxel into emulsion droplets might make it avoid p-gp-mediated efflux to some extent. In the present study, tributyrin was selected through solubility study and sub-micron sized tributyrin emulsion was prepared and evaluated to show potentials as alternative carrier for paclitaxel without using Cremophore EL.

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DOI : 10.4333/KPS.2011.41.5.295

Experimental

Materials

Tributyrin, soybean oil and verapamil were purchased from Sigma Chemical Co, (St. Louis, MO, USA) and Labrafac Lipophile WL1349 which is medium chain triglyceride oil (MCT) was provided by Gattefosse (Cedex, France). Egg phosphatidylcholine (eggPC) and distearoyl phosphatidyl ethanolamine-N-poly (ethylene glycol)$_{2000}$ ($PEG_{2000}PE$) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). MCF-7 was from Korean Cell Line Bank (Seoul, Korea) and MCF-7/ADR was kindly gifted by Dr. S.J. Lim in Sejong University, Seoul, Korea. All the reagents for cell culture were purchased from Invitrogen Corp. (CA, USA). All other chemicals were reagent grade and used without further purification.

Measurement of solubility of paclitaxel in various oils

Excess amount of paclitaxel was added to 1 mL of tributyrin, MCT oil (Labrafac Lipophile WL1349) and soybean oil and followed by constant mixing using magnetic stirrer for 24 hours at room temperature. The mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through syringe filter of 0.45 μm pore size to remove undissolved paclitaxel. The filtrate was subjected to HPLC determination of the dissolved paclitaxel after dilution with methanol.

Preparation of tributyrin emulsion

The emulsion was prepared using high pressure homogenizer as follows. Tributyrin (100 mg), eggPC (50 mg), $PEG_{2000}PE$ (5 mg) and pacilitaxel (5 mg) were weighed into glass tube followed by sonication for approximately 1 hr at 65°C in bath type sonicator (Branson® ultrasonic cleaner, 3210R-DTH, Branson Ultrasonics Corp., CT, USA) to dissolve paclitaxel in oily mixture. Preheated (65°C) water for injection was added to make 1 mL and sonicated for more than 3 hours until milky, homogeneous crude emulsion was obtained. The crude emulsion was homogenized for 5 cycles at 100 MPa using a high pressure homogenizer (Emulsiflex® EF-B3, Avestin Inc., Canada). The hot ultra fine emulsions obtained by high pressure homogenization (HPH) were cooled by dipping into water bath at room temperature. The resultant dispersion was stored at 4°C.

Particle size analysis of emulsion

The particle size of tributyrin emulsion was measured using submicron particle sizer, ELS-Z (Photal Otsuka Electronics, Japan). The emulsion was diluted with pre-filtered water through 0.22 μm before measurement.

HPLC analysis of paclitaxel

Paclitaxel in various oils and in emulsion was determined using HPLC. Fifty microliter of oils or emulsion was mixed with methanol to make 2 mL and filtered through 0.45 μm before injection of 5 μL onto the HPLC column. HPLC system was Shiseido Nanospace SI 2. The column was CD-C18 (ODS, 3 μm, pore size 12 nm, 150 × 4.6 mm, Imtakt Corp., Japan) and kept in column oven set at 35°C. The mobile phase consisted of a mixture of acetonitrile (550 mL) and 2 mM phosphoric acid (450 mL). The flow rate was 0.5 mL/min and the eluting paclitaxel was detected at 227 nm.

In vitro assay of anticancer activity

In vitro anticancer activity paclitaxel-containing TB emulsion was measured against human breast cancer cell line, MCF-7, and its MDR variant, MCF-7/ADR by MTT assay. Cancer cells were cultured in PRMI 1640 medium supplemented with 10% of heat-inactivated FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin under 5% CO_2 at 37°C. The cells were inoculated to a 96-well plate at a density of 10^4 cells in 200 μL medium per well and incubated for 12 hours. The medium was then replaced with emulsion-containing media and incubation was continued for 48 hours. When co-treated with verapamil, the medium containing 20 μM of verapamil was added to the wells 30 min before adding paclitaxel. After incubation, the emulsion-containing media were removed to avoid emulsion-induced interference in the MTT assay, and 180 iL of fresh medium and 20 μL of MTT solution (5 mg/mL in PBS) were added to the wells. The cells were incubated for another 3 hours. MTT internalization was terminated by aspiration of the media, and the cells were lysed with DMSO. The optical density at 570 nm was determined using a microplate spectrophotometer (SPECTRAmax® 340PC; Molecular Devices Corp., Sunnyvale, CA, USA). Anticancer activity was expressed as % survival of the cancer cells compared to the untreated control cells (100% survival).

Results and Discussion

Solubility of paclitaxel in various triglycerides

Tributyrin showed the greatest solubility of paclitaxel (26.6 mg/mL) among the triglycerides tested as presented in Table I. As we proposed, longer chain triglycerides showed significantly reduced solubility, which were 8.3 mg/mL and 0.3 mg/mL in MCT oil (Labrafac WL1349) and LCT oil (soybean oil), respectively. Tarr et al. suggested that Intralipid, a commonly used parenteral emulsion, would be inadequate as a vehicle for paclitaxel due to the poor solubility of paclitaxel in

Table I. *Solubility of paclitaxel in various triglycerides*

Triglycerides	Solubility (mg/mL)*
Tributyrin	26.6±3.2
MCT oil (Labrafac WL1349)	8.3±1.1
Soybean oil	0.3±0.2

*Mean±standard deviations of 3 measurements

soybean oil and tried 50% triacetin emulsion instead of fat emulsion of soybean oil because paclitaxel dissolved in triacetin as much as 75 mg/mL (Tarr et al., 1987). However, 50% triacetin emulsion containing paclitaxel showed toxicity when administered intravenously in animal study according to their report. MCT and LCT oils have been used for intravenous calorie supply and as vehicles for several water-insoluble but lipid-soluble drugs, which means they are biocompatible and safe when clinically used (Hippalgaonkar et al., 2011; Mirtallo et al., 2011). Generally, water-insoluble drugs have been considered to be solubilized sufficiently into MCT- and LCT-based emulsion. It is true only for oil-soluble drugs. Unfortunately, many water-insoluble drugs are not soluble in lipids as well, and thus MCT- and LCT-emulsions may not be satisfying solutions for clinical formulation. Even so, triglycerides are usually regarded to be safe and used as additives for food, cosmetics and pharmaceuticals, which led us to propose that triglycerides with matched chain length can be good vehicles for water-insoluble drugs. As we proposed, tributyrin, a triglyceride with moderately shorter chain, could dissolve paclitaxel, insoluble in both of water and LCT oils. Based on the results, tributyrin was chosen as inner non-aqueous phase for intravenous submicron-sized emulsion carrying paclitaxel and subjected to the further study.

Particle size of paclitaxel-containing tributyrin emulsion

TB emulsion was prepared using egg PC and pegylated phospholipids as stabilizers of the TB droplets. To make injectable submicron-sized emulsion, the crude emulsion was subjected to high pressure homogenization. The resulting emulsion showed mean particle size of 395.5 nm after high pressure homogenization as shown in Table II. The particle size and polydispersity index were dramatically reduced by high pressure homogenization. Through high pressure homogenization, submicron emulsion was successfully prepared.

***In vitro* anticancer activity**

In vitro anticancer activity was evaluated against human breast cancer cell line, MCF-7. TB emulsion containing paclitaxel showed dose-dependent cytotoxicity and higher anti-

Table II. *Particle size of paclitaxel-loaded tributyrin emulsion before and after high pressure homogenization (HPH)*

	Mean particle size	Polydispersity index
Before HPH	1840.9 nm	0.370
After HPH	395.5 nm	0.174

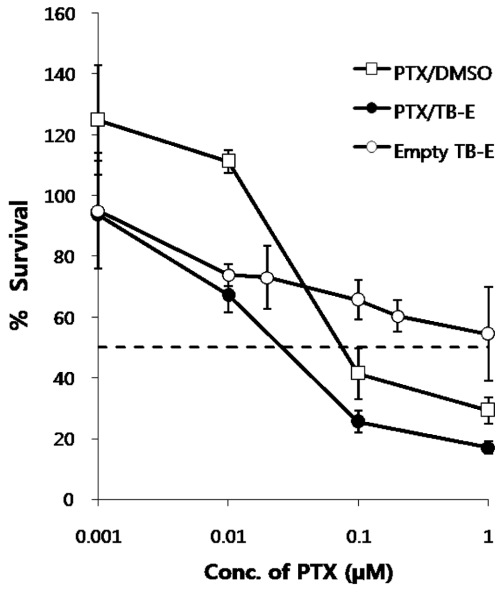

Figure 1. Percent cell survival of human breast cancer cell line MCF-7 when incubated with empty tributyrin emulsion (TB-E) and paclitaxel (PTX) delivered in DMSO and TB-E for 48 hours. For empty TB-E, doses are expressed as corresponding deliverable PTX dose with TB-E if PTX incorporated.

cancer activity to the paclitaxel solution in DMSO (Figure 1). Against MCF-7, paclitaxel delivered in TB emulsion gave lower IC_{50} (approx. 0.05 μM) compared to that in DMSO did (approx. 0.09 μM). The stronger anticancer activity of paclitaxel in TB emulsion appeared to be due to the cytotoxicity of empty TB emulsion itself according to Figure 1. Empty TB-E showed cytotoxicity to some extent. This may be explained by the fact that TB can act as prodrug for butyric acid which has been reported as histone deacetylase (HDAC) inhibitor and anticancer agent (Ooi et al., 2011; Yin ,Chow 2009). At lower dose of paclitaxel (0.001~0.01 μM), the contribution of TB emulsion itself was predominant in anticancer activity compared to that of paclitaxel as shown in significantly lower rate of cell survival than paclitaxel delivered in DMSO. However, anticancer activity of TB emulsion itself was slowed down with increasing dose as presented in Figure 1. As increasing dose, dose-dependent rise of cytotoxicity of paclitaxel in TB emulsion became much steeper than those of empty TB emulsion and that in DMSO implying more efficient delivery into cells using emulsion system. Some studies have reported that

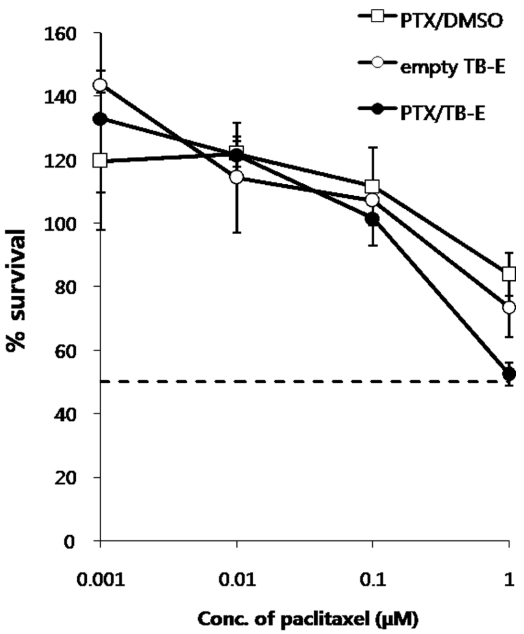

Figure 2. Percent cell survival of MCF-7/ADR when incubated with empty tributyrin emulsion (TB-E) and paclitaxel (PTX) delivered in DMSO and TB-E for 48 hours. For empty TB-E, doses are expressed as corresponding deliverable PTX dose with TB-E if PTX incorporated.

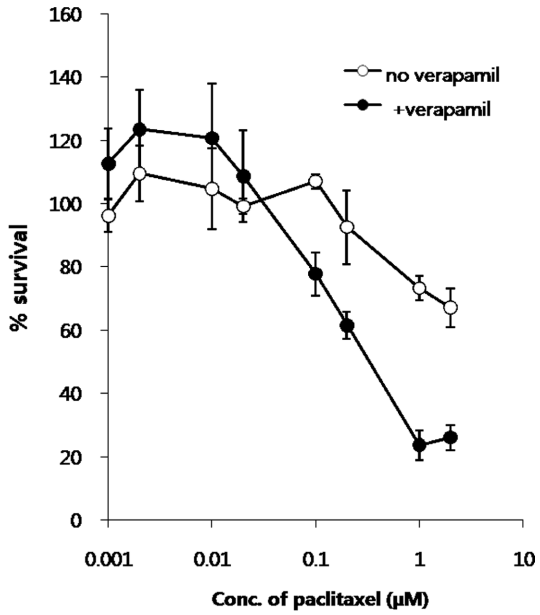

Figure 3. Percent cell survival of MCF-7/ADR when incubated with paclitaxel in tributyrin emulsion for 48 hours in the absence or presence of verapamil.

drug delivered in particulate systems were taken up into cells more than that in free form (Fang et al., 2011; Parveen et al., 2011; Wong et al., 2006), which may be also the case for the TB emulsion and a possible explanation for the enhanced anticancer activity of paclitaxel in TB emulsion.

In recent years, there have been extensive studies to overcome multi-drug resistance caused by p-gp efflux protein through drug-encapsulation into particulate systems (Zhao et al., 2011; Garcion et al., 2006; Wong et al., 2006). TB emulsion would be also an efficient delivery system for overcome the drug efflux by p-gp, and thus were tested for anticancer activity in MCF-7/ADR, which has been known as a p-gp over-expressing MDR variant cancer cell line of MCF-7 (Chavanpatil et al., 2006). When delivered as free form in DMSO, paclitaxel was almost inactive against MCF-7/ADR cells as expected because paclitaxel is a well known substrate for p-gp (Figure 2). Likewise, the anticancer activity of paclitaxel in TB emulsion against MCF-7/ADR was not as strong as that shown in MCF-7. However, paclitaxel in TB emulsion showed significantly higher anticancer activity than PTX in DMSO considering the cell survival rate at 1 μM of paclitaxel (approx. 50% vs. 84% for paclitaxel in TB emulsion and DMSO, respectively, $p<0.05$) (Figure 2). Empty TB emulsion did not kill the MCF-7/ADR cells either showing the percent cell survival rates of 85% and 54% against MCF-7/ADR and MCF-7, respectively. Considering the reduced activity in MDR cells, there might be interaction between p-gp and tributyrin, a prodrug of butyric acid. There have been several studies which reported the induction of p-gp by sodium butyrate in colorectal and leukemia cell lines (Shibata et al., 1990; Bates et al., 1992; Frommel et al., 1993; Marks et al., 1995; Massart et al., 2005). However, there is no clear relationship between sodium butyrate and p-gp function. According to Marks et al, sodium butyrate induced p-glycoprotein in leukemic cells, but sodium butyrate treatment caused an increase in P-glycoprotein without increased drug resistance or without decreased rhodamine-123 accumulation suggesting that the p-gp induced by sodium butyrate was nonfunctional (Marks et al., 1995). Even though the suggested non-functionality of p-gp, there is a study showing that sodium butyrate potentiated doxorubicin activity in thyroid carcinoma cell lines independently ABC transporters (Massart et al., 2005). The potentiating effect might be due to the HDAC activity of butyric acid itself. There have been no reports yet to show the abolished activity of butyric acid in MDR cancer cell lines. It needs further studies to address the reason of the reduced cytotoxicity of TB emulsion in MDR cells.

The diminished anticancer activity of paclitaxel in TB emulsion was almost fully recovered by verapamil, an inhibitor of p-gp (Figure 3). As proposed by many other studies, particulate systems encapsulating drugs could avoid p-gp-mediated efflux to a certain extent, but not completely. Mahesh et

al showed that nanoparticle-encapsulated paclitaxel was susceptible to p-gp-mediated drug efflux and its cytotoxicity was increased by the presence of verapamil (Chavanpatil et al., 2006). Likewise, TB emulsion could also help the encapsulated paclitaxel avoid p-gp-mediated efflux in MCF-7/ADR. Even though it was not full evasion, the result suggested that it would be more difficult for p-gp to remove the drug molecules from the cells when the molecules are associated with nanoparticles (Wong et al., 2006). It appears to be worth trying to co-incorporate into TB emulsion to overcome MDR in the future.

Conclusion

Tributyrin was found to be a good vehicle for solubilization of water-insoluble paclitaxel and formulated into injectable submicron-sized o/w emulsion using phospholipids as stabilizers. Paclitaxel incorporated in tributyrin emulsion showed higher anticancer activity than free form in DMSO against human breast cancer cell line, MCF-7, even though significantly reduced activity in MDR variant, MCF-7/ADR due to the p-gp mediated efflux. Tributyrin emulsion showed great potential as a promising vehicle for water-insoluble anticancer agents.

Acknowledgements

This study was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2006-331-E00440).

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