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**Original Research Article** 

# Cytotoxicity of Gemcitabine-Loaded-Microemulsions in Breast and Colon Cancer Cells

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# Abstract

**Purpose:** To evaluate the antitumor activity of gemcitabine (GEM), incorporated in microemulsions with varying surfactant-to-oil (S/O) ratio, against MCF-7 breast cancer cells and HCT 116 colon cancer cells. **Methods:** The microemulsion formulations consisted of Tween 80, Span 20, isopropyl myristate (IPM) and aqueous ethanol (40 %). Anticancer assessment involved determination of hemolysis activity, screening for cytotoxicity using sulphorhodamine B assay and determination of the mechanism of cell death using light microscope and ApopNexin FITC apoptosis detection kit.

**Results:** Hemolysis activity of all the microemulsion formulations, either blank or drug-loaded, was significantly less than that of GEM solution. On average, MCF-7 cell viability significantly (p < 0.05) decreased from  $38.53 \pm 6.04$  to  $30.1 \pm 4.66$  % when the administered microemulsion concentration in modified eagle medium (MEM), increased from 0.03 to 0.3 % v/v but significantly (p < 0.05) increased by 1.4-fold when exposed to GEM solution at equivalent concentrations. In contrast, the cytotoxicity of the microemulsion formulation against HCT116 cells was similar to that of 0.03 % v/v GEM solution but greater than that of GEM solution by 1.5-fold when their concentration in MEM increased to 0.3 % v/v. Microscopic studies show that the microemulsions stimulated apoptosis in MCF-7 and HCT116 cell within 48 h and at low concentration (0.03 % v/v).

**Conclusion:** Microemulsion formulations improved the efficacy of GEM and induced apoptosis in MCF-7 and HCT116 cells.

*Keywords:* Apoptosis, MCF-7 breast cancer cells, HCT116 colon cancer cells, Hemolysis, Sulphorhodamine B assay, Microemulsion

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# INTRODUCTION

Chemotherapy involves the use of low molecular weight drugs to selectively destroy tumor cells or at least limit their proliferation. Antitumor drugs can be classified according to their mode of **DNA-interactive** action. such as agents, antimetabolites, antitubulin agents, molecular targeting agents and other biological agents [1]. In spite of the advancement in the pharmaceutical industries of producing

chemotherapeutic agents, cancer treatment is still challenging. In fact, the chemotherapeutic agents have several delivery issues including poor selectivity for target tissues, severe side effects in healthy tissues, rapid clearance, and susceptibility to induce drug resistance [2].

Gemcitabine (GEM), dFdC 2', 2'-difluoro-2'deoxycytidine, is a low molecular weight, deoxycytidine analogue, inhibitor of cellular DNA synthesis [3] and is effective against many solid tumors [4]. However, it has a very short half-life of 8 min elimination because it gets degraded by the plasma deaminases. Therefore, it is used in high doses and hence, cause severe side effects [5]. Many research studies have recently proposed delivery systems for GEM that would improve its efficiency and reduce its side effects [2, 6-10]. One of these delivery systems was microemulsion [11].

Microemulsion, a single optically isotropic and thermodynamically stable liquid solution, is a colloidal system that consists of water, oil and amphiphile. There are three types of microemulsions which are most likely to be formed depending on the compositions: oil-inwater (o/w), water-in-oil (w/oil) and bicontinuous microemulsions. Microemulsions have recently played a great role in cancer therapy, either by delivering anticancer agents or by producing the nanoparticulate of the chemotherapeutic agents [12]. The objective of this study was to evaluate the microemulsion system, proposed by Tsai et al [11] for delivering GEM to bladder cancer, for GEM activity against MCF-7 breast and HCT116 colon cancer cells.

### **EXPERIMENTAL**

#### MATERIALS AND CELL LINES

Polyoxyethylene sorbitan monooleate (tween 80) was purchased from El-Nasr Pharmaceutical Chemicals Co (Egypt). Isopropyl Myristate (IPM) was obtained from Jamjoom Pharma (Jeddah, KSA) while ethanol was purchased from Fisher Chemical (UK). Sorbitan monolaurate (Span 20), modified eagle medium (MEM), vitamins solution, fetal calf serum (FCS), non-essential amino penicillin streptomycin, phenol acid. red. phosphate buffered saline, 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid buffer solution (HEPES), trypsin, sulforhodamine B (SRB), trichloroacetic acid (TCA) and pure gemcitabine were purchased from Sigma-Aldrich Chemical Co. (MO, USA). The distilled water was purified using a water purification system from Bibby sterilin Itd, (UK). ApopNexin Annexin V FITC Apoptosis Kit (Lot no. 2053919) was purchased from Millipore (MA, USA). All other reagents were of analytical grade. MCF-7 and HCT-116 cell lines were generously donated by Tissue Culture Bank of King Fahd Medical Research Center, Jeddah, Saudi Arabia.

#### Formation of the microemulsion

Three microemulsion systems were produced by mixing Tween 80, Span 20, IPM and distilled water containing 40 % ethanol, as described by

Tsai *et al* [11]. The surfactant-to-oil (S/O) ratio of the three microemulsions, M1, M2 and M3, were 4:1, 0.8:1 and 3:1 respectively. GEM was incorporated in the microemulsions by directly adding 1 mg/ml of GEM to the microemulsion, and labeled as M1-D, M2-D and M3-D.

# Hemolysis assay of microemulsion formulations

Hemolytic assay was carried out using the method of Bulmus et al [13]. Freshly collected human red blood cells (5 ml) were washed three times with 150 mM NaCl and centrifuged at 2500 rpm for 10 min. The serum was removed and the cells were suspended in 100 mM sodium phosphate buffer (pH 7.4). An appropriate amount of the test samples, i.e., 10 µl of microemulsion (M1, M2 and M3), 10 µl of 1 mg/ml of GEM-loaded microemulsion (M1-D, M2-D and M3-D) and 10 µl of 1 mg/ml of GEM dissolved in water, were mixed with 200 µl of red blood cells solution and the final reaction mixture volume made up to 1 ml by adding sodium phosphate buffer (pH 7.4). The reaction mixture was placed in a water bath at 37 °C for 1 h. After the incubation period, the reaction mixture was centrifuged again at 2500 rpm for 15 min. The supernatant was collected and its optical density was measured at 541 nm, using sodium phosphate buffer (pH 7.4) as blank. Deionized water was used as a positive control. The experiment was performed in triplicate and the hemolytic activity (%) was calculated as in Eq 1.

Hemolytic activity (%) =  $\{(As - Ab)/Ac\}100$  .....(1)

where As, Ab and Ac are the absorbance of sample, blank and positive control, respectively.

#### **Cell culture**

The human cell lines, MCF-7 breast cancer and HCT-116 colon cancer, were cultivated, as described by Alkhatib and Albishi [14], in a cell culture flask (25 cm<sup>2</sup>) containing 10 ml of MEM media supplemented with vitamins solution, nonessential amino acid, penicillin streptomycin, phenol red, HEPES and 10 %v/v heat inactivated fetal calf serum (FCS) at 37 °C in a 95 % air and 5 % humidified CO<sub>2</sub> incubator. Medium was changed every 48 h intervals until confluence. Then, confluent cells were obtained by trypsinization, washed and passaged every 3 days. The experimental cells were incubated in 10 % of MEM culture medium for 24 h in a 95 % air and 5 % humidified CO<sub>2</sub> incubator at 37 °C.

#### Scanning of cell toxicity using SRB assay

The assay was performed according to the method of Skehan *et al* [15]. Cells were cultivated at a density of 1 x10<sup>4</sup> cells in flatbottomed tissue culture plates containing 0.1 ml of growth medium. A 200 µl of 0.03 and 0.3 %v/v of the test samples, diluted in MEM culture medium, microemulsions (M1, M2 and M3), 1 mg/ml of GEM loaded in microemulsion (M1-D, M2-D and M3-D) and 1 mg/ml of GEM dissolved in water were added to the cells. Experiments for each test sample were implemented in triplicate. Untreated cells were used as control. The cell toxicity of the tested sample against the MCF-7 and HCT-116 cells was assessed by measuring the ratio of dead cells to vital cells as in Eq 2.

Cell viability (%) = (As/Ac)100 .....(2)

where As is the absorbance of the cells treated with the test sample and Ac is the absorbance of the untreated cells. Both preparations were measured at 490 nm using MR 7000 ELISA reader (Dynatech Laboratories Inc., Chantilly, Va.)

#### Evaluation of mechanism of cell death

Amounts (each of 1 x10<sup>5</sup>) of MCF-7 and HCT-116 cells were seeded into flat-bottomed tissue culture plates containing 0.1 ml of growth medium. Quantities (200 µl each) of 0.03 and 0.3 %v/v of microemulsion (M1, M2 and M3), 1mg/ml of GEM-loaded microemulsion (M1-D, M2-D and M3-D) and 1 mg/ml of aqueous GEM solution were administered to the cells and left for 48 h. The treated cells were washed twice with phosphate buffer (pH 7.2) for 5 min and fixed by the addition of 4 % formaldehyde. The fixation solution was discarded and the cells stained with 10 % coomassie blue dye for 10 min. Finally, the stain was washed with tap water twice, and left to dry overnight at 25 °C. Morphological changes were observed by phase-contrast inverted microscope (model 1X17, Olympus, Japan).

#### Detection of early signs of apoptosis

ApopNexin FITC assay was used to detect early signs of apoptosis by staining the cells with a green fluorescent system obtained from the binding between Annexin V and phosphatidylserine translocate from the inner membrane of the cell to the cell surface during apoptosis. The experiments were performed as mentioned in the protocol of ApopNexin FITC kit (Lot no. 2053919). An aliquot (200 µl) of 0.3 %v/v of microemulsion (M1), 1 mg/ml of GEM-loaded in microemulsion (M1-D) and 1 mg/ml of aqueous GEM were introduced into the cells. Untreated cells were used as control.

#### **Statistical analysis**

All values were expressed as mean  $\pm$  standard deviation, each experiment being performed in triplicate. Statistical analyses were performed with one-way analysis of variance (ANOVA) test, two-way ANOVA test and independent sample t-test using MegaStat. Differences were considered significant when P < 0.05.

### RESULTS

HEMOLYTIC EFFECT OF MICROEMUL-SION FORMULATIONS

As illustrated in Figure 1, the highest hemolysis  $(36.77 \pm 4.20 \%)$  was recorded when agueous GEM solution was introduced, indicating that it has the largest effect on red blood cells. The microemulsions, M1 and M2, have hemolytic activity of 9.72 ± 2.47 and 7.18 ± 1.93 %, respectively, which are significantly less than the hemolytic effect of M1-D and M2-D with values of 17.27 ± 0.14 and 21.37 ± 3.70 %, respectively. On the other hand, M3 and M3-D showed similar hemolytic activities of 21.38  $\pm$  3.49 and 21.53  $\pm$ 3.41 %, respectively. It is worth noting that among the GEM-loaded-microemulsions, M1-D with the least S/O ratio had the least effect on the red blood cells hemolysis activity of 17.27 ± 0.14 %.



**Figure 1:** The percentages of hemolysis activity of 10  $\mu$ l of microemulsion solutions (M1, M2, M3), 10  $\mu$ l of 1mg/ml of GEM loaded in microemulsions (M1-D, M2-D and M3-D) and 10  $\mu$ l of 1mg/ml of GEM dissolved in water. All of the experiments were performed in triplicate and the values of the *error bars* represent the standard deviations

| Microemulsion | MCF-7       |            | HCT-116    |             |
|---------------|-------------|------------|------------|-------------|
|               | 0.03%       | 0.3%       | 0.03%      | 0.3%        |
| M1            | 35.84±4.68  | 26.18±1.76 | 53.35±2.66 | 31.29±7.80  |
| M1-D          | 30.27±7.41  | 26.08±3.59 | 62.93±5.95 | 32.85±5.89  |
| M2            | 46.45±10.39 | 29.22±1.19 | 63.00±7.55 | 36.93±4.74  |
| M2-D          | 37.80±4.79  | 34.71±7.71 | 57.33±6.92 | 37.12±3.37  |
| M3            | 45.95±4.51  | 31.29±7.80 | 54.76±2.15 | 32.17±11.81 |
| M3-D          | 34.92±4.46  | 32.85±5.89 | 65.64±7.52 | 37.03±3.64  |
| GEM           | 54.28±3.17  | 42.3±5.70  | 51.22±2.89 | 50.84±1.31  |

**Table 1:** Cytotoxic activity of 0.03 and 0.3% v/v of microemulsions (M1, M2 and M3), GEM-loaded-microemulsions (M1-D, M2-D and M3-D), and aqueous GEM solution against MCF-7 and HCT-116 using SRB assay (mean  $\pm$  SD, n = 3)

#### Cell toxicity of microemulsions

As demonstrated in Table 1, the viability of MCF-7 and HCT-116 cells varied treatment with 0.03 %v/v and 0.3 %v/v of the tested microemulsion samples. The anti-proliferative effect of all of the microemulsion formulations against MCF-7, either drug-free or drug-loaded, were significantly (P < 0.05) more than the GEM solution. Changing the concentration in MEM from 0.03 to 0.3 %v/v did not improve the cytotoxic effect of all of the GEM loaded-microemulsions (M1-D, M2-D and M3-D) and GEM solution against MCF-7 cells. In contrast, increasing the concentration in MEM of all the microemulsion formulations (M1/M1-D, M2/M2-D and M3/M3-D) 10-fold significantly (P < 0.05) improved their cytotoxicity effect against HCT116 cells while varying the concentration of GEM solution did not change its cytotoxicity. In fact, the cytotoxic effect of 0.03 % aqueous GEM solution against HCT116 was similar to that of the microemulsion formulations (M1/M1-D, M2/M2-D and M3/M3-D) but significantly (P < 0.05) less than when applied at a concentration of 0.3 %. It is worth noting that there were no significant differences  $(P \ge 0.05)$  in cytotoxicity against both MCF-7 and HCT116 for M1/M1-D, M2/M2-D and M3/M3-D when applied at the same concentration.

#### Mechanism of cell death

Morphology is an important indicator of the status of cells. In order to understand the mechanism of cell death of the drug-free and drug-loaded microemulsion formulations at a concentration of 0.03 and 0.3%v/v when administered into MCF-7 and HCT-116 cells, the cells morphologies were visualized using light microscope. In general, slight changes in cells shape, decreased total number of cells and increased intracellular space of the cells treated with 0.03% (v/v) of drug-free and drug loaded-microemulsion formulations were observed compared with the untreated cells whereas cells treated with 0.3 %v/v of the tested microemulsion formulas have shown dramatic changes in shape, extremely increased intracellular space and clearance of cells (Figures 2 and 3). On the other hand, there was no noticeable difference between the cells treated with either 0.03 or 0.3 %v/v of GEM solution except little changes in the morphology of the cells.

MCF-7 cells were not obviously affected by all of the microemulsion formulations at 0.03 %v/v as shown in Figure 2A. They only displayed earlier stages of apoptosis as more intracellular spaces were observed without noticeable changes in the shape of the cells. In contrast, they were more affected by the entire formulations at 0.3 %v/v. As shown in Figure 2B, they exhibited late stages of apoptosis through the complete formation of fragmented apoptotic bodies resulted from the enormous killed cells.

On the other hand, HCT-116 cells treated with the drug-free and drug-loaded microemulsion formulations showed varying levels of cell death as shown in Figure 3. The cells treated with 0.03 %v/v of each of the microemulsion formulations showed earlier stages of apoptosis as they shrunk and their chromatin became more condensed. On the other hand, 0.3 %v/v microemulsions clearly killed most of the cells, suggesting that the cells had undergone late stages of apoptosis. On the other hand, aqueous GEM solution did not display signs of apoptosis as clearly as the microemulsions.

# Effect of GEM-loaded microemulsions on apoptosis induction of cancer cells

As shown in Figure 4, the untreated cells did not stain positively which indicates the viability of cells, while MCF-7 and HCT116 cells treated with 0.3 % of M1, M1-D and GEM solution were stained positively with green fluorescent which implies signs of apoptosis due to the externalization of phosphatidylserine caused by the cell surface outbreak.



**Figure 2:** Light microscopic images showing morphological changes in MCF-7 cells treated with (A) 0.03 %v/v and (B) 0.3 % of microemulsion and aqueous GEM solution. Images were magnified at x 200.



**Figure 3:** Light microscopy images showing morphological changes in HCT116 cells treated with (A) 0.03 %v/v and (B) 0.3 %v/v of microemulsion formulas and aqueous GEM solution. Images were magnified at x 200.



Figure 4: Fluorescent microscopic images of (A) MCF-7 cells and (B) HCT116 cells labeled with Annexin V. All images were magnified at x 200.

# DISCUSSION

GEM is a chemotherapeutic agent that is used to treat various types of carcinomas, such as breast cancer, ovarian cancer, non-small cell lung cancer, head and neck cancers, pancreatic cancer and colon cancer [3]. The synergistic mechanism of action of GEM is based on destroying cancer cells undergoing DNA synthesis which result in apoptosis induction. Therefore, it has several side effects [8] and has to be used in high concentrations and in combination with other agents [9,10]. Many studies have suggested delivery systems that would improve the efficiency of GEM against various cancers and reduce its side effects [2, 6-8].

In this study, it has been found that the hemolysis activities of all of the microemulsion formulations, either drug-free or loaded with drug, were significantly less than GEM solution. Therefore, incorporation of GEM in microemulsion reduced its cytotoxicity against erythrocytes, thus minimizing what is considered one of the major drawbacks of this drug [16]. Our findings were in agreement with those of Singh *et al* [17] who reported that GEM-loaded folic acid conjugated multi-walled carbon nanotubes have significantly less hemolytic toxicity. In fact, Wilk *et al* [18] found out that surfactant structure and the critical micelle concentration play a critical role in damaging the red blood cells.

The cytotoxicity of the drug-free microemulsions was similar to the drug-loaded microemulsions which indicate that the components of the microemulsion are interfering with the biochemical assay results. Li et al [19] have demonstrated that Tween 80 affected the results of MTT assay, which is similar to SRB assay, when screening for the cytotoxicity of drugs by detecting biochemical changes on the cell membrane. When Tsai et al [11] administered the drug-free microemulsions into the rats, they found out that the microemulsions caused inflammation according to their histological evaluation of rat bladder tissues. They also have showed that GEM release from the microemulsion formulations is within 8h, which indicates the complete release of the drug from the microemulsion formulations into the cells within 48 h incubation, the time used in our study.

Moreover, the cytotoxicity of the microemulsion formulations and GEM solution against MCF-7, determined by SRB assay, were not sensitive to concentration variations. There was a significant change in the morphologies of the cells when the concentration of the microemulsion formulations increased from 0.03 to 0.3 %v/v, while the for GEM solution matched with what was visualized light microscope. In other words, under increasing the concentration of GEM solution did not enhance its efficiency as much as was the case with GEM-loaded-microemulsion. On the other hand, the cytotoxicity of the microemulsion formulations against HCT116 cells were very sensitive to concentration variation, and this was confirmed by the results of light microscopy and the ApopNexin assay. Similarly, the cytotoxicity of aqueous GEM solution against HCT 116 was not sensitive to concentration change even when concentration increased 10-fold.

A previous study showed that encapsulating GEM in liposomes improved its antitumor effect by 4.6- and 7.9-fold within 72 h when its concentration increased from 1 to 100  $\mu$ M, respectively [6]. Another study found that chitosan nanoparticle of GEM demonstrated improved antiproliferative effect over free GEM when it was administered to mice-bearing L1210 wt subcutaneous tumor [2]. The nanoparticle formulation enhanced the permeability of GEM into the tumor which resulted in a superior tumor regression. Furthermore, Yang *et al* [20] have delivered GEM in multiwalled carbon nanotubes with magnetic properties which would selectively transport GEM into the lymph nodes.

#### CONCLUSION

Incorporation of GEM in microemulsions decreases cytotoxicity against erythrocytes. microemulsion improves the efficiency of GEM and induces apoptosis in cancer cells. Consequently, recommended to establish *in vivo* studies are recommended to determine if a similar finding can be replicated in the body tissues. Further studies are also required to elucidate the mechanisms responsible for the improved antitumor activity of the microemulsions.

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