ENHANCED CELL KILLING BY METHOTREXATE ENCAPSULATED IN FOLATE TARGETED THERMOSENSITIVE LIPOSOMES

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Abstract. The exploitation of folate receptor as Trojan horse to deliver folate-targeted liposomes bearing diverse cargo represents a novel therapeutic strategy to target folate receptor-expressing cells. In this paper, thermosensitive liposomes made of synthetic lipids (distearolphosphatidylcholine, DSPC and dipalmitoylphosphatidylcholine, DPPC) showing gel to liquid phase transition at 41º C, were used for encapsulation of methotrexate. The liposomes were prepared by thin film hydration method, the liposome binding constant K_b for the drug was measured using a spectroscopic assay and was found to be 57.18 $(mg/ml)^{-1}$. We have studied the liposome-mediated delivery of methotrexate to breast tumor cells *in vitro*, the cell sensitivity study was performed at normal physiological temperature (37º C) as well as hyperthermia increased temperature of 42º C. Moreover, a targeting moiety was used by modifying the liposome surface with folate using polyethyleneglycol (PEG) as a spacer. The ability of methotrexate to inhibit tumor cell growth is increased dramatically when encapsulated in targeted (folated) thermosensitive liposomes, but decreased when encapsulated in liposomes deficient folate. The index of cell Killing expressed as IC_{50} was reduced dramatically from 7 μg/ml to 0.864 μg/ml upon using folate as a targeting moiety. Hyperthermia was not effective when used with non-specific targeted liposomes. However, the cytotoxicity of the drug increased dramatically upon heating folate targeted thermosensitive liposomes (the IC_{50} was reduced to 0.34 μg /ml).

Key words: methotrexate, liposomes, hyperthermia, folate, targeting, tumour cells, light scattering, fluorescence, cytotoxicity.

INTRODUCTION

Liposomes, the phospholipid vesicles, are spherical lipid bilayers capable of entrapping water-soluble solutes within an aqueous domain or alternatively lipid molecules within the lipid bilayers. They are biodegradable, biocompatible and non-immunogenic in nature, which makes them ideal drug carrier systems in therapeutics [15]. The efficacy of liposomes as drug delivery systems would be increased dramatically if it were possible to deliver their contents selectively to particular cells or anatomical sites. Several types of liposomal formulations have

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been explored; such as those bearing carbohydrate determinants (lecithins), monoclonal antibodies, glycoproteins and antigens with a view of targeting the liposomes to selective tissues or anatomical sites [3, 4]. However, most of these formulations have failed to show any remarkable therapeutic efficacy because of several reasons such as antigenicity of the ligands, inability of the drug to reach the appropriate cellular compartment in active form and the presence of the endothelial or other histological barrier between the liposomes and its cellular binding site.

These difficulties can be minimized by using temperature sensitive liposomes. These liposomes were developed by Yatvin *et al*. [26], Weinstein *et al*. [22] and others [20, 24] for better orientation to targeted sites. These liposomes are formed by a mixture of synthetic lipids such as dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC), that exhibits a gel to sol transition at temperature few degrees above the physiological temperature, a range easily obtainable by local hyperthermia.

Hyperthermia has been used to modify the local tumor environment to increase liposomal drug delivery to tumors. Although classically viewed as a form of adjuvant therapy to increase the efficacy of radiation and chemotherapy, hyperthermia can be applied to augment liposomal drug delivery by increasing tumor blood flow and microvascular permeability. At temperatures of 41–43°C, hyperthermia has been shown to increase the blood flow and oxygenation. It has also been shown to increase permeability of tumor vessels to antibodies, ferritin, and Evans blue dye. More specifically, hyperthermia has been shown to increase permeability of tumor vessels to liposomes. Additionally, it can be used as a modality for increasing liposomal drug.

It was postulated that such liposomes would remain reasonably stable in the vascular system at the normal body temperature (37 °C), and when passing through the heated area they would release their contents when the transition temperature was attained [11]. These authors found that specific release of antitumour drugs from liposomes in response to hyperthermia occurring in the heated regions resulted in a delay in the growth of the tumour. The present study was aimed at studying the delivery of methotrexate as an anticancer drug encapsulated in targeted thermosensitive liposomes to breast cancer tumor cells *in vitro*. The targeted moiety used in our study was folate synthesized on a polyethylene glycol spacer. The physicochemical properties of the resultant liposomes were studied using a light scattering assay as well as fluorescence measurements. The binding constant of the drug to the liposomal membrane was followed using a spectroscopic method.

The purity of lipids is an important factor; contaminated phospholipids may yield liposomes with an undesirable phase transition temperature (Tg). Therefore, the lipids were checked for purity by thin-layer chromatography using silica gel as a stationary phase and chloroform: methanol: water (65:25:4) as a mobile phase. Visualisation was done by iodine vapors. Impure lipids were discarded.

MATERIALS AND METHODS

Distearolyphosphatidylcholine (DSPC) of > 98% was obtained from Nattermann phospholipid GmbH, Koln, Germany. Dipalmitoyl-phosphatidylcholine (DPPC) of > 98% purity was purchased from Avanti polar lipids, Inc. (Alabaster, Alabama). Distearoyl-phosphatidyl-ethanolamine derivatized at the amino position with a 1900 molecular weight segment of polyethylene glycol (PEG-PE) was obtained from Avanti Polar lipids, Inc. (Alabaster, Alabama). Methotrexate (MTX) used was a commercially available injection (biotexate, Biochem Pharmaceutical Industries, Mumbai). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co (St. Louis, Missouri). Cell culture media, 0.5% trypsin solution, 10,000 units / ml penicillin, and fetal calf serum were obtained from Gibco (Grand Island, New York). Sephadex G 75 was obtained from pharmacia (Piscataway, New Jersey). N-[2-Hydroxyethyl] Piperazine-N-[2-ethanesulfonic acid] buffer (HEPES) was obtained from Sigma Chemical Co (St. Louis, Missouri). All other chemicals and reagents were of analytical grade.

PREPARATION OF LIPOSOMES

Thermosensitive liposomes were prepared by reverse phase evaporation method as reported by Szoka and Papahadjopoulos [19]. To get a lipid composition with the phase transition temperature near HT temperature, we preferably used the mixture of DPPC and DSPC [22]. Briefly, DPPC (221 μM) and DSPC (23 μM) (9:1 v/v) were dissolved in 50 ml of isopropyl ether-chloroform mixture (1:1 v/v). The lipid solution was mixed with 10 ml of phosphate buffered saline (pH 7.4) containing MTX in a 250 ml round bottom flask by a mixture. The obtained emulsion was homogenized with a Cell Sonicator, and the organic solvent in the emulsion was evaporated gradually by a rotary evaporator at 60 °C to form a liposomal suspension. The entrapment efficiency of the liposomal formulation was determined by separating the unentrapped drug from liposomes by gel filtration on Sephadex G-50 column [17]. Drug content was determined spectrophotometrically at 300 nm.

MEASURING THE BINDING CONSTANT

Binding constants were measured using a modification of a spectroscopic assay previously used [2]. The intensity of the main absorption bands of methotrexate changes upon partitioning into a lecithin environment. The absorbance at λ_{max} was measured in an aqueous solution, A_{init}, and in the presence of increasing amounts of lipid, $A_{obs,}$ on a Perkin Elmer (Norwalk, CT, USA) Lambda 20 spectrophotometer. By monitoring the absorption after the addition of

liposomes it was found that a 5-min incubation period was sufficient to achieve equilibrated binding. This equilibration time was maintained after the addition of an increased amount of lipid. It has been shown that the ratio A_{obs}/A_{init} depends on the lipid concentration as follows [13]:

$$\frac{A_{obs}}{A_{init}} = A_{comp} - \frac{\left(A_{obs}/A_{init}\right)-1}{K_b \cdot [lpd]} \tag{1}$$

where [*lpd*] is the lipid concentration and A_{comp} is the ratio of A_{obs}/A_{init} at complete binding. Algebraic manipulation results in the hyperbolic function:

$$A_{obs} = \frac{A_{init}\left(1 + A_{comp} K_b [lpd]\right)}{1 + K_b \cdot [lpd]} \tag{2}$$

Fitting the data to this natural non-linear function with the available non-linear regression algorithms is preferred over linearization of Eq. (1) and linear least square analysis. The reason is that linearization of Eq. (1), by using 1/ [*lpd*] as the independent variable, gives too strong weight to the value and to the spectral reading at low lipid concentrations, which are less accurate. The absorbance-versus.-[*lpd*] data were transferred to the graphics program Origin (Microcal Software, Northampton, MA, USA) and fitted to Eq. (2), whereby "*K"* and "*A"* were extracted.

CYTOTOXICITY ASSAY OF METHOTREXATE

The cells were grown in a cell culture medium without folic acid. Growth medium was supplemented with 10% fetal bovine serum and 100 units / ml of Penicillin. Cells were normally grown in 175 ml polystyrene tissue culture flasks (Corning, NY, USA) in a humidified incubator at 37º C and 4% CO_2 atmosphere until they became confluent. Confluent flasks were trypsinized to remove the cell monolayer and cells were resuspended in a fresh medium for plating.

Cell concentration and viability were determined by hemocytometer counts of cells. Tumor cells at 4000 cells / well were plated in 96-well flat-bottomed plates. The tumor cells were plated one day prior to the addition of liposomes encapsulating methotrexate. Methotrexate, either free or encapsulated, was added at different concentrations starting at 0.08 μg /ml and proceeding with different dilutions with a factor of two. The drug or liposome was incubated with the cells for one hour at 37 ºC under gentle shaking conditions to ensure uniform distribution and homogeneous mixing. The medium containing drug was then aspirated, and several washing steps took place using a phosphate saline buffer solution. One group of the plates were transferred to a 42 ºC incubator for one

hour, and then returned to 37 ºC afterwards. The other group was kept at 37 ºC. The two groups were incubated at 37 ºC in the incubator for three days, and the MTT assay was performed as before [6]. The optical density was then measured at 590 nm wavelength, and the percentage of cell viability was calculated and plotted against drug concentration.

FLUORESCENCE SPECTROSCOPY

Fluorescence emission intensity of liposome encapsulating methotrexate solutions was measured using spectrofluorometer (Schimadzu RF-1501). Liposome solutions were excited at 370 nm and the emission spectra were recorded from 400 to 600 nm.

RESULTS

Artificial liposomes are very often used as a model for biological membranes. This is justified, based on the similarity between the lipid content of natural membranes and the lecithin that we used. Obviously, additional parameters of cells membranes, such as membrane-bound proteins and active internalization, are important to the uptake of external materials in living cells. Therefore, a comparative binding to specific cells that are being studied will be necessary. The binding constant, $K_{b,}$ was measured for methotrexate, using a modification of a spectroscopic technique that was described in the *Materials* section (2, 13).

Fig. 1.A shows the absorption spectral changes of methotrexate upon binding to liposomes. To determine K_b, the absorbance of the longest wavelength peak, A_{obs}, was measured in aqueous solution, A_{init}, and in the presence of increasing amounts of lipids. The data are fitted to the hyperbolic equation, Eq. (2) (Fig.1.B), from which the fitted parameter, K_b, is obtained. It should be remembered that this binding constant reflects the equilibrium between the aqueously dispersed aggregates and membrane-bound monomers. The value of K_b for methotrexate was found to be 57.18 $(mg/ml)^{-1.}$

Figure 2 (A&B) shows the effect of folate-PEG-PE as a targeting moiety on the size distribution of liposomes. It is clear from the graph that incorporation foliate-PEG-PE in the liposome membrane affected the total size of liposomes and caused a second population with a larger size to take place.

Figure 3 shows the fluorescence intensity of methotrexate encapsulated in liposomes before and after the addition of Triton X100 detergent. It is clear from the figure the enhanced fluorescence intensity upon detergent addition, which is a good indicator for drug encapsulation.

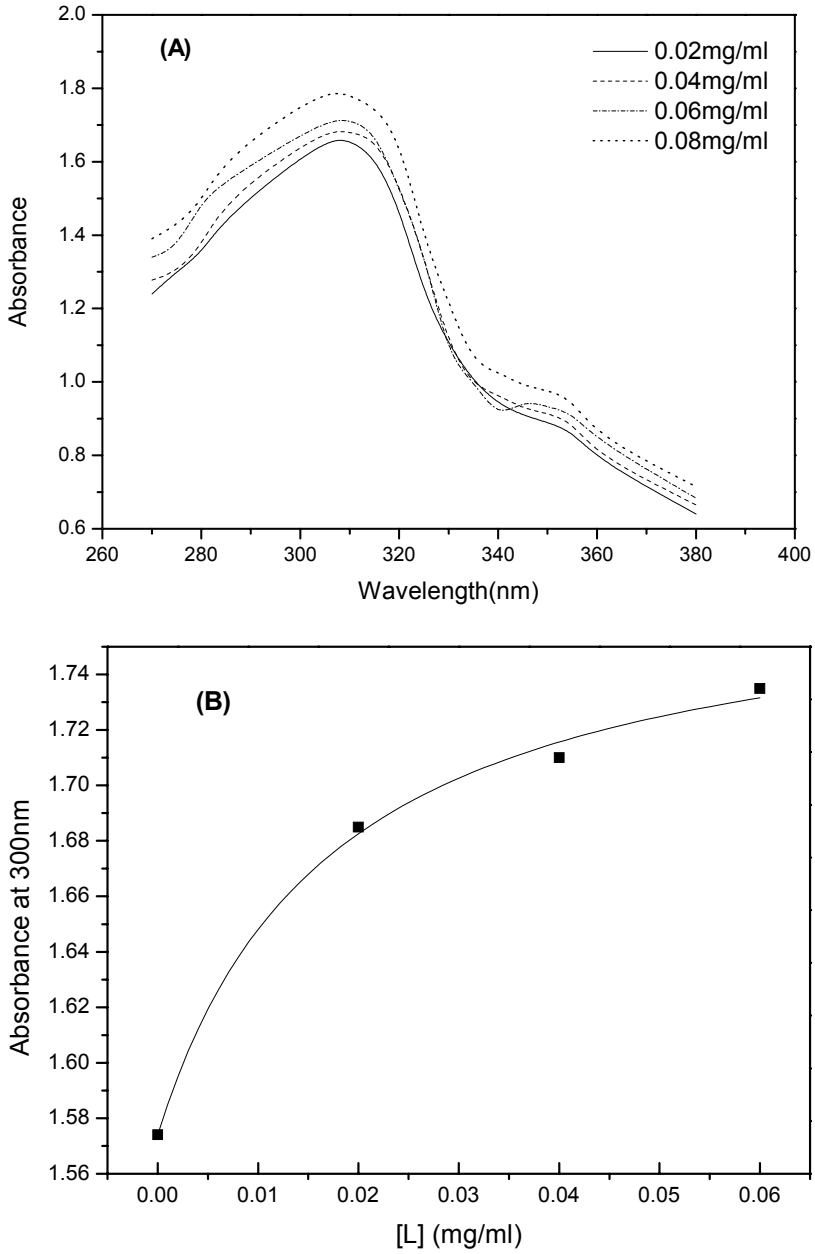

Fig. 1. – Absorption spectra (graph A) of methotrexate (5 mM) in water and in the presence of increasing concentrations of lipid (0.02, 0.04, 0.06, 0.08 mg/ml). Graph B shows the increasing absorbance at 300 nm upon binding to liposomes and a line fitted according to Eq. (2).

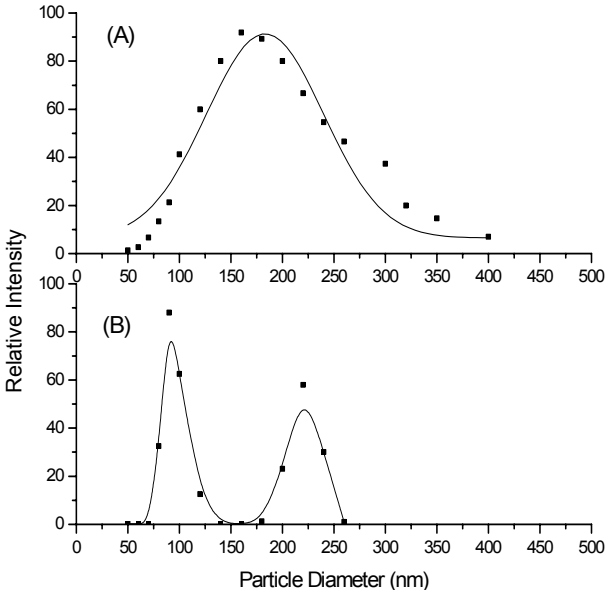

Fig. 2. – The effect of folate-PEG-PE as a targeting moiety on the size distribution of liposomes in the absence of folate (graph A), and in the presence of folate (graph B).

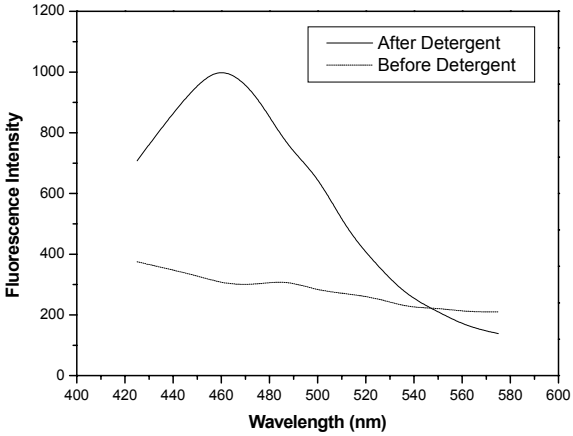

Fig. 3. –Fluorescence emission spectrum of methotrexate encapsulated in liposomes. Liposome solutions were excited at 370 nm and the emission spectra were recorded from 400 to 600 nm.

To measure the antitumor activity of these liposomes against breast cancer cell type, and to test the influence of hyperthermia on such antitumor activity, the cytotoxicity of folate targeted thermosensitive liposomes encapsulating methotrexate was compared with the cytotoxicity of non-targeted liposomes as well as free drug, (at 37 ºC and 42 ºC). The results are presented in Figure 4. Figure 5 shows the IC_{50} for both types of liposomes (targeted and non targeted) at 37 ºC and 42 ºC. It is clear from the graphs that the index of cell Killing expressed as IC_{50}

was reduced dramatically from 7 μg/ml to 0.864 μg/ml upon using folate as a targeting moiety. Hyperthermia was not effective when used with non-specific targeted liposomes. However, the cytotoxicity of the encapsulated drug increased dramatically upon heating folate targeted thermosensitive liposomes (the IC_{50} was reduced to 0.34 μg /ml).

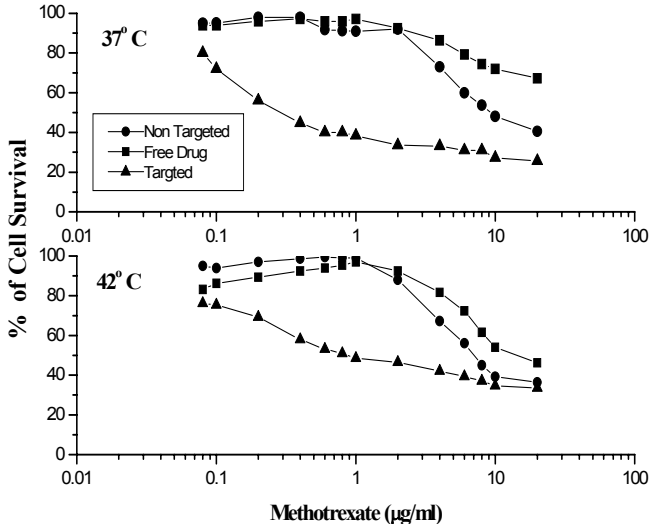

Fig. 4. – Percentage of cell (breast cancer) survival as a function of increasing concentration of methotrexate (encapsulated in folate targeted liposomes, non-targeted liposomes and in a free form) at 37 ºC (upper graph) and at 42 ºC (lower graph).

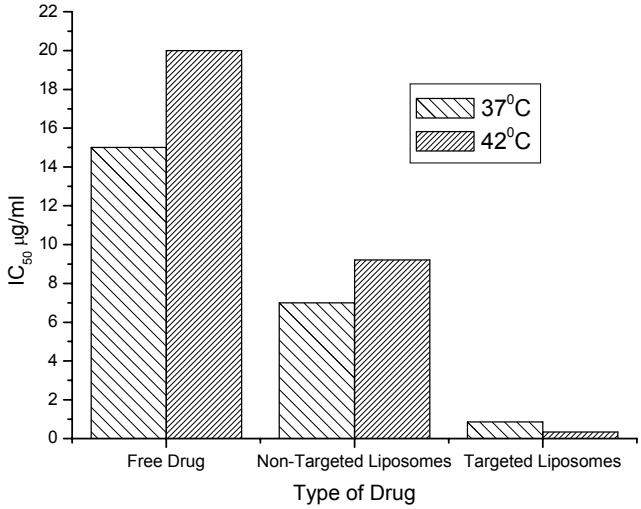

Fig. 5. –The IC_{50} for free drug and encapsulated in both types of liposomes (folate-targeted and non targeted) at 37 ºC and 42 ºC.

DISCUSSION

The combination of hyperthermia (42–44 °C) and chemotherapeutic agents frequently results in increased cytotoxicity over that predicted for an additive effect [5]. Several researchers have proved the effectiveness of this bimodality approach in tumour treatment. Combination of alkylating agents (nitromin) or antibiotics (adriamycin, bleomycin, actinomycin), or nitrosoureas (BCNU) [18] with hyperthermia (42–43 °C) also enhanced the tumour response in animals. The efficacy of this bimodality approach (chemotherapeutic agents and hyperthermia) could be increased dramatically if one could deliver the chemotherapeutic agents selectively to tumour cells and then apply localized hyperthermia to tumour site. This can be achieved in two ways; first one can administer the drug intratumourly and apply hyperthermia or alternatively one can use specialized drug delivery systems to selectively target the drug to tumour site and apply the hyperthermia. The former approach has certain inherent complexities. There may be necrosis of the cells following intratumoural injection of antineoplastic drug and the application of hyperthermia may be difficult. The use of liposomes as a drug delivery system coupled with local hyperthermia has been suggested as an effective strategy to achieve the preferential release of the drug in a target area. This approach of using thermoresponsive liposomes has been tested using various antitumour drugs in combination with local hyperthermia of the tumour and the results obtained were indicative of the preferential accumulation of the drug in heated tumours [20, 27]. The present study attempts at evaluating the antitumour effect of methotrexate by encapsulating it in temperature sensitive liposomes and using it in combination with hyperthermia for targeted delivery in management of solid tumour, breast cancer cells.

The folate Receptor (FR) is represented by a homologous family of glycoproteins [7, 12], two of which (FR-α and FR-ß) are attached to the cell surface by a glycosyl-phosphatidylinositol anchor [25]; the third isoform (FR-γ) and its truncated version (FR-γ') are constitutively secreted because of a lack of an efficient signal for glycosyl-phosphatidylinositol modification [16]. FR-α is expressed in some normal epithelial cells and is elevated in certain carcinomas, whereas FR-ß is a myeloid differentiation marker and is elevated in some nonepithelial malignancies [14]. FR-γ/γ' is expressed in hematopoietic tissues [16]. At present, FR is a major focus as a tumor target for multiple experimental approaches in cancer therapy. One novel approach uses bifunctional antibodies to target T cells to the FR on the surface of ovarian carcinoma cells. Selective growth inhibition of the tumor cells was obtained by this approach [10].

Folate-coated liposomes were shown to selectively target FR-rich tumor cells [8], and selective killing of the malignant cells was obtained by encapsulating doxorubicin in the liposomes [9]. By a similar strategy, it was possible to deliver antisense oligonucleotides against the epidermal growth factor receptor to FR-rich tumor cells. Furthermore, selective targeting of an adenoviral vector to FR-rich

tumor cells has been achieved in the presence of an antibody to ablate the endogenous viral tropism [1]. Finally, several studies have shown that FR, when expressed at high levels, could offer the preferred uptake route of novel classes of antifolate drugs that target glycineamide ribonucleotide formyltransferase and thymidylate synthase [23].

In the present study, a triple modality was used to achieve better therapeutic effect for breast cancer tumor cells and to get better understanding for the mechanism of hyperthermia in killing tumor cells *in vitro*. The combination of thermosensitive liposomes encapsulating methotrexate and coated with folate as a targeting moiety and hyperthermia further enhanced the tumor response, which may be because of the selective release of the methotrexate to tumour cells from liposomes in response to hyperthermia and the interaction of the hyperthermia and MTX, subsequently resulting in increased cytotoxicity to tumor cells. Our results have shown that targeting moiety is a very important and necessary factor in enhancing hyperthermia effect, since non-targeted liposomes did not show an enhanced effect before and after hyperthermia.

The results of the present study suggest that hyperthermia in combination with folate targeted temperature sensitive liposomes encapsulating methotrexate may serve as a useful targeted drug delivery system for more effective management of breast tumors.

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