The Apoptotic Effect of Gemcitabine-Loaded-Microemulsion (Isopropyl Myristate/Tween 80/Span 20/Water/Ethanol) on A549 Non-Small Cell Lung Cancer Cells

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Summary Gemcitabine (GEM) is a chemotherapeutic agent with several dose limitation aspects. It was encapsulated in three microemulsion (ME) formulations with nanoparticles that differ in polarity: hydrophilic MEa, hydrophobic MEb and relatively neutral MEc. The apoptotic effect of the ME formulations was evaluated in the A549 non-small cell lung cancer cells, whereas the side effects of the formulas on the healthy cells were tested in the HFS human foreskin cells. The cell toxicity of all GEM-loaded-MEs (MEa+, MEb+ and MEc+) and free GEM solutions at concentrations of 1 and 10 \( \mu \text{M} \) was determined by using sulphorhodamine B (SRB) assay, while the mechanism of cell death was assessed by using ApopNexin FITC apoptosis detection kit and viewing the ultrastructure of treated A549 cells by using transmission electron microscope (TEM). It has been found that 10 \( \mu \text{M} \) of MEb+ (MEb10+) has the best antiproliferative and apoptotic effect compared to all of the ME formulations and GEM solutions. MEb10+ reduced the percentages of A549 cell viabilities to 11.15 \( \pm \) 1.4 whereas 10 \( \mu \text{M} \) of GEM (GEM10) decreased the percentages of A549 cell viabilities to 58.09 \( \pm \) 2.5. This study verified that ME formulations with hydrophobic droplets improved the therapeutic potential of GEM as an anticancer drug.

Key words Antitumor activity, Cytotoxicity, Nanoparticle, Transmission electron microscope.

One of the most common worldwide fatal diseases is cancer (Anand et al. 2008, Torre et al. 2016). The anti-proliferative chemotherapeutic agents that are extensively involved in treating several cancers with high mortality rates are more connected to unpleasant side effects that reduce their success in treating cancer (Khdair et al. 2010). Therefore, there are critical needs for combining anticancer drugs with other agents (Hu and Zhang 2012).

One of these anticancer agents is GEM which is a deoxycytidine analogue, an antagonist of DNA synthesis with an efficient antitumor activity. In fact, GEM is rapidly metabolized into the inactive uracil derivative when subjected intravenously, which means that it has to be administered in high doses (40 mg mL\(^{-1}\)) and thereby severe side effects would develop (Reddy and Couvreur 2008). Therefore, there has been a drive to design and develop new drug delivery systems which allow efficient passage and high bioavailability of the drug. In order to reduce GEM’s toxicity, it was linked to various drug delivery systems such as liposomes (Celano et al. 2004), gold nanotubes (Patra et al. 2008), carbon nanotubes (Arsawang et al. 2011), and chitosan (Arias et al. 2011).

A recent form of drug delivery for GEM was proposed by Tsai et al. (2010) which was ME formulations that differ in composition. MEs are defined as a mixture of water, oil, and an amphiphile which is optically isotropic and thermodynamically stable and does not require energy for the dispersion process. The production of ME structure is affected mainly by the fraction of oil and water. The structure can be oil-in-water (o/w) when more fractions of water are used; water-in-oil (w/o) at which the amount of oil is more than the water; or bicontinuous at which equal amounts of both of water and oil are utilized. The interfacial surfactant in each structure separates the water and oil domains (Bagwe et al. 2001). These peculiar behaviors of MEs have grabbed the attention of many different kinds of industries. MEs are themselves reactors since they are used for synthesizing nanomaterials, polymers and can hold organic, inorganic and enzymatic processes (Bonacucina et al. 2009).

Many research studies and reviews have demonstrated that MEs could be subjected intravenously because of their thermodynamic stability, low viscosity, small droplet sizes that enhance their blood circulation time and applicability to be sterilized (Ma et al. 2013). Additionally, MEs do not cause emboli formation in the blood and are less painful on injections relative to other co-solvent formulations. In fact, Tsai et al. (2010) used a catheter to administer GEM-loaded-MEs for treating bladder cancer, which is the regular method used in treating such cancer.

In case of non-small cell lung cancers, MEs as a deliv-
ery vehicle would be a good choice for such cancers that are usually treated with anticancer drugs intravenously. Furthermore, it is worth noting that a phase II study of intravesical GEM dissolved in alkaline solution demonstrated that GEM underwent crystallization under certain conditions, high pH, addition of sodium bicarbonate and storage at low temperature, which minimized the amount of GEM subjected into the syringes (Manners et al. 2011). The objective of the present study was to evaluate the ME formulations, designed by Tsai et al. (2010), in vitro. The ME formulas consisted of isopropyl myristate (IPM) as the oil phase, a surfactant mixture of polyoxyethylene sorbitan monooleate (tween 80) and sorbitan monolaurate (span 20), and an aqueous phase of distilled water and cosurfactant of ethanol. They were o/w (MEA) with hydrophilic nanoparticles, w/o (MEb) with hydrophobic nanoparticles and bicontinuous (MEc) with equivalent hydrophilic and hydrophobic properties of the nanoparticles.

Materials and methods

Materials and cell lines

IPM was purchased from Jamjoom Pharma, Jeddah, KSA. Polyoxyethylene sorbitan monooleate (tween 80) was procured from El Nasr Pharmaceutical Chemicals Co., Egypt. Span 20, modified eagle medium (MEM), phosphate buffered saline, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer solution (HEPES), trypsin, SRB, trichloroacetic acid (TCA) and pure GEM were purchased from Sigma-Aldrich Chemical Co., MO, U.S.A. Ethanol was obtained from Fisher Chemical, U.K. The distilled water was purified using a water purification system from Bibby sterilin Ltd., UK. ApopNexin FITC Apoptosis Kit was purchased from Millipore (Lot No. 2053919, Billerica, MA, U.S.A.). The human cell lines of A549 and HFS were obtained from the American Type Tissue Culture Collection (Manassas, VA, U.S.A.).

Methods

Preparation of the microemulsion formulations

Blank and GEM loaded-ME formulations were prepared using a method described by Tsai et al. (2010).

As demonstrated in Table 1, the blank ME formulations, MEa, MEb and MEc were produced. The GEM loaded-ME formulas were prepared by adding directly 1 mg mL⁻¹ of GEM to the blank ME formulas, and were designated as MEa+, MEb+, and MEc+.

In vitro evaluation of antitumor activity

Cell culture

Both A549 and HFS cell lines were seeded in a cell culture flask (25 cm²) containing 10 mL of MEM media at 37°C in a 95% air and 5% humidified CO₂ incubator. The medium was discarded from the cell culture flask and changed at 48 h intervals. Cells were fed until confluence and confluent cells were collected by trypsinization, washed and passaged every three days. They were dissociated with 2 mL of trypsin (0.15%) added to the cell culture flask, left for a few seconds and then the trypsin solution was discarded. The addition of trypsin was repeated, but the time was extended to 3 min. The tested cells were incubated in 10% of MEM culture medium for 24 h in a 95% air and 5% humidified CO₂ incubator at 37°C.

Determination of antiproliferative effect using SRB assay

The colorimetric SRB assay was employed to examine the viability of cells treated with blank and drug-loaded MEs formulations by staining the total cellular protein. Higher percentages of cell viability are an indication of low cytotoxicity of the formula, and vice versa. The SRB assay was implemented as described elsewhere (Skehan et al. 1990). In brief, each well containing 100 μL of growth medium in a 96-well, flat-bottomed tissue culture plate was seeded with 1×10⁴ cells. After that, cells were subjected into 200 μL of 1 and 10 μM concentrations of blank MEs (MEA, MEb and MEc), GEM loaded-MEs (MEA+, MEb+ and MEc+) and GEM solutions followed by incubation for 48 h at 37°C in a humidified 5% CO₂. It should be noted that all of the formulations were designated by 1 and 10 when used at 1 and 10 μM, respectively, and each tested formula was performed in triplicate. Untreated cells were used as control. The number of living cells was measured according to the color intensity at a wavelength of 490 nm. The cytotoxicity effect was determined by measuring the percentages of cell viability through dividing the absorbance of the treated cells by the absorbance of the untreated cells and multiplying it by 100.

Detection of apoptosis using ApopNexin FITC kit

ApopNexin FITC assay detects the translocation of the phosphatidylserine (PS) from the inner to the outer membrane of the cells undergoing early signs of apoptosis. In particular, annexin V conjugated with fluorescein isothiocyanate (FITC) gets attached to the exposed PS and gives a green fluorescence. Cultured cell lines, A549 and HFS, were seeded in 24-well plates (2×10⁴ cells per well) and incubated for 24 h. MEb10, MEb10+, GEM10

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<th>Microemulsion type</th>
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and the media as control were subjected to the cells and left to grow for 48 h. The supernatant containing detached cells was removed and put into a tube, centrifuged at 1600 rpm for 5 min. After that, the adherent cells were washed twice with cold buffer solution while the supernatant containing the detached cells was re-suspended in ApopNexin FITC diluted in 1 × binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and transferred back into the container of adherent cells and incubated for 15 min at room temperature. All cells were viewed using a fluorescence microscope (BX61, DP72 Olympus, U.S.A.). Cells that fluoresced were identified as apoptotic whereas cells that did not fluoresce were considered viable.

**Cell morphology and ultrastructure of A549 using transmission electron microscope (TEM)**

A549 cells were placed in a 6-well plate (2 × 10⁵ cells per well). MEb10, MEb10+ and GEM10 were introduced separately to cells in culture medium with a final concentration of 200 µg mL⁻¹ and incubated 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 A549 cells, thin sections of the cells were investigated using TEM. After 48 h incubation, the cells were digested with trypsin, harvested by centrifugation (1400 rpm × 5 min) at 4°C, 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 was viewed with MOR-GAGNI 268 TEM.

**Statistical analysis**

All values were expressed as mean ± standard deviation as each experiment was performed in triplicate. Statistical analyses were performed with one-way analysis of variance (ANOVA) test, two-way ANOVA test and the independent sample t-test for the pairwise test using the MegaStat. The difference was considered significant when p-value < 0.05.

**Results**

**Cytotoxicity screening using SRB assay**

As shown in Fig. 1a, MEb10+ has the best antiproliferative effect compared to all of the subjected formulas as the percentage of cell viability was 11.15 ± 1.4. In contrast, MEb1+ and MEd1+ and MEd1+ have similar cytotoxic effects to GEM1. It is noteworthy to mention that there was a significant reduction in the percentages of cell viabilities of all of the drug-loaded-MEs when their concentrations increased from 1 to 10 µM, whereas the cytotoxicity of GEM1 did not considerably differ from GEM10. In addition, all of the blank MEs at both 1 and 10 µM have a slight antiproliferative effect on the A549 cells.

Furthermore, the cytotoxicity of the drug formulations was scanned against HFS cells with the aim to find out the safest formula that has minimized side effects (Fig. 1b). Interestingly, MEb10+ has the least cytotoxic effect among all of the drug formulas. In addition, all of the blank MEs have a slight cytotoxic effect on HFS cells. On the other hand, both GEM1 and GEM10 were more cytotoxic than all of the ME formulas.

**Effect of ME formulations on apoptosis induction of cancer cells**

As shown in Fig. 2, the untreated cells did not stain with green fluorescent which signifies the viability of cells, whereas all the cells treated with GEM10, MEd10 and MEd10+ were stained positively, which indicates...
signs of apoptosis. MEb10+ strongly induces apoptosis (% apoptosis=66±2.1) compared to GEM10 (% apoptosis=35±1.3) when applied into A549 cells. On the other hand, the cytotoxic effect of MEb10+ (% apoptosis=10±1.1) was significantly less than GEM10 (% apoptosis=25±1.2) when subjected into HFS cells. It should be noted that the blank ME, MEb10, has a more apoptotic effect on A549 cells (% apoptosis=20±1.1) than HFS cells (% apoptosis=6±1.3).

Cell morphology and ultrastructure of A549 cells using TEM

In order to elucidate the exact mechanism of cell death caused by the exposure of A549 cells to 10 µM of GEM10, MEb10 and MEb10+ for 48h, TEM was utilized. Untreated cells have been considered as a control (Fig. 3a). Cells were rounded and contained few autolysosomes (AL) which are mature autophagosomes, membrane-bound compartment containing cytoplasmic...
material and/or organelles, fused with lysosomes, which are vesicles, contain digestive enzymes that break down waste and foreign materials. The structure of the cells treated with MEb10 was similar to the control as displayed in Fig. 3b.

In contrast, few cells were obtained when treated with GEM10 (Fig. 4), one of which revealed late signs of apoptosis that included the observation of the chromatid fragments (CH), the condensed mitochondria (M), the formation of early autophagosomes (EAP), which are cytoplasmic constituents of cells engulfed within a cytoplasmic vacuole, small vesicles (sv), pre-autophagosomes (PAP), which are a punctuate structure localized in the vicinity of the vacuole that is necessary for the production of autophagosomes, AL's that included swollen mitochondria and endoplasmic reticulum, and Whorl, which is a large degradative endosome, featured by a point of entry from an external, neighboring structure and multiple convoluted membranes, which usually spans many sections.

A few A549 cells treated with MEb10+ were ultrastructurally visualized (Fig. 5). One of the cells displayed late signs of apoptosis integrated obviously with the autophagy pathway as the nucleolus disappeared, leaving few chromatid fragments and the development of multi-vesicular bodies (MVBs), AL's, Whorl, EAP's, condensed M's, membrane blebbing, pinocytic invagination and apoptotic bodies were observed.

Discussion

Lung cancer has been one of the most challenging cancer chemotherapy. Therefore, there has been a need to find and design new cancer therapies that are more effective and less toxic (Clegg et al. 2001). Many new cytotoxic drugs have been successfully discovered that can be used for cancer treatment. However, severe side effects and acquired drug resistance are usually accompanied by chemotherapy. GEM is a drug used in treating cancer; primarily the active GEM diphosphate and GEM triphosphate kills cells enduring DNA synthesis (S-phase) and also impedes the growth of cells through the G1/S-phase boundary by the incorporation of GEM triphosphate into DNA, leading to inhibition of DNA synthesis and apoptosis induction. However, this would cause serious side effects to normal cells, such as bone marrow toxicity. Accordingly, it had been suggested to develop target therapy that allows greater tumor specificity and less toxicity.
Target drug delivery to malignant cells can be employed by using nanotechnology that will reduce the toxicity and side effects of the anticancer drug to non-target cells (Arsawang et al. 2011). As demonstrated by some studies, using GEM alone does not have a significant cytotoxic effect against A549 cells. Clegg et al. (2001) stated that GEM alone did not lead to significant improvements in survival rates for patients with non-small cell lung cancers. Denlinger et al. (2004) found out that administering doses of GEM ranging from 1 to 200 µM into A549 cells did not have any noticeable antitumor effect whether applied in vitro or in vivo.

In the present study, which involved SRB assay in screening the cytotoxicity, there was a low cytotoxic effect of GEM against A549 cells compared to the ME formulations, and it was not sensitive to the change in concentration from 1 to 10 µM. However, 10 µM of the drug-loaded-MEs (MEa10+, MEb10+ and MEc10+) has had significantly more cytotoxic effect than 1 µM of the same ME formula (MEa1+, MEb1+ and MEc1+). The best formula was the hydrophobic delivery, MEb10+, which increased the mortality of the cells when compared with all of the drug formulas. Tsai et al. (2010) produced three GEM-loaded-ME formulas that release GEM at different rates depending on the fraction of the ME components. The release rates of MEa, MEb, and MEc were 630.1±55.0, 390.3±96.2, and 141.1±9.6 mg cm⁻² h⁻¹/², respectively. In other words, they have showed that GEM release from the ME formulations is within 8 h, which implies the complete discharge of the drug from the ME formulations into the cells within 48 h of exposure, which shows that the ME formulations enhance the efficacy of GEM and have a strong antitumor activity.

Moreover, nanoparticles of GEM, which were fabricated using chitosan polymer, have exhibited significant improved antitumor activity compared to the free GEM in mice bearing L1210 wt subcutaneous tumor (Arias et al. 2011). Another study has found out that hydrophilic multi-walled carbon nanotubes attached to magnetite nanoparticles have extravagated to the lymph vessels and delivered GEM to lymph nodes with high efficiency under the control of a magnetic field (Yang et al. 2009).

In this study, it was noticed that there were no considerable discrepancies in the cell viabilities of HFS measured by SRB assay when treated with blank MEs. In fact the constituents of the ME have safe effect; 10 mg kg⁻¹ of tween 80 was recommended for use in preclinical safety studies when examined for three months in mice, rats, dogs, and monkeys (Thackaberry et al. 2010). The other component of the ME, span 20, was found to have limited toxicity in rats (Cater et al. 1978), while isopropyl myristate was nontoxic (CIR Expert Panel 1982). The co-surfactant, ethanol, would cause different types of cancer when consumed by humans regularly over time (Nelson et al. 2013). However, mixing a very small amount of ethanol with the MEs would help the drug in permeating the cancer cells. It should be mentioned here that Tsai et al. (2010) did not assess the antitumor activity of the blank ME formulations. Interestingly, their histological examination of bladder tissue revealed that the blank ME formulation did not have any systemic side effect.

The present study has shed light on how the ME vehicle delivers GEM inside the A549 cells, which is considered a very important step in understanding the mechanism of action of this new drug-loaded formula against the cancer cells. The ultrastructure of the treated A549 cells has shown that the ME formulas have enhanced the antitumor effect of GEM through the formation of endosomes, which means that the cancer cell resistance, which is one of the major issues of the marketed GEM (Gemzar), is significantly reduced. The same cell death mechanism was seen recently in tumor cells treated with 4-(N)-stearoyl GEM solid lipid nanoparticle and was further explained by the clathrin-mediated endocytosis (Wonganan et al. 2013).

In order to clarify the cellular uptake of the ME formulations, the ultrastructures of A549 cells treated with GEM10, MEb10 and MEb10+ for 48 h were viewed by TEM. In brief, MEb10 did not have an antiproliferative effect on the cells, whereas MEb10+ has caused a significant induction of apoptosis that was integrated with autophagocytosis. It was very clear that GEM10 got more resistance from the cancer cells than MEb10+.

In particular, early and late signs of apoptosis were seen individually when treated with GEM10 and MEb10+. It was very obvious that there was an integration of autophagy with apoptosis, as was extensively observed in the cell treated with MEb10+. In other words, GEM10 induced apoptosis, whereas MEb10+ formula stimulated the autophagocytosis along with the apoptosis induction in the cancer cells which resulted in the considerable enhancement in the antitumor activity. In fact, macroautophagy and apoptosis cause cell death in different mechanisms (Benbrook and Long 2012). The autophagic cell death occurs in the presence of the digestive vesicles, whereas apoptosis occurs through the activation of the caspases. Although the relationship between macroautophagy and apoptosis is not clear, previous studies validated that some of the autophagic vesicles would induce apoptosis through the intrinsic mitochondrial route (Pattingre et al. 2005, Yousefi et al. 2006, Wei et al. 2008).

Conclusion

In this study, three ME formulations, o/w (MEa) with hydrophilic nanoparticles, w/o (MEb) with hydrophobic nanoparticles and bicontinuous (MEc) with equivalent hydrophilic and hydrophobic properties of the nanoparticles, were evaluated in vitro against A549 and HFS.
cells. The cytotoxicity study revealed a low antiproliferative effect of GEM10 against A549 cells compared to MEb10+. However, the ME formulations had a significant cytotoxic effect, especially at a dose of 10µM. The best formula was the hydrophobic delivery, MEb10+. The ultrastructure of the A549 cells revealed that the improved antitumor activity of MEb10+ is due to the combined effect of the ME formula, which stimulated the autophagocytosis, and GEM that induced apoptosis.

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