Design and Characterization of Liposomal Methotrexate and Its Effect on BT-474 Breast Cancer Cell Line

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Abstract
Background: Breast cancer is the most common type of cancer among women worldwide. Traditional treatments, including chemotherapy, surgery, mastectomy, and radiotherapy, are commonly used. Because of the limitation of the aforementioned methods, novel treatment strategies are needed. Methotrexate is a chemotherapeutic drug, which is commonly used to treat breast cancer. Because of the side effects of the free drug, the liposomal form of the drug is suggested.

Methods: Liposomal methotrexate was prepared and the encapsulation efficiency was measured. Moreover, the particle size and the zeta potential were measured. The liposome morphology was confirmed using transmission electron microscopy. The MTT assay was done to examine the cytotoxicity of free and encapsulated methotrexate on BT-474 cell line. The Annexin-V/PI dual staining assay was performed to assess the apoptosis in BT-474 breast cancer cells via the flow cytometry method.

Results: The transmission electron microscopy results confirmed the integrated and spherical structure of the nanoparticles. The results of drug release showed that in acidic pH (5.4), more than 90% of the drug was released after 24 hours, which was higher than 2 other pHs. Furthermore, the IC50 value of liposomal methotrexate was determined as 2.15 and 0.82 mg/mL for 24 and 48 hours. The flow cytometry results confirmed that liposomal methotrexate had a greater cytotoxic effect on cancer cells compared with free methotrexate.

Conclusion: Because of the advantages of liposomal based nanocarriers, in this study, liposomal methotrexate could be suggested as an appropriate candidate to treat breast cancer.

Keywords: Liposome, Methotrexate, BT-474, Breast Cancer, Nanocarriers

Introduction

Breast cancer is the most prevalent type of cancer among women worldwide (1). The effective treatment is remained as a great clinical challenge, although currently several treatment options are available (2, 3). Breast cancer is classified into 3 main subtypes based on the presence or absence of molecular markers for estrogen or pro-

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↑*What is "already known" in this topic:*
Cancer is one of the leading causes of mortality worldwide. Among women, breast cancer is the most common one. Most of these treatments have severe adverse effects. Chemotherapy has a narrow therapeutic window and requires high dosage treatment in patients with advanced stage cancers who need further innovative treatment strategies.

→*What this article adds:*
Methotrexate (MTX) is an effective drug that might impair malignant growth without irreversible damage to normal tissues. In the current study, we aimed to prepare the liposomal MTX and compare the effects of this drug in free (MTX) and encapsulated forms (MTX-Lip) on BT-474 breast cancer cell line.

gesterone receptors and human epidermal growth factor 2 (ERBB2; previously HER2): positive hormonal receptor/negative ERBB2 (70% of patients), positive ERBB2 (15%-20%), and triple negative breast cancer, which are the tumors that have none of the 3 standard molecular markers (15%) (4, 5).

Treatment usually includes breast conserving surgery (Tumor removal and a margin of surrounding tissue, sometimes called lumpectomy) or mastectomy (complete breast tissue removal), which depends on the characteristics of the tumor and the patient's priority. Furthermore, treatment includes radiotherapy, chemotherapy, hormonal therapy, targeted therapy, and recently immunotherapy (6). Chemotherapy is a process in which, cancerous cells are killed by means of specific drugs (7). The main defects of chemotherapeutic methods at the moment are the non-specific distribution of chemotherapeutic drugs, which causes the drug to affect both normal and tumor cells (8), and their low solubility in aqueous environment, leading to incomplete treatment and serious side effects for the patients (9). Furthermore, cancerous cells are often resistant to these compounds. Multiple drug resistance is related to a wide range of pathological modifications at various cellular and intracellular levels (10). This process includes the reduction of drug transmission, increased efflux, availability of alternative targets, apoptosis prevention (11, 12), lipid membrane alteration, metabolic conversion of drugs, and changes in the main points of the cell cycle and microtubule associated proteins and β-tubulin mutation (13). For these reasons, the therapeutic outlook for these drugs is low (11). MTX is an antimetabolite (folate antagonist), which is used extensively in cancer chemotherapy (14). MTX is a dicarboxylic acid (15), which is competitively bound to the dihydrofolate reductase (DHFR) enzyme, which inhibits the enzyme, depletes tetrahydrofolate cellular storage, and finally leads to thymidylate synthesis cessation (16-18). The DHFR is an essential enzyme that converts dihydrofolate to tetrahydrofolate (19). The cell that does not have enough thymidine cannot synthesize DNA; therefore, it inhibits cell proliferation and growth, which causes the cell arrest in the G1/S phase (16, 17).

MTX is used for the treatment of various solid tumors (eg, osteosarcoma, breast and lung cancer) and also for autoimmune and inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, and psoriasis (16, 20). MTX has been reported to cause apoptosis in various cell lines, but its low accumulation at the tumor site limits its effects on the cells (16). Moreover, MTX causes several side effects such as hepatotoxicity, ulcerative colitis, nephrotoxicity, gastrointestinal disorders, bone marrow toxicity, and pneumonitis, which limit its therapeutic applications (15, 16).

Because of chemotherapeutic methods limitations and the side effects of these drugs, new treatment strategies are required. Nanocarriers are one of the promising new treatment strategies (21). Drug-containing nanocarriers accumulate in target tissues or organs because of various factors, such as barrier's penetration, tissue damage, and environmental pH (22).

Liposomes are one type of nanocarriers that have been widely used for targeted drug delivery. These are vesicles composed of one or more concentric phospholipid bilayers separated by aqueous compartments (23). When used as a carrier, hydrophobic molecules are located in bilayer membrane, and hydrophilic molecules are located in the aqueous center of the liposome (24).

In the current study, we aimed to prepare the liposomal MTX and compare the effects of this drug in free (MTX) and encapsulated forms (MTX-Lip) on BT-474 breast cancer cell line. Furthermore, the drug release was measured in physiological conditions (pH: 7.4), tumor tissue (pH: 6.4), and endosomal compartments (pH: 5.4), respectively.

Methods
MTX (25 mg) was obtained from Sigma (Aldrich). Phospholipon 90 G and cholesterol were obtained from Avanti Polar Lipids (Alabaster). The BT-474 cell line was obtained from the Leibniz institute of Germany. The RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, insulin, glutamine (50x), methanol solution, chloroform solution phosphate buffer saline (PBS), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution, dimethyl sulphoxide, and annexin V-FITC apoptosis detection kit were obtained from Sigma (Aldrich).

Cell Culture
The BT-474 was utilized as HER2-positive cell line. To culture the cells, the RPMI-1640 medium was supplemented with 20% FBS. Then, the streptomycin (100 μg/mL), penicillin (100 μg/mL), and insulin (10 μg/mL) were applied and the cells were incubated at 37°C in a humidified incubator containing 5% CO_2.

Preparation of Nanoparticles and Encapsulation of MTX
Preweighed lipids (phospholipon 90G) and cholesterol (70%: 30%) were dissolved in methanol and chloroform (1:1 v/v) solution. The resulting solution was desiccated to shape a thin film layer in a round-bottom flask on a rotary evaporator under low pressure at 33°C until total removal of solvents (25). After adding 1 mL MTX (10 mg/mL) at room temperature, the rehydrated lipid film was extruded (21× at 33 °C) through 2 stacked 200 nm polycarbonate membranes (Nucleopore) (Liposofast R) to form unilamellar vesicles. The liposomes were incubated at ambient temperature to cool down and then stored at 4°C. A zetasizer apparatus was used to analyze the diameter of produced liposomes.

Encapsulation Efficiency Determination
Separation of encapsulated & free drugs: For this process, the Amicon Ultra-3 (molecular weight cutoff of 3 kDa) was applied. The filter was centrifuged at 5000 g for 15 minutes at 15 °C (26). In this step, the free and encapsulated drugs that were separated from each other were collected in 2 different tubes and stored at 4°C.
Encapsulation efficiency: The free MTX absorption

values were read by the UV/Vis spectrophotometer at a wavelength of 300 nm (27). The efficiency of encapsulation was calculated by the following equation (28):

EE stands for encapsulation efficiency:

EE% = (Total MTX- free MTX/ Total MTX) x 100%.

Liposome Characterization

Particle Size and Zeta Potential: The mean size and the zeta potential of liposomal MTX were measured using a dynamic light-scattering detector (Zeta-sizer ZS). A minimum of 3 different batches were assessed to render a mean value and standard deviation for the particle diameter and zeta potential.

Liposome Morphology: Liposome formation was confirmed by transmission electron microscopy (TEM, AB-FETEM Leo 9112). Samples were fixed by applying a drop of the mixture to a carbon-coated copper grid and leaving it for 2 minutes to allow some of the particles to attach onto the carbon substrate. The excess dispersion was removed using a piece of filter paper and then, a drop of 1% phosphotungstic acid solution was applied for 1 minute and left to be air-dried. The samples were observed by TEM (29).

Drug Release Evaluation: The drug release process of liposomal MTX was tested immediately after the drug was loaded into the liposomes. One mL of drug-loaded liposome solution was suspended inside a dialysis bag (10 kDa) immersed in 20 mL PBS solution at 3 pH levels: 7.4, 6.4, and 5.4. Dialysis bag was continuously stirred in release medium at 300 rpm in a shaker incubator at 37°C. Samples of the released liposomes were taken at 1 to 7 and 24 hours after dialysis started. The free drug concentration was measured using UV/Vis spectrophotometer at 300 nm. The experiments were repeated triplicate and the results were reported.

Cell Cytotoxicity: The MTT assay (30) was applied to examine the cytotoxicity of free and encapsulated MTX on BT-474 cell lines. The cells were treated with an increasing concentration (0-5 mg/mL) of each compound for 24 and 48 hours in a 5% CO_2 incubator at 37°C (repeated triplicate). It should be noted that untreated cells were considered as controls. Following incubation, 20 μL MTT solution (5 mg/mL) was added in each well. The result was incubated at 37°C for 24 and 48 hours, and finally the medium was removed, and formazan salt crystals were dissolved by adding 200 μ of dimethyl sulfoxide to each well. Plates were evaluated using an ELISA plate reader (Labsystems multiskan RS-232C, Finland) at 570 and 630 nm.

Flow Cytometry: The flow cytometry method was done to confirm the results of MTT assay. The method showed the number of alive, apoptotic, or necrotic cells. The Annexin-V/PI dual staining assay was performed to assess the apoptosis in BT-474 breast cancer cells induced by treatment with free and liposomal MTX (MTX-lip) using fluorescein isothiocyanate (FITC), Annexin V Apoptosis kit and PI (BioLegend). In this process, cells were cultured (5 ×10^5 cells) in 25 cm² cell culture flasks for 48 hours before treatment with free and liposomal MTX. The cells were then treated with IC_{50} value of the free and

MTX-lip for 24 and 48 hours. The cells were collected and washed twice with cold PBS, and resuspended in binding buffer (1× 10^6 cells/mL). In this step, 1 mL of the cells was transferred into a tube, and then 10 μL of FITC conjugated Annexin V (Annexin V-FITC) and 10 μl of propidium iodide (PI) were added and incubated for 15 minutes at room temperature in dark. The stained cells were diluted by the binding buffer and immediately analyzed using the flow cytometer (Becton Dickinson FACSCanto II (BD FACSCanto II, BD Biosciences)). Data from 10,000 cells were collected in each data file. Four populations of the cells are clearly distinguished, including unlabeled (viable) cells, cells that have bound Annexin V-FITC only (early apoptotic), cells that have been stained with PI (necrotic), and the cells that have bound Annexin V-FITC, and been labelled with PI, which demonstrate the late apoptotic/necrotic cells. All samples were assayed in triplicate and each test was performed 3 times.

Results

Preparation and Characterization of Liposomal MTX

The unilamellar vesicles containing MTX were prepared by the thin layer evaporation method. The liposomal MTX nanoparticles were characterized by dynamic light scattering (DLS) and TEM. The TEM observations (Fig. 1) confirmed that these nanoparticles have an integrated, spherical structure. The DLS measurements showed a mean hydrodynamic particle size (Table 1), polydispersity index (PDI), and also zeta potential of empty liposomes (Lip), and liposomal MTX (Table 1).

The results of DLS showed that the addition of MTX to liposomes does not have a significant effect on the size of the nanoparticles. Moreover, the PDI of (MTX/Lip) nanoparticles are desirable and their surface charge was negative. Furthermore, the (MTX/Lip) nanoparticles exhibit an MTX-loading capacity of ~98%.
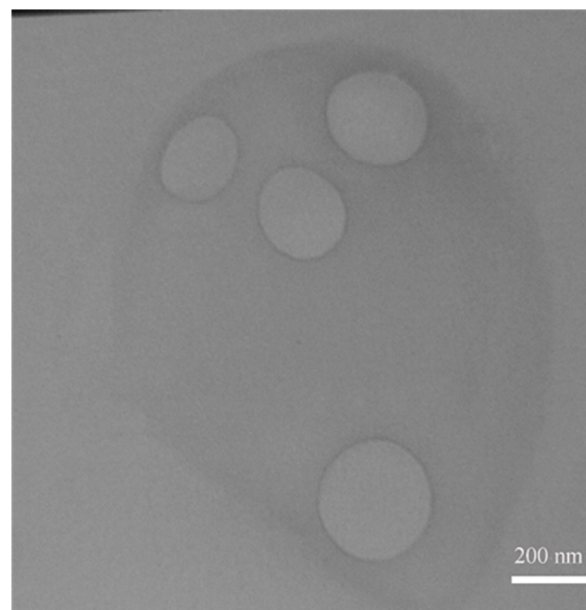

Fig. 1. TEM image of (MTX/Lip) nanoparticles

Table 1. Mean hydrodynamic particle size, polydispersity index (PDI) and zeta potential of liposomes (Lip) and liposomal MTX (MTX/Lip)

Formulation	Mean hydrodynamic particle size (nm)	Polydispersity index (PDI)	Zeta potential (mv)
Lip	191.9 ± 2.3	0.140 ± 0.1	-3.18 ± 0.5
MTX/Lip	195 ± 2.2	0.163 ± 0.1	-3.26 ± 0.2

All data are expressed as the mean ± SD (n= 3).

In Vitro Drug Release

As depicted in Figure 2, the release of the drug (MTX) from liposomes, increased in all 3 pH levels over the time. Furthermore, the MTX release rate was promoted by a decrease in environmental pH. Thus, at pH = 5.4, more than 90% of the drug was released after 24 hours (Fig. 2). These results confirmed that the higher amount of drug could be released at the tumor site, which has the lower pH and it also should be noted that the lowest amount of drug is released at the physiological pH (7.4), which was confirmed by other studies (6, 25).

In Vitro Cytotoxicity

The cytotoxicity of free and encapsulated MTX were evaluated on BT-474 breast cancer cell line using MTT assay. Different concentrations (0.3 to 5 mg/mL) of free and encapsulated MTX were applied to these cells for 24 and 48 hours (Fig. 3). The cells in the culture medium that were not affected by the drug were considered as controls. Based on Figure 3, the IC_{50} value of free drug (MTX) was calculated as 4.85 and 3.62 mg/mL for 24 and 48 hours, respectively. Also, the IC_{50} value of the encapsulated drug (MTX/Lip) was determined as 2.15 and 0.82 mg/mL for
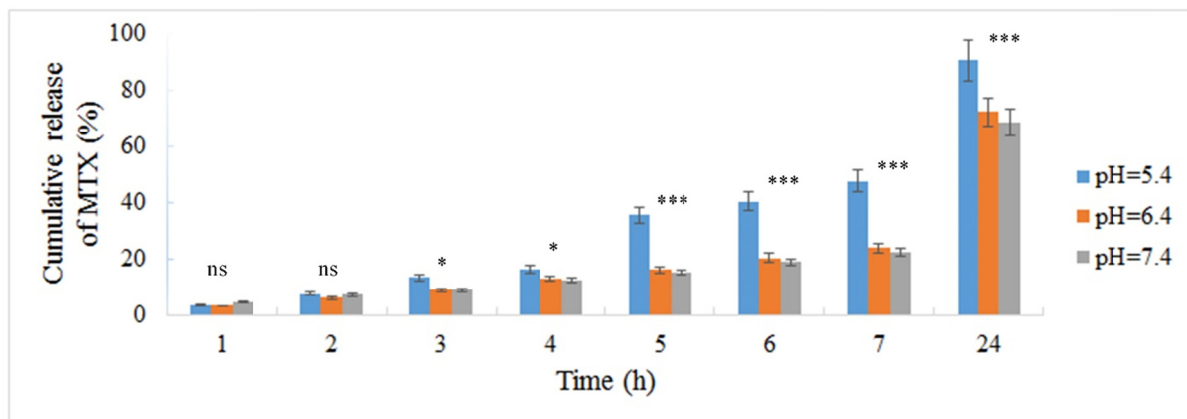

Fig. 2. In vitro release profiles of MTX from MTX/Lip in PBS buffer (pH 7.4, 6.4 and 5.4) at 3 7°c. (Blue, Orang and Grey colors represent pH: 5.4, pH: 6.4 and pH: 7.4). Results of the MTX release were mean ± SD (n=3). ***p < 0.001 and ns: not significant, it is a comparison between pH: 5.4 and two other pHs (pH: 6.4 and pH: 7.4).
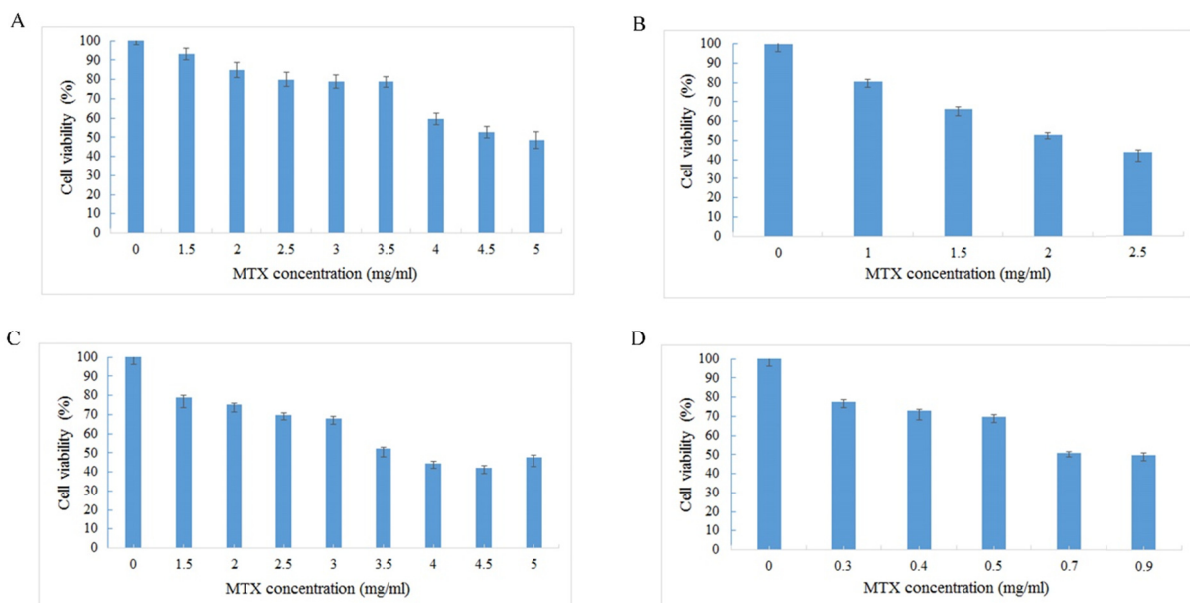

Fig. 3. In vitro cell viability of BT-474 cell line incubated with free MTX after 24 hours (A), liposomal MTX after 24 hours (B), free MTX after 48 hours (C) and liposomal MTX after 48 hours (D).
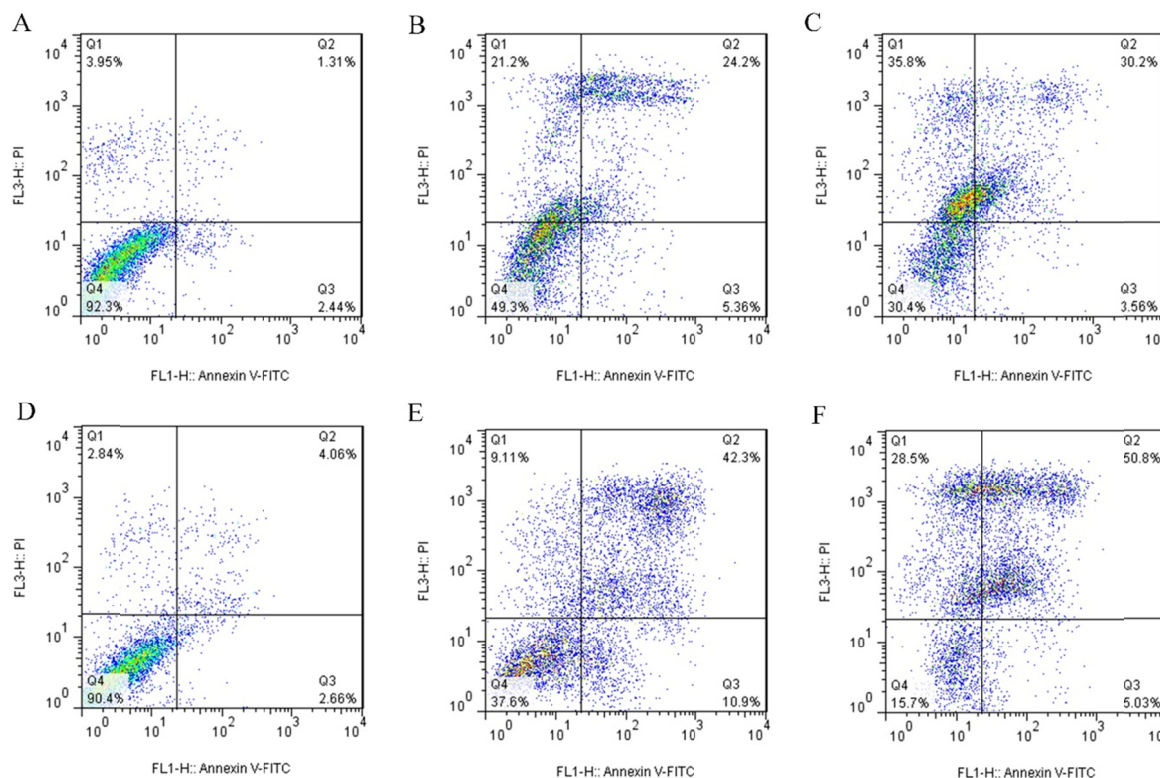
Fig. 4. Flow cytometry profiles of, controls after 24 hours (A), free MTX after 24 hours (B), liposomal MTX after 24 hours (C), controls after 48 hours (D), free MTX after 48 hours (E) and liposomal MTX after 48 hours (F).

24 and 48 hours, respectively. These results demonstrated that the amount of IC_{50} in encapsulated form is less than the free drug form; also this value for 48 hours is <24 hours. Therefore, it should be mentioned that after 48 hours, the liposomal drug (MTX/Lip) has a greater cytotoxic effect on this cell line.

Flow Cytometry

Flow cytometry was conducted to determine the cellular uptake efficacy of the nanoparticles. The BT-474 cells were incubated with free and liposomal MTX at 37° C for 24 and 48 hours. It should be noted that the early apoptosis of free and liposomal MTX after 24 hours was 5 ± 0.5% and 3.5 ± 0.6%) and after 48 hours it was 10 ± 1% and 5 ± 0.4 %. Furthermore, the late apoptosis of free MTX and liposomal MTX after 24 hours was 24 ± 1.1% and 30 ± 1% and after 48 hours it was (42 ± 0.6% and 50 ± 1%), respectively. As shown in Figure 4, the liposomal MTX could be successfully internalized by BT-474 breast cancer cell line. These results confirmed that MTX in liposomal form had a greater cytotoxic effect on these cells compared with free MTX. Furthermore, its cytotoxic effects were more significant after 48 hours than 24 hours.

Discussion

Liposomes are a type of drug delivery system that improve the pharmacological properties of chemotherapeutic drugs by altering the biodistribution and pharmacokinetic properties of these drugs. The aforementioned nanoparti-

cles have specific properties that make them appropriate candidates for the drug delivery system. For example, liposomes cause slow and stable drug release, reduce the cytotoxicity of the drugs, and increase the accumulation of the drugs at the tumor site because of their special characteristics (31). Based on the aforementioned characteristics, in this study, liposomes were used as nanocarriers to treat breast cancer cells. In the present study, MTX was loaded into liposomal nanoparticles and its anti-cancer effect was evaluated in free (MTX) and liposomal (MTX/Lip) forms on BT-474 breast cancer cell line.

The mean hydrodynamic size of empty liposomes and MTX/Lip was 191.9 and 195 mm, respectively. The results of DLS showed that the addition of the MTX to liposomes did not have a significant effect on the size of the nanoparticles and this size was suitable to target breast cancer cells by passive targeting. Furthermore, the PDI of MTX/Lip nanoparticles was desirable and their surface charge was negative. This negative surface charge may prevent the accumulation of nanoparticles by creating electrostatic repulsion and also reduces adverse reactions between nanoparticles with plasma proteins and/or red blood cells (32).

Abdelbary et al determined the encapsulation efficiency (EE%) of MTX in nanostructured lipid carriers (NLCs), which was calculated above 60% (28). Ekinci et al calculated the drug loading content of chitosan nanoparticles loaded with methotrexate. One of the formulations had a drug loading content of 28 mg and EE% of 35%, and an-

other formulation had a drug loading content of 13 mg and EE% of 63% (33). In our study, the %EE was calculated to be about 98% which confirmed that the encapsulation efficiency of MTX in liposomes was desirable enough. Jin et al investigated the release of MTX from various micelle formulations in 2 different pH levels: physiological (pH = 7.4) and acidic (pH = 5). In one particular formulation and under similar conditions, the release of MTX from nanocarriers was only 20% at physiological pH (7.4) and approximately 60% at acidic pH (5) after 48 hours. Thus, the release of MTX was dependent on pH (34). In our study, the release of MTX from liposomal nanoparticles (MTX/ Lip) was investigated at 3 different pH levels: physiological (pH = 7.4), tumor surrounding (pH = 6.4), and endosomal environment (pH = 5.4) for 24 hours. At a particular time, the drug release increased with decreasing the pH. Thus, the drug release at physiological pH (pH = 7.4) was less than the other 2 pHs. It should be noted that the highest drug release rate was in acidic pH (pH = 5.4). As shown in Figure 2, after 24 hours, 90% of the drug was released at (PH = 5.4). As a result, it could be suggested that drug release in tumor surrounding environment is higher than healthy tissues, and this causes the maximum amount of drug to reach the cancer cells, and the healthy cells are less likely to be affected by the drug (Fig. 2).

Gorjikhah et al studied the cytotoxic effects of free and encapsulated MTX in polymer nanoparticles on the T47D breast cancer cell line at different concentrations. The IC_{50} values of free MTX on T47D cells after 24, 48, and 72 hours were 0.391, 0.361, and 0.285 mg/mL, respectively. On the other hand, the IC_{50} values of encapsulated MTX in polymer nanoparticles on T47D cells after 24, 48, and 72 hours were 0.318, 0.294, and 0.241 mg/mL, respectively. Thus, the cytotoxic effect of encapsulated MTX was greater than that of the free MTX (35). In this study, the cytotoxic effects of MTX and MTX/Lip at various concentrations (0.3-5 mg/mL) were investigated by the MTT assay on BT-474 breast cancer cell line. As shown in Figure 3, the IC_{50} values of MTX after 24 and 48 hours were 4.85 and 3.62 mg/mL, respectively. Furthermore, the IC_{50} values of MTX/Lip after 24 and 48 hours were 2.15 and 0.82 mg/mL, respectively. These results demonstrated that free MTX had lower cytotoxic effect on BT-474 breast cancer cell line than MTX/Lip, and for a particular drug form, cytotoxicity increased over time. These data confirmed that the differences in cytotoxicity for free and encapsulated MTX were significant.

In one study, Anne Marie Ciobanu et al created the liposomal MTX. The effect of MTX incorporated in liposomes was investigated in vitro on human lymphoblastic cell line K562. Their study showed that MTX incorporated into liposomes moderately reduced the proliferation of K562 cells, but significantly inhibited RNA synthesis (36).

Flow cytometry was used to confirm the uptake of free and liposomal MTX (MTX/Lip) by BT-474 breast cancer cells. The cytotoxic effect of these 2 forms increased over time. This Figure (Fig. 4) confirmed that the cytotoxicity effect of MTX/Lip was greater than MTX. Several studies demonstrated that the rate of apoptosis in liposomal MTX

was higher than free MTX, which was confirmed by our results (37-39).

Conclusion

Nowadays, studies on nanoparticle formulations are ongoing. Moreover, liposome-based formulations have entered the clinical trials to treat a variety of cancers. Methotrexate is an anticancer drug that is commonly used to treat different cancers, including breast, lung, head and neck, et cetera. Because of special side effects of this chemotherapeutic drug, the liposomal MTX can be a promising choice for the treatment of breast cancer; however, more investigations are needed.

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