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The Antiproliferative Effect of Docetaxel-Microemulsion Formulation against HepG2 Liver Cancer Cells

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Docetaxel is a chemotherapeutic agent that is used against various cancers. However, it has several side effects due the solvent used in its formulation. In this study,the antitumor activity of docetaxel-loaded-microemulsion against HepG2 liver cancer cells was assessed by studying the cell toxicity and mechanism of cell death. The microemulsion formula consisted of weight percentages of 37 of cremophor/transcutol mixture (2:1, w/w), 29 of capryol 90 and 34 of water. Three microemulsion formulas were evaluated, freshly prepared docetaxel-loaded-microemulsion (F-ME), stored formula of F-ME for two weeks (O-ME) and blank microemulsion formula (B-ME). The antiproliferative screening using sulphorhodamine B (SRB) assay revealed that the average cytotoxicities of all of the microemulsion formulations (F-ME, O-ME and B-ME) at all micromolar concentrations (1, 5 and 10) against HepG2 cells were more than Taxotere by 3 folds. The three formulas have induced apoptosis through altering the cell membrane in HepG2 cells as revealed by the images of ApopNexin FITC detection kit, light microscopy and transmission electron microscopy. Our findings demonstrated that blank microemulsion as well as the docetaxel-loaded microemulsions was having greater cytotoxicity than Taxotere against HepG2 cells.

Key words: Antitumor activity, ApopNexin FITC detection kit, Sulphorhodamin B assay, Cytotoxicity, Cell viability.

Liver cancer or hepatic cancer is the cancer that begins in the liver. It is a malignant tumor that grows on the surface or inside the liver. It is usually treated by surgical interventions. Since un-resectable tumors shorten patient's survival, it is of utmost importance to find appropriate treatments. Taxanes have shown to inhibit cell growth, induce apoptosis either alone or in combination with other cytotoxic agents[1]. Additionally, using taxanes in the targeted interventional therapies like Ultrasound and Intrarterial Paclitaxel on localized or metastasized tumors have been tried in preclinical and clinical investigations [2]. Therefore, they may represent novel strategies for chemotherapy in liver cancer.

Docetaxel, an antineoplastic agent belonging to the second generation of the taxoid family, was formulated recently in a microemulsion formula and evaluated for its solubilization capacity and oral bioavailability. It is about twice as potent as paclitaxel as an in vitro inhibitor of microtubule depolymerisation. However, the only licensed indication for docetaxel is in the treatment of locally advanced or metastatic breast cancer with a first-line chemotherapy regimen. Currently,

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parenteral formulations are available for clinical use. While oral administration is still limited because of its low oral bioavailability [3], which is in part due to its practically insoluble property 4.93 µg/ml in water [4], as well as its high affinity to the multidrug efflux pump P-glycoprotein (P-gp) and hepatic first-pass metabolism. To improve patient compliance and for efficient combination therapy with other antineoplastic agents, an oral formulation of docetaxel would be useful since oral chemotherapy could ease the use of more chronic regiments [5]. Studies have shown that cyclosporine A (CsA) or interferon-α could increase the oral bioavailability of docetaxel by inhibiting P-gp. When co-administrated with 15 mg/kg CsA, the bioavailability of oral docetaxel (5 mg/m^2), given as an oral drinking solution of the formulation, increased from 8 ± 6% (monotherapy) to 90 ± 44% [6]. In case of interferon-α co-administration in rats, oral bioavailability was improved from 10.4% to 37.2% [7]. However, pre-treatment with a P-gp inhibitor before the oral administration of docetaxel is far from improving patient compliance and may lead to more side effects. Microemulsion is a lipid based delivery system demonstrating absorption enhancement. Several excipients commonly used in these systems, including Cremophor EL, Tween 80, Labrasol and Transcutol, could inhibit the function of P-gp[8] which make the microemulsion system an attractive choice for docetaxel oral delivery. A recent study has showed that microemulsions of about 30 nm in mean diameter were formulated with improved solubilization capacity towards the docetaxel [9]. The oil-in-water microemulsion formulation composed of Capryol 90 (oil), Cremophor EL (surfactant) and Transcutol (co-surfactant) enhanced the solubility of docetaxel up to 30 mg/ml, which maintained solubilization capacity for 24 h, even after it was diluted 20 times with normal saline. Moreover, the oral bioavailability of the formulation in rats rose dramatically compared to the current commercial formula of docetaxel (Taxotere). This increase in bioavailability was probably due to the combined effect of the enhancement in solubility, the inhibition of P-gp efflux system and the increase in permeability. These results encourage further development of docetaxel microemulsions as oral drug delivery systems. The main objective of the present study was to assess the anticancer activity of docetaxel- loaded-microemulsion formulations when applied into HepG2 liver cancer cells.

MATERIAL S AND METHODS

Materials

Cremophor EL, transcutol, capryol 90 and docetaxel were purchased from Jassomah Establishment (Jeddah, Saudi Arabia). Taxotere was generously gifted from King Abdulaziz University Hospital. Modified eagle medium (MEM), vitamins solution, fetal calf serum (FCS), non-essential amino acid, penicillin streptomycin, phenol red, phosphate buffered saline, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution (HEPES), trypsin, sulforhodamine B (SRB) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Chemical Co, St Louis, MO, USA. The distilled water was purified using a water purification system from Bibbysterilin ltd, UK. ApopNexin FITC Apoptosis Detection Kit was purchased from Millipore, MA, USA. All other reagents were of analytical grades. The human cell lines of HepG2 liver cancer was obtained from the Tissue Culture Bank at King Fahd Medical Research Center, Jeddah, KSA.

Methods

Preparation of docetaxel-loaded-microemulsion

Docetaxel-loaded-microemulsion formulations were prepared using the method described by Yin et al.[9]. The microemulsion formula composed of weight percentages of 37 of cremophor/transcutol mixture (2:1, w/w), 29 of capryol 90 and 34 of water. It was produced by mixing the components in subsequent steps. First, the surfactant, cremophor EL, was mixed with the cosurfactant, transcutol, at fixed weight ratio of 2:1. Then, the oil, capryol 90, was added slowly to the desired weight fraction of the surfactant and cosurfactant until the phase was formed. After that, the fixed fraction of the aqueous phase was added drop wise. Finally, the mixture was vortexed and shaken at room temperature to be clear and transparent. The microemulsion formula was stored at 25°C. To prepare docetaxel-loaded-microemulsion, sufficient amount of 1mg/ml of docetaxel was dissolved directly and vortexed well in the liquid microemulsion formula. The examined microemulsion formulations involved in this study were blank microemulsion (B-ME), freshly

prepared docetaxel-loaded-microemulsion (F-ME) and stored docetaxel-loaded-microemulsion for around two weeks (O-ME).

Cell Culture

HepG2 cell lines were cultivated in a tissue culture flask (75 cm^2) containing 10 ml of MEM media supplemented with 10% (v/v) heat inactivated fetal calf serum at 37°C in a 95% air and 5% humidified CO_2 incubator. Medium was discarded from the tissue culture flask and changed at 48 h intervals. Whenever the cells get confluent after continuous feeding, they were collected by trypsinization, washed and passaged every 3 days. Cells, used for experiments, were between passages 7 and 11. They were dissociated with 2 ml of trypsin (0.15M) added to the tissue culture flask, left for few seconds and then trypsin solution was discarded two times with expanding the second time to three min. The experimental cells were incubated in a MEM media (10% FBS) for 24 h in a 95% air and 5% humidified CO_2 incubator at 37°C.

Screening of the antiproliferative effect using SRB assay

SRB assay was performed according to method of Skehan et al. [10]. Cultured HepG2 cells were counted using hemocytometer and seeded at a density of 1 $x10^5$ cells per well containing 0.1 ml of growth medium. After that, cells were incubated with 0.1 ml of media containing (1, 5 and 10) µM of B-ME, F-ME, O-ME and Taxotere solubilized in the media (triplicate wells were prepared for each individual concentration) and re-incubated for additional 48 h at 37°C in a humidified 5% CO_2. Untreated cells were used as control. After the time of incubation, cells were fixed by gentle layering with 50 µl of cold 50% TCA on the top of growth media in each well. The cultures were incubated at 4ºC for one hour and then washed five times with tap water to remove TCA and left for drying at room temperature. TCA-fixed cells were stained for 30 min with 0.4% (wt/vol) of SRB dissolved in 1% acetic acid. At the end of the staining period, SRB was removed and cultures were quickly rinsed four times with 1% acetic acid to remove unbound dye. Bound dye was solubilized with 10 mMunbufferedTris-EDTA (100 µl/well) for 5 min on a gyratory shaker. The numbers of living cells were assayed by measuring the color intensity using enzyme-linked immunosorbent assay, ELISA, reader at wave length of 490 nm. The ratios of vital cell to dead cells were determined to evaluate the cytotoxicity of B-ME, D-ME, O-ME and Taxotere against HepG2 cells.

The cytotoxicity effect was determined by measuring the percentages of cell viability using the following equation:

Cell viability (%) = (Absorbance of the sample)/ (Absorbance of the control) ×100

where the absorbance of the sample and the absorbance of the control were defined as the absorbance of the treated and untreated cells, respectively.

Characterization of cell morphology using light microscope

Cultured HepG2 cells were counted and plated at a density of 1 $x10^5$ cells per well into 96-well, flat-bottomed tissue culture plates containing 0.1 ml of growth medium per well. Cells were incubated with 200 µl of media containing (1, 5, 10) µM of B-ME, F-ME and O-ME. Then, they were washed with 100 µl of PBS for 5 min. After that, 4% of formaldehyde was added for 5 min and then, discarded and stained with a 100 µl of 10% Coomassie blue dye for 10 min. Finally, the stain was discarded and cells were washed with tap water five times, and left to dry overnight at room temperature. Morphological changes were observed by phase contrast inverted microscope (1X17 Olympus, Japan).

Apoptosis detection using ApopNexin FITC assay

The signs of apoptosis induced by 5 µM of B-ME, F-ME, O-ME and Taxotere were inspected by ApopNexin FITC Apoptosis Detection kit (Millipore, Lot. No. 2053919, Billerica, MA, USA). This kit uses a staining protocol in which the apoptotic cells are stained with annexin V conjugated with fluorescein isothiocyanate (FITC) (green fluorescence) which stains phosphatidylserine (PS). All cultured HepG2 cells were plated in 24-well plates (2×10^4 cells per well) and incubated for 24 h. The formulations of 5 µM of B-ME, F-ME, O-ME and Taxotere were introduced to the cells and incubated for another 48 h. The supernatant containing detached cells was removed and put into a tube, centrifuged at 400 x g for 5 min. Then, the supernatant was

taken and the adherent cells were washed twice with cold buffer solution. After that, the detached cells were re-suspended in ApopNexin FITC diluted in 1X binding buffer (10 mM HEPES/ NaOH, pH 7.4, 140 mMNaCl, 2.5 mM $CaCl_2$) and transferred back into the container of adherent cells and incubated for 15 min at room temperature. The supernatant containing detached cells was removed and spun down at 400 x g for 5 min; the cells were re-suspended in 1X binding buffer. Adherent cells were harvested by scraping with a rubber policeman in 200 µl of 1X binding buffer, centrifuged at 400 x g for 5 min and re-suspended in 1X binding buffer (1×10^4 cells/ml). After transferring 100µl of adherent and detached cells to a tube, 2 µl of propidiumiodied (PI) were added followed by incubation for 15 min in ice-cold and dark. All cells were viewed using fluorescence microscope (BX61, DP72 Olympus, USA). The positive of Annexin V-FITC indicates the out-releasing of phospholipid phosphatidylserine (PS), which happens in the early stage of apoptosis. Therefore, the apoptotic cells were identified as Annexin V-FITC+ and PI-. The nonviable cells were identified as Annexin V-FITC+ and PI+ and viable cells as Annexin V-FITC- and PI-.

Cell morphology and ultrastructure of HepG2 cells using Transmission Electron Microscope (TEM)

HepG2 cells were placed in a 6-well plate (2×10^5 cells per well). A 5µM of B-ME, F-ME, O-ME and Taxotere were introduced separately to cells in culture medium for 48 h. The untreated cells were cultured in the MEM cultured medium and were considered the control. To look at the cellular ultrastructure of treated HepG2 cells, thin-sections of cells were investigated using TEM. After 48 h exposure, the cells were digested with trypsin, harvested by centrifugation (1400 rpm×5 min) at 4ºC. Then, cells were collected, prefixed with 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, washed three times, dehydrated in a graded alcohol series, embedded in resin, and cut with an ultramicrotome. Thin sections post stained with uranyl acetate and lead citrate were inspected with MORGAGNI 268 TEM.

Statistical analysis

All values were expressed as mean ± standard deviation of the obtained data from the experiments (each experiment was performed in triplicate). Statistical analyses were performed using one-way analysis of variance (ANOVA) test, two-way ANOVA test and independent sample t-test using the MegaStat. The statistical significance difference was considered when p-value≤05.

RESULTS

Screening of the antiproliferative effect using SRB assay

Different micromolar concentrations (1, 5 and 10) of B-ME, O-ME, F-ME and Taxotere were applied into HepG2 cells. As illustrated in Table 1, it has been found that all of the microemulsion formulations were having similar cytotoxic effect at different concentrations. However, they were having more antiprofilerative effect than Taxotere at all of the selected concentrations. On average, the cell mortalities, when subjected into microemulsion formulations, were more than Taxotere by around 3 folds at all concentrations. According to the statistical analyses using one –factor ANOVA and Fisher's LSD post –hoc test, there were highly significant differences between all of the microemulsion formulations and Taxotere

Table 1. The percentages of cell viability of HepG2 liver cancer cells subjected for 48 h into different micromolar concentrations (1, 5 and 10) of B-ME, F-ME, O-ME and Taxotere. The percentages of cell viability were expressed as mean ± standard deviation

Formula	% Cell Viability at certain micromolar concentration		
	1	5	10
F-ME	16.64 ± 1.70	15.58 ± 2.52	22.99 ± 3.54
O-ME	21.01 ± 3.07	16. 94 ± 1.77	17.17 ± 3.48
B-ME	22.57 ± 1.43	17.60 ± 3.04	18.62 ± 4.51
Taxotere	66.07 ± 26.54	55.07 ± 10.94	62.03 ±11.56

at different concentrations (P = .006), whereas the differences between the microemulsion formulations at all of the concentrations were not significant (P = .293).

Characterization of cell morphology using light microscope

As exhibited in Figures 1-3, it was very noticeable that the chromatids of HepG2 cells were condensed, when treated with 5 µM of B-ME, O-ME and F-ME. However, at 1 and 10 µM of all of the microemulsion formulations membrane blebbing and more intracellular spaces took place between the treated cells.

Apoptosis detection using ApopNexin FITC assay

In order to clarify the mechanism of cell death whether it occurred due to apoptosis or necrosis, ApopNexin FITC apoptosis detection kit was employed. After the treatment of HepG2 cells with 5µM of F-ME, B-ME and Taxotere for 48 h,

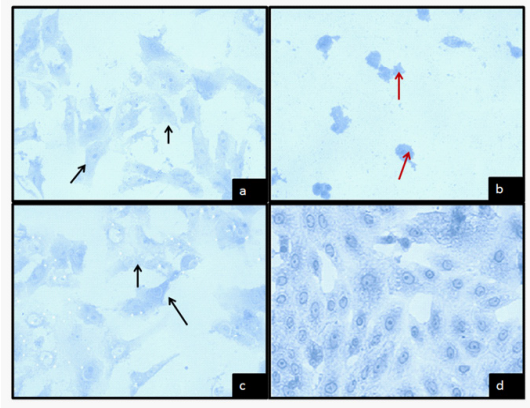

Fig. 1. Light microscopic images of HepG2 liver cancer cells treated with B-ME at different concentrations of (a) 1 µM, (b) 5 µM, (c) 10 µM and (d) 0 µM (control). Images were magnified at 400 µm. *Black arrows* represent the membrane blebbing while the *red arrows* represent the condensed chromatid

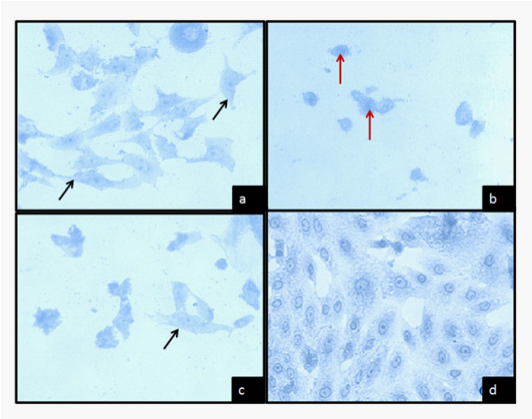

Fig. 2. Light microscopic images of HepG2 liver cancer cells treated with F-ME at different concentrations of (a) 1 µM, (b) 5 µM, (c) 10 µM and (d) 0 µM (control). Images were magnified at 400 µm. Black arrows represent the membrane blebbing while the red arrows represent the condensed chromatid.

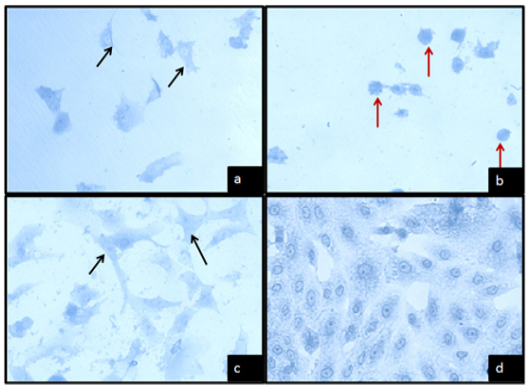

Fig. 3. Light microscopic images of HepG2 liver cancer cells treated with O-ME at different concentrations of (a) 1 µM, (b) 5 µM, (c) 10 µM and (d) 0 µM (control). Images were magnified at 400 µm. *Black arrows* represent the membrane blebbing while the *red arrows* represent the condensed chromatid

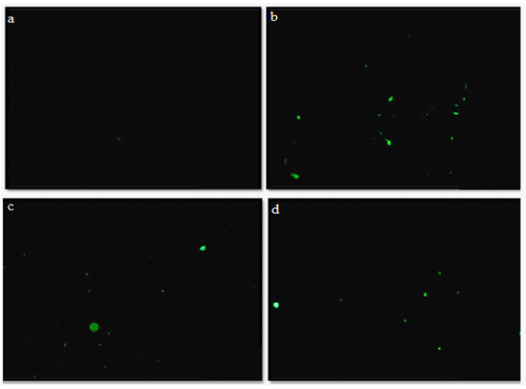

Fig. 4. Fluorescent microscopic images of HepG2 liver cancer cells labelled with Annexin-V-FITC and propidium iodide. Images were magnified at 200 µm. (a) Untreated cells, cells treated with 5 µM of (b) B-ME,(c) F-ME and (d) Taxotere

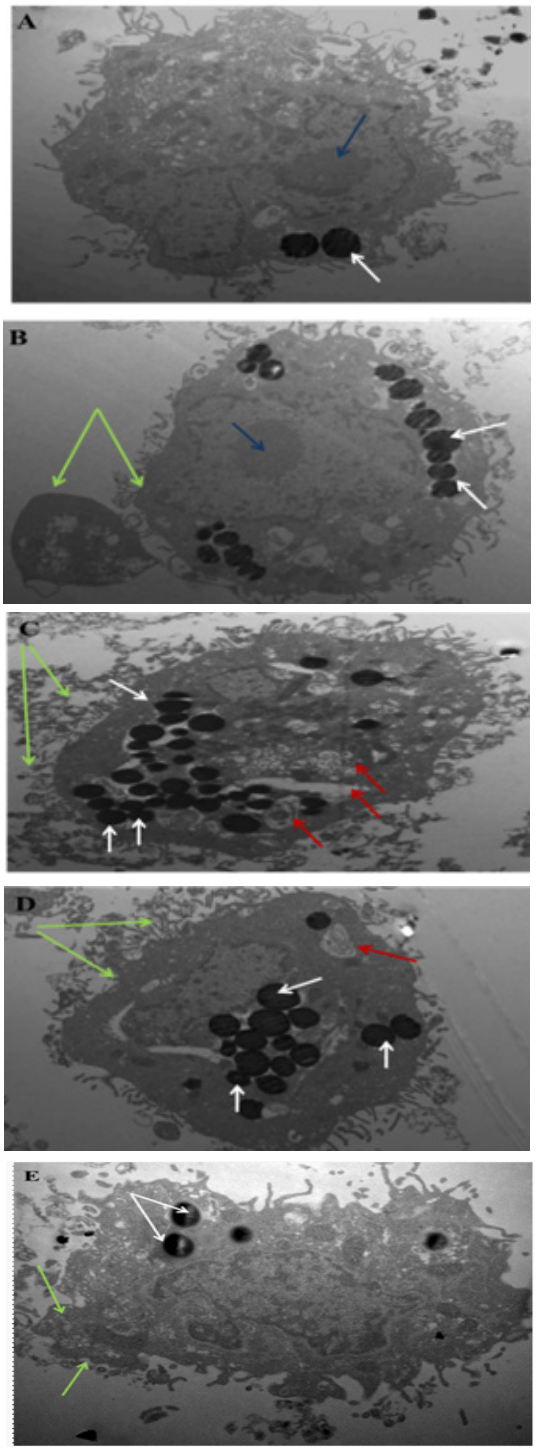

Fig. 5. Electron micrographs of untreated (A) and treated HepG2 cells with 5 µM of (B) B-ME, (C) F-ME, (D) O-ME and (E) Taxotere.*Blue, white, green and red arrows* represent the nucleolus, lysosomes, membrane blebbing and vacuoles, respectively

the cells were labeled with annexin V conjugated with FITC and PI, then viewed by fluorescence microscope. As shown in Figure 4, untreated cells didn't stain positively with neither dyes which indicates the viability of cells, while all the treated cancer cells with F-ME, B-ME and Taxotere were stained positively green fluorescent with annexin-V-FITC but not with PI which implies early signs of apoptosis with no detectable necrotic effect due to the externalization of PS caused by the cell surface outbreak.

As exhibited in Figure 5, the late signs of apoptosis were observed in the cells that were treated with F-ME, O-ME and Taxotere as the nucleolus vanished. However, the mechanism of cell death in the cells treated with both of F-ME and O-ME was different from the Taxotere as more lysosomes and vacuoles containing cell debris and organelles were seen in F-ME and O-ME treated cells. On the other hand, B-ME disturbed only the cell membrane of HepG-2 cells without affecting the nucleus.

DISCUSSION

Docetaxel, a toxoid chemotherapeutic agent, is mainly applied intravenously to cure various cancers. Docetaxel was loaded in several delivery systems that improved its efficiency [9, 11-18]. It has been encapsulated in a Tween 80-free liposomal formulation with better pharmacokinetic properties than free docetaxel, while maintaining the same in vitro cytotoxicity [11]. Additionally, drug plasma concentration as well as drug disposition up to 24 h was considerably increased in docetaxel liposomal formulation than in Tween 80. Moreover, docetaxel was recently formulated in a liposome that has a pH-dependent release behavior, which would be favorable for selectivity against tumor cells [18]. Compared with Duopafei®, the liposomal formula of docetaxel gave a prolonged residence time of the drug in mice and an improved efficiency to reticuloendothelial system (RES) organs, such as the liver and spleen, but not to non-RES organs, which might potentially contribute to decrease the risk of toxicity. Furthermore, a 0.8 mg/ml of docetaxel was encapsulated in emulsion that composed of 10% oil phase (Soya oil and Miglyol 812), 1.2% soybean lecithin and 0.3% Pluoronic F68 [4]. The submicron lipid emulsion

showed promising intravenous carrier in place of the available commercial ones with more efficiency. Zhao et al. [16] have also formulated docetaxel in lipid emulsion that exhibited higher plasma concentrations in rats than Taxotere but bioequivalent to it in beagle dogs. The docetaxel loaded-lipid emulsion displayed safe effect and exhibited antitumor activities against the A549, BEL7402 and BCAP-37 cell lines in nude mice, similar to Taxotere. Hwang et al. [13] have produced a nano-sized drug carrier, consisted of hydrophobically modified glycol chitosan, which easily loaded the docetaxel. The docetaxel-loaded nanocarriers showed higher antitumor efficacy such as reduced tumor volume and increased survival rate in A549 lung cancer cells-bearing mice and strongly reduced the anticancer drug toxicity compared to that of free docetaxel in tumor-bearing mice.

Some researches attempted to encapsulate docetaxel in micelles in order to improve its efficiency and reduce its side effects. Liu et al. [14] have evaluated the novel docetaxel-loaded micelle which was based on the biodegradable thermosensitive copolymer poly(N-isopropylacrylamide-co-acrylamide)-b-poly(dl-lactide). Docetaxel-loaded- micelle showed reduced toxicity and higher antitumor efficacy in mice as well as enabling a prominent higher docetaxel concentration in tumor than conventional docetaxel formulation. It also caused less body weight loss of mice. Another recent study have incorporated docetaxel into mixed micelles that comprised of methoxypoly(ethylene glycol)–poly (lactide) polymer and Pluronic copolymers. The micelle formula have enhanced the bioavailability (3.6 fold) and overcome the multidrug resistance of docetaxel in rats [17]. Docetaxel was recently formulated in a solid lipid nanoparticle. Xu et al. [15] produced a new docetaxel-loaded hepatoma-targeted solid lipid nanoparticle (tSLN) that was prepared with galactosylateddioleoylphosphatidyl ethanolamine. Cytotoxicity of tSLNs against hepatocellular carcinoma cell line BEL7402 was superior to Taxotere. The tSLNs also showed better tolerant and antitumor efficacy in murine model bearing hepatoma compared with Taxotere.

Microemulsion (MEs) is a lipid based delivery system demonstrating absorption enhancement. The major advantages include high solubilization potential, thermodynamic stability, improved dissolution of lipophilic drugs and surfactant-induced permeability enhancement[19, 20]. Additionally, several excipients commonly used in these systems including Cremophor, Tween 80, Labrasol and Transcutol could inhibit the function of P-gp making the microemulsion system an attractive choice for docetaxel oral delivery [8, 21, 22]. The MEs have recently attracted much attention in pharmaceutical research areas. High thermodynamic and kinetic stability, low viscosity and optical transparency make them very attractive as a pharmaceutical application form to improve the solubility, the dissolution and the oral absorption of poorly water-soluble drugs [23]. Further advantages of using MEs as drug delivery systems include a better drug solubilization and the protection against enzymatic hydrolysis, as well as the potential for an enhanced absorption due to a surfactant-induced improvement in the permeability. In addition, MEs represent an interesting and potentially quite powerful alternative carrier system for drug delivery because of their high solubilization capacity, transparency, ease of preparation, and high diffusion and absorption rates, compared to solvents without the surfactant system [9]. The combination of surfactants with oils to form MEs offers an advantage with a low free energy and a large surface area, which were considered to be responsible for transporting drugs to cancer tissue membrane for absorption [18].

In this study, the selected microemulsion formula that consisted of weight percentages of 37 of cremophor/transcutol mixture (2:1, w/w), 29 of capryol 90 and 34 of water was based on the maximum solubilization of docetaxel (30 mg/ml)[9]. The mean diameter of the microemulsion droplet, measured by the electrophoretic light-scattering spectrophotometer, was 35.1 ± 3.1. In other words, the small droplet size of microemulsion formulations enhances the permeability of docetaxel into HepG2 cells and stimulates the lysosomes and vacuoles to produce the digestive enzymes responsible to degrade the cancer cell organelles as confirmed by TEM.

According to the SRB assay, the average cytotoxicities of the entire drug microemulsion formulations at all concentrations, were more than Taxotere by 3 folds. There were no significant differences in their effect on the viability of the

cells. In fact, SRB or kiton red ($C_{27}H_{30}N_2O_7S_2$) is a red, solid, water-soluble dye that binds to the basic amino acid of the cellular proteins when the cell membrane get altered [10]. As confirmed by images of the TEM, it has been found that the cell membrane has changed when subjected into all of microemulsion formulas.

ApopNexin FITC Apoptosis Detection Kit was employed to study the mechanism of cell death of HepG2 treated with 5µM of F-ME, B-ME and Taxotere as they revealed their apoptotic effect according to the cell membrane blebbing. Similar to the results of SRB assay, there were no significant differences between the three formulas. The ApopNexin FITC Apoptosis Detection Kit is designed to detect a specific biochemical change in the cell surface membrane that is considered to be a signature event of early apoptosis [24]. It contains Annexin V conjugated with FITC, allowing for convenient quantitative assays. The counterstain, PI, is used to distinguish apoptotic cells with intact membranes from lysed, necrotic cells. Early in the apoptotic process, the characteristic of cell surface phospholipid asymmetry is disrupted. This leads to the exposure of PS on the outer leaflet of the cell membrane. Annexin V is an anticoagulant protein that preferentially binds PS and, when conjugated to a reporter molecule, can be used as an indicator of apoptosis. The Annexin V conjugate is involved to facilitate the rapid and early detection of cell surface changes associated with apoptosis. Annexin V binding to PS occurs only in dying cells.

As elaborated by the SRB, ApopNexin FITC Apoptosis Detection Kit and TEM images, encapsulating docetaxel in microemulsion improved its diffusion into the cell and hence, induced apoptosis. The microemulsion formula did not show instability as there were no differences between F-ME and O-ME in their cytotoxicities, determined by SRB assay, and their apoptotic effect observed by TEM.

CONCLUSION

In this study, the microemulsion formula, consisted of weight percentages of 37 of cremophor/ transcutol mixture (2:1, w/w), 29 of capryol 90 and 34 of water, was produced. The cytotoxicity screening using SRB assay revealed that the average cytotoxicities of all of the microemulsion formulations (F-ME, O-ME and B-ME) at all micromolar concentrations (1, 5 and 10) against HepG2 cells were more than Taxotere by 3 folds. The apoptotic effect of O-ME and F-ME on the HepG2 cells were observed under the TEM as the nucleolus was vanished and more digestive vacuoles were stimulated. In conclusion, all microemulsion formulations (free or loaded with drug) were having greater cytotoxicity that Taxotere. It is recommended to make further in vivo studies on all of the microemulsion formulations.

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REFERENCES

1. Okano, J.I., Nagahara, T., Matsumoto, K., Murawaki, Y. The growth inhibition of liver cancer cells by paclitaxel and the involvement of extracellular signal-regulated kinase and apoptosis. *Oncology Rep.,* 2007; **17**: 1195-200.
2. Kang, J., Wu, X., Wang, Z., Ran, H., Xu, C., Wu, J., Wang, Z., Zhang, Y.Antitumor effect of docetaxel-loaded lipid microbubbles combined with ultrasound-targeted microbubble activation on VX2 rabbit liver tumors. *J. Ultrasound Med.,* 2010; **29**: 61-70.
3. Kuppens, I.E., Bosch, T.M., van Maanen, M.J., Rosing, H., Fitzpatrick, A., Beijnen, J.H., Schellens, J.H.Oral bioavailability of docetaxel in combination with OC144-093 (ONT-093). *Cancer Chemother.Pharmacol*., 2005; **55** (1): 72-78.
4. Gao, K., Sun, J., Liu, K., Liu, X., He, Z.Preparation and characterization of a submicron lipid emulsion of docetaxel: submicron lipid emulsion of docetaxel. *Drug Dev. Ind. Pharm*., 2008; **34:** 1227–1237.
5. Pfeiffer, P., Mortensen, J.P., Bjerregaard, B., Eckhoff, L., Schønnemann, K., Sandbergd, E., Aabo,K., Jakobsen, A.Patient preference for oral or intravenous chemotherapy: a randomised cross-over trial comparing capecitabine and Nordic fluorouracil/leucovorin in patients with colorectal cancer. *Eur. J. Cancer.,* 2006; **42**:

2738-2743.

6. Malingré, M.M., Richel, D.J., Beijnen, J.H., Rosing, H., Koopman, F.J., Ten BokkelHuinink W.W., Schot, M.E., Schellens, J.H.Coadministration of cyclosporine strongly enhances the oral bioavailability of docetaxel. *J.Clin.Oncol.,* 2001; **19**:1160–1166.
7. Ben Reguiga, M., Bonhomme-Faivre, L., Farinotti, R. Bioavailability and tissular distribution of docetaxel, a P-glycoprotein substrate, are modified by interferon-alpha in rats. *J. Pharm. Pharmacol*., 2007; **59 (**3):401-8.
8. Lin, Y.L., Shen, Q., Katsumi, H., Okada, N., Fujita, T., Jiang, X.H., Yamamoto, A.Effects of Labrasol and other pharmaceutical excipients on the intestinal transport and absorption of rhodamine123, a P-glycoprotein substrate, in rats. *Biol. Pharm. Bull.,* 2007; **30**: 1301–1307.
9. Yin, Y.M., Cui, F.D., Mu, C.F., Choi, M.K., Kim, J.S., Chung, S.J., Shim, C.K., Kim, D.D.Docetaxel microemulsion for enhanced oral bioavailability: preparation and in vitro and in vivo evaluation. *J. Control.Release.,* 2009; **140**: 86-94.
10. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, MR.New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.,* 1990; **82**: 1107-1112.
11. Immordino, M.L., Brusa, P., Arpicco, S., Stella, B., Dosio, F.,Cattel, L. Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing docetaxel. *J. Control. Release*., 2003; **91**(3): 417-29
12. Musumeci, T., Ventura, C.A., Giannone, I., Ruozi, B., Montenegro, L., Pignatello, R., Puglisi, G.PLA/PLGA nanoparticles for sustained release of docetaxel. *Int. J. Pharm*., 2006; 325(1-2):172-179.
13. Hwang, H.Y., Kim, I.S., Kwon, I.C., Kim, Y.H. Tumor targetability and antitumor effect of docetaxel-loaded hydrophobically modified glycol chitosan nanoparticles. *J. Control. Release.,* 2008; **128**(1): 23–31.
14. Liu, B., Yang, M., Li, R., Ding, Y., Qian, X., Yu, L., Jiang, X. The antitumor effect of novel docetaxel-loaded thermosensitive micelles. *Eur. J. Pharm.Biopharm.,* 2008; **69**(2): 527-34.
15. Xu, Z., Chen, L., Gu, W., Gao, Y., Lin, L., Zhang, Z., Xi, Y., Li, Y.The performance of docetaxel-loaded solid lipid nanoparticles targeted to hepatocellular carcinoma. *Biomaterials,* 2009; **30**(2): 226-232.
16. Zhao, M., Su, M., Lin, X., Luo, Y., He, H., Cai, C., Tang, X.Evaluation of docetaxel-loaded intravenous lipid emulsion: pharmacokinetics, tissue distribution, antitumor activity, safety and toxicity. *Pharm. Res.,* 2010; **27**(8):1687-1702.
17. Mu, C.F., Balakrishnan, P., Cui, F.D., Yin, Y.M., Lee, Y.B., Choi, H.G., Yong, C.S., Chung S.J., Shim, C.K., Kim, D.D.The effects of mixed MPEG-PLA/Pluronic copolymer micelles on the bioavailability and multidrug resistance of docetaxel. *Biomaterials,* 2010; **31**(8):2371-2379.
18. Zhang, Y.T., Huang, Z.B., Zhang, S.J., Zhao, J.H., Wang, Z. In vitro cellular uptake of evodiamine and rutaecarpine using a microemulsion. Int. *J.Nanomedicine*, 2012; **7**: 2465-2472.
19. Shah, N.H., Carvajal, M.T., Patel, C.I., Infeld, M.H., Malick, A.W. Self-emulsifying drug delivery systems (SEDDS) with polyglycolyzed glycerides for improving in vitro dissolution and oral absorption of lipophilic drugs. *Int. J. Pharm.,* 1994; **106**:15–23.
20. Constantinides, P.P. Lipid microemulsions for improving drug dissolution and oral absorption: physical and bio- pharmaceutical aspects. *Pharm. Res*., 1995; **12**:1561–1572.
21. Rege, B.D., Kao, J.P., Polli, J.E. Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers. *Eur. J. Pharm. Sci*., 2002; **16**: 237–246.
22. Takahashia, T., Sutherlanda, S.C., Sweeneya, C., Poissonb, A., Metzlb, N., Tilbrookc, B., Bates, N., Wanninkhof, R., Feely, RA.Global sea–air CO_2 flux based on climatological surface ocean pCO_2, and seasonal biological and temperature effects. *Deep-Sea Research II*, 2002; **49**:1601–1622
23. Tsai, Y.H., Hsieh, Y.H., Huang, Y.B., Chang, J.S., Huang, C.T., Wu, P.C. Microemulsions for intravesical delivery of gemcitabine. *Chem. Pharm. Bull*., 2010; **58**: 1461-1465.
24. Casciola-Rosen, La., Rosen, A., petri, M., Schlissel, M. surface blebs on apoptotic cells are sites of enhanced procoagulant activity: Implications for coagulant events and antigenic spread in systemic Iupuserythermatosus. *Proc. Natl. Acad. Sci.,* 1996; **93**: 1624-1629.