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Research Article

In vivo antitumor activity study of targeted chlorambucil-loaded nanolipid carrier for breast cancer

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Abstract

Chlorambucil (CBL) is an efficient anticancer drug. It is a lipophilic agent with serious adverse effects. The objective of this study was to formulate a CBL-loaded nanolipid carrier and target breast cancer using folic acid as a targeting probe. Characterizations of the optimum formulation were 79.9±3% EE after the addition of 4mg CBL, 119±6nm particle size which is considered appropriate for parenteral use, 0.3±0.02 PDI, -42±1mV ZP that stabilized the formulation. Tumor volume, body weight, and tumor mass weight were recorded to evaluate tumor volume doubling time, tumor growth inhibition rate, and systemic toxicity. It appeared there was a significant antitumor activity of targeted formulation compared with non-targeted one and free CBL. Moreover, the systemic toxicity was less after body weight evaluation concerning the targeted formulation when compared with other formulations.

Keywords

Chlorambucil, DSPE, Antitumor Activity, Targeting, Folic Acid Moiety, Nanolipid carrier, Breast Cancer

Introduction

Breast cancer (BC) is common cancer that affects approximately 2–2.5 million people worldwide each year. More than 600,000 women died from breast cancer in 2018, and one in every eight women in the United States will develop advanced breast cancer during their lifetime. BC is the most common cancer in Indian women, with an estimated 170,000 women affected, which is 14% more than the total number of cancer cases in India (WHO 2020). Chlorambucil (CBL) is an alkylating agent orally administered in chronic lymphocytic leukemia therapies, lymphosarcomas, and occasionally in serious auto-immune disorders like rheumatoid arthritis, uveitis, and nephrotic syndrome (Chen et al. 2018). Also, it is used for the management of solid tumors such as advanced ovarian and breast carcinomas (Descôteaux et al. 2010). Chlorambucil shares common side effects with other alkylating agents such as nausea, vomiting, diarrhea, alopecia, oral ulcers, bone marrow suppression, hypersensitivity reactions, and rash. Long-term therapy may be associated with an increased rate of acute myelocytic leukemia, interstitial pneumonitis, and pulmonary fibrosis (Medicine 2017). Tumor-targeted drug delivery systems have emerged as a viable cancer therapy approach since they reduce the patient's harmful adverse reactions while improving the

therapeutic effectiveness (Fernández et al. 2018). Breast cancer cells overexpressed with folate receptors higher than healthy normal cells about 100–300 times and there are about 10 million copies of folate receptors in each cancer cell (Vlahov and Leamon 2012). This project was developed employing a PEGylated folate moiety for drug delivery targeting BC. Biocompatible lipids for nanolipid carrier formulation with PEGylation and folate targeting, hoping to result in dose modification, reduction in toxicity, and improving the effectiveness of the CBL.

Materials and methods

Materials

Chlorambucil (CBL) was purchased from Beijing Yibai Biotechnology Co., Ltd., (Beijing, China), distearoyl phosphatidyl ethanolamine (DSPE), PEGylated (MW 2000) DSPE (DSPE-PEG2000), and folate PEGylated DSPE (DSPE-PEG-Folate) were purchased from Xi'an Ruixi Biological Technology (Xi'an, China), lutrol F 68 (poloxamer 188) was purchased from BASF (Ludwigshafen, Germany), soybean lecithin (using parenterally) was purchased from Beiya Corp. (Tieling, China), soybean oil, Amicon 15 centrifugal filters and Millex syringe filter (0,45μm and 0,22 μm) were purchased from Sigma-Aldrich International GmbH (Taufkirchen, Germany). All other reagents and chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Preparation of CBL-loaded nanolipid carrier

Using high-pressure homogenization and ultrasonication techniques with some modifications, a CBL-loaded nanolipid carrier was formulated (Ganta et al. 2010). Specific amounts of CBL (4mg), soybean oil (3mg, 5mg, 6.5mg, and 10mg), DSPE (10mg, 13.5mg, 15mg, and 17mg), and PEG-DSPE (0.8mg), with or without folate-PEG-DSPE (80μg) were added to a glass beaker after using a suitable organic solvent system. After evaporation of the organic solvent through using a hot plate magnetic stirrer rotating until 900rpm at 75 °C. At the same temperature an aqueous phase containing lutrol F68 as surfactant with or without soya lecithin as co-surfactant was added drop by drop onto the melted lipid film resulting in an organic phase. After the formation of coarse emulsion, it was homogenized for 5 minutes at 12000rpm with an Ultra-Turrax Digital Homogenizer (IKA T25 basic, IKA Werke GmbH and Co., Germany). Finally, the formulation was nanosized using high-energy ultrasonication (Misonix ultrasonic liquid processor S-4000, Hielscher, GmbH, Germany) for 10 minutes (on-off 4-1 sec). The temperature of the sample was controlled by placing it in an ice bath.

Finally, the formulated dose (after adjusting the volume with deionized water to the required limit) was filtered through a 0.45μm MF-Millipore Membrane Filter (Merck, Darmstadt, Germany) to remove any contamination from the homogenization and ultrasonication methods, and stored in a clean and sealed vial. All the formulations to be injected were adjusted for osmolality (up to ~ 300 mOsml/kg) and pH (7.4) and filtered by 0.22μm filters (Millipore Express PES Membrane, Merck Millipore Ltd.) for sterilization (Chinsriwongkul et al. 2012). The blank formula was made in the same way as the test formula but without the chlorambucil.

Characterization of nanolipid carrier

Evaluation of particle size, polydispersity index, and zeta potential

The average diameter, polydispersity index, and zeta-potential were measured by photon correlation spectroscopy (PCS) and zeta potential measurement employing a Nano-ZS90 laser particle analyzer (Zetasizer Nano; Malvern Instruments; Malvern UK). Dilution of nanocarriers 10-fold using deionized water and bath sonication for several minutes to get a homogenous formulation and eliminate any aggregations before the examination (Pan et al. 2008). Standard deviations were calculated at room temperature and 90^0 scattering angle, and all the results were estimated three times.

Drug Loading Capacity and Encapsulation Efficiency Determination

The percentage of the drug quantity embedded in nanostructures is referred to as the drug entrapment efficiency (EE) and loading capability (LC). Briefly, five milliliters of each formulation were poured into the upper chamber of a centrifuge tube (Amicon Ultra, MWCO 10KDa, Sigma-Aldrich, Germany) and centrifuged for 15 minutes at 4000 rpm and 4 °C. The process was repeated for washing with deionized water at the same parameters of centrifugation. From the lower chamber of the Amicon tube, 50 μL was diluted with 5ml of ethanol and assayed spectrophotometrically at 258 nm wavelength (Saedi et al. 2018).

Drug LC and EE were evaluated by direct and indirect methods, by measuring encapsulated and unencapsulated drug, respectively. The LC and EE of the drug were calculated using the following equations (Liu et al. 2011):

$$EE=(Wt - Wf) / Wt \times 100$$
$$LC=(Wt - Wf)/ Wl \times 100$$

Where Wt is the total drug added, Wf is the free unentrapped drug, and Wl is the total lipid added.

Osmolality and pH measurements

The osmolality measurement of NLCs depended on the depression of the freezing point method illustrated in the user's manual (Advanced Instruments). Briefly, firstly put 100 μl of ultrapure water (from WFI group) in Eppendorf for pro-

be washing and calibration of the osmometer apparatus (Osmometer 800 CLG, Gonotec GmbH, Germany), then repeat this step using 100µl of reference standards (Osmoref 290 mOsm/kg, Gonotec GmbH, Germany) for final calibration before measurement. The osmolality was recorded with 100µl of sample and calculated after adjusting the correct reading compared with the calibration reading step. A pH meter (Mettler Toledo GmbH, Switzerland) was used for pH measurement and adjustment (Sharma et al. 2009). These tests adjusted significantly for the nano-formulations and CBL solution dose for animal study and parenteral injection.

In vivo animal study

Animals and tumor models

The Kunming strain mice (22±3 g/weight) were purchased from the Laboratory Animal Center of the Tehran University of Medical Science (TUMS). All animals were acclimated to standard circumstances at 25±2 °C and relative humidity of 70%±5% with reasonable access to food and water. All animals were maintained in a pathogen-free environment and treated according to institutional guidelines of animal care (Gao et al. 2004). Four groups (4 mice for each group) were randomly selected for *in vivo* antitumor activity study for injection of four different formulations. All groups were under the induction of cancer cell lines, where a suspension containing 2×10^6 cells of the 4T1 cell line was implanted by subcutaneous injection in the right flank. All of the animal experimental processes were accomplished following the Animal Ethical Committee's guidelines at the University of Baghdad, college of pharmacy, with an ethical code of RECAUBCP-11262019A.

In vivo antitumor activity study

The efficacy of CBL-loaded nano-formulations with and without folic acid targeting agents was investigated after injection intravenously in the tail of Kunming mice implanted with 4T1 cells for tumor induction. After seven days of tumor inoculation, when the tumor volume approached 100–200 mm^3, the treatments began, and that day was marked as day 1. Each group of mice was randomly assigned to one of four treatment groups with four mice in each group, and each group of mice was treated twice weekly for three weeks by tail vein injection of various formulations; (A) group 1 normal saline (0.9% NaCl) as a control group, (B) group 2 CBL solution (prepared in a specific method will be explained later), (C) group 3 CBL loaded non-targeted formulation, and (D) group 4 CBL loaded targeted formulation. The dose was adjusted to be 10mg/kg/week (Ganta et al. 2010). Tumor volume development, survival time, and body weight changes in mice were checked after treatment.

Every three days, the volume of the tumor was detected by a digital vernier caliper and calculated based on the following formula:

$$Tumor\ volume=[L\times W^2]/2\ ,$$

where (W) is the shortest width perpendicular to the length and (L) is the longest length. The tumor volume doubling time (DT) was calculated using the following equation:

$$Dt=(Tf-Ti)\times log2/(logVf-logVi)\ ,$$

where Vi means the initial tumor volume, Vf is the final volume, and (Tf-Ti) is the day's number between initial and final measurements (Nerli et al. 2014; Olerile et al. 2017).

Each animal was weighed using an electronic balance at the time of treatment to adapt dosages to obtain the dose per kg amounts recorded. The animal weights were assessed every three days during the trial and the findings were reported as an indicator of systemic toxicity. The mice were euthanized by cervical spine dislocation at the end of the investigation (day 21), and tumor masses were harvested. Following the acquisition of the tumors, the weights of the tumors were determined, and the tumor growth inhibition rate (I.R.) was measured, as seen in the equation below (Li et al. 2010):

$$\%\ IR=[(Wc-Wt)/Wc]\times 100$$

Where *Wc* refers to the mean tumor weight of the negative control group, while *Wt* refers to the mean tumor weight of the tested group (CBL solution, targeted, and non-targeted CBL loaded nanolipid formulations).

Preparation of chlorambucil solution for injection

CBL solution for tail vein administration was prepared according to the method reported by Lee et al.(Newell et al. 1983). Briefly, 20mg of CBL was added to 1ml of acidified ethanol (4.8ml of concentrated HCL added to 95%(v/v) ethanol in 100ml volume) until dissolving and then diluted to 10 ml with propylene glycol/dipotassium hydrogen phosphate buffer (20 g of K2HPO4 plus 450 ml propylene glycol diluting to 1000ml with water for injection), the final pH is 7.4. This solution was injected immediately into the mouse tail vein at a dose determined previously and in a very slow manner.

Results and discussion

Particle size, PDI, and zeta potential

Different factors concerning the best CBL solubility in different solid and liquid lipids, the ratio of liquid to solid lipid, and the ratio of surfactant to lipid ratio were studied. The best liquid to solid ratio was obtained in the range of 1:3 which is compatible with the results of Sahib et al (Sahib et al. 2021), and the optimum ratio of surfactant to lipid phase was 1:2. According to the literature reviews, the ratio of PEGylated DSPE used was 1:25 (PEG2000-DSPE: lipids) to the total amount of lipids used in the formulation.

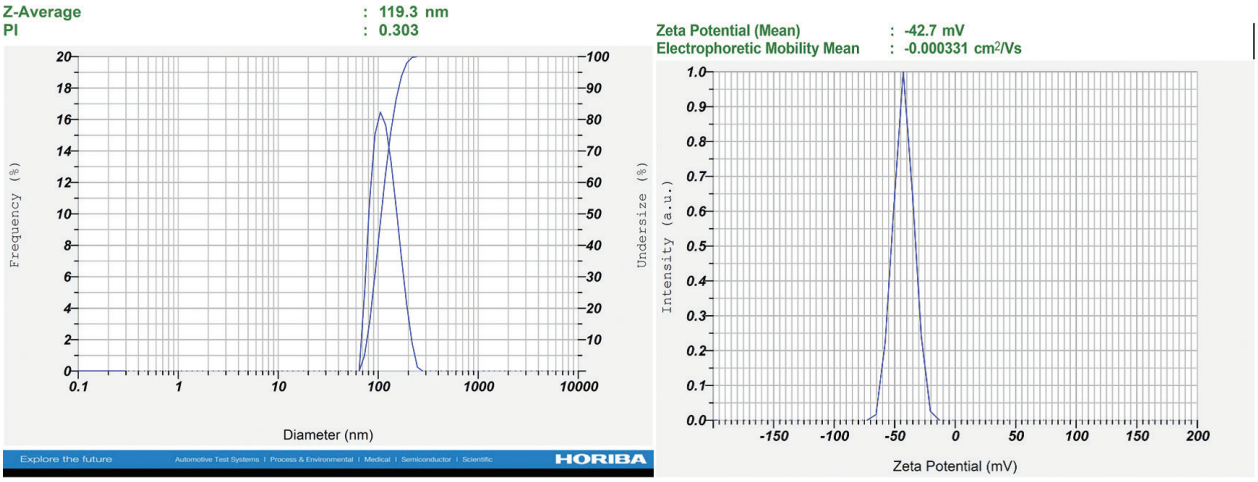

Figure 1. Average size distribution and zeta potential of the best selected formulation.

Simultaneously, the ratio of folated PEG2000-DSPE was 1:250 to the total amount of lipids used (Suk et al. 2016).

The particle size of the best-selected formula after adding PEGylated and folate-based DSPE was 119±6nm with 0.3±0.02 PDI, also the high stable formula due to the high zeta potential value toward negative sign (-42±1.0), Fig. 1. These data reflect the proper characteristics of the best-selected formula to be used for parenteral administration concerning sterilization step by filtration and high stability for lyophilization and storage.

Drug loading capacity and entrapment efficiency

Concerning the drug entrapment efficiency and loading capacity, there were good results for the best formula. Different ratios of liquid to solid lipid (1:6, 1:3, 1:2, and 1:1) were studied, and the best ratio depending on entrapment efficiency was 1:2 ratio with 99.1±0.7 EE when adding 1mg of CBL. Furthermore, there were good results when increasing the amount of drug; the values were 94±2.0 EE and 8.6±0.20 DL, or 79.9±3.0 EE and 13.8±0.6 DL for loading of 2mg CBL and 4mg CBL, respectively.

In vivo antitumor activity study

Various CBL-containing formulations were studied to evaluate their efficacy as antitumor agents. The different formulations of the groups 2 to 4 as a parenteral dose were administered to the mice and comparatively assessed according to the untreated group (normal saline administered as a control group) by measuring the tumor volume and mice body weight continuously. The changes in the tumor volume corresponding to the time for the four different groups are shown in Fig. 2.

Assessment of these results indicated a significant tumor growth suppression for all the CBL formulations compared to the control group. Moreover, for the targeted formulation, there was a significant ($P < 0.05$) antitumor activity compared with the non-targeted one. Specifically, the enhanced permeability and retention effect of the CBL-nanocarrier explained its preference as an antitumor agent compared to CBL solution; whereas, the folic acid receptor targeting ability of the targeted formula was granted the superior effect against the tumor mass. These findings are, to some extent, consistent with the results obtained by Han et al. concerning the effect of anticancer-loaded nanocarrier and folate-tagged nanocarrier (Han et al. 2020).

The data analysis documented in Table 1 confirms the superiority and antitumor efficacy of the various CBL formulations shown in Fig. 2. These data were obtained after evaluating the formulations by measuring the volume and weight of the tumor and estimating the tumor volume doubling time and tumor growth inhibition rate. Tumor volume doubling time (Dt) is the mean time required for the tumor to be doubled in volume. The longer Dt values suggest the more benign lesion and more beneficial efficacy of the therapy administered as an antitumor(Koike et al. 2014). Tumor growth inhibition rate (%IR) is used to quantify the response of the tested treatment compared to the control one at the end of the experiment(Transl et al. 2019). There was a significant ($P < 0.05$) effect of the targeted formulation concerning the time for the tumor volume to be doubled and the inhibition rate of the tumor growth compared with other formulations.

The high values of Dt for both targeted and non-targeted formulations (9.77±0.98 days and 7.38±0.49 days, respectively) compared with CBL solution (5.90±0.04 days) could be explained by the prolonged systemic circulation time of these formulations. This circulation time prolongation may be due to the surface grafting of the formulations (CBL-DPF and CBL-FPF) by PEG moieties resulting in reducing reticuloendothelial system (RES) clearance. Moreover, the targeting ability of the folate-grafted formulation (CBL-FPF) enhanced its affinity towered the cancer cells leading to higher efficacy. Hence, PEGylated and targeted formulations were assessed with better activity compared with CBL solution. Also, the enhanced permeability and retention (EPR) effect and the prolonged time of systemic circulation leading to steady drug concentration in the tumor microenvironment were considered a vital

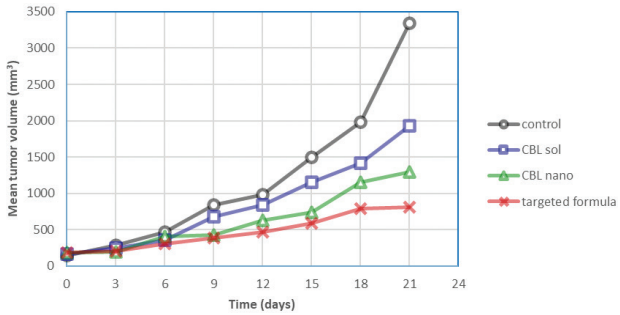

Figure 2. Changes in the tumor volume as a function of time in 4T1 cell line mice model after the tail vein administration of normal saline (control), CBL solution, non-targeted formulation, and targeted formulation, CBL dose adjusted to be 10mg/kg/week.

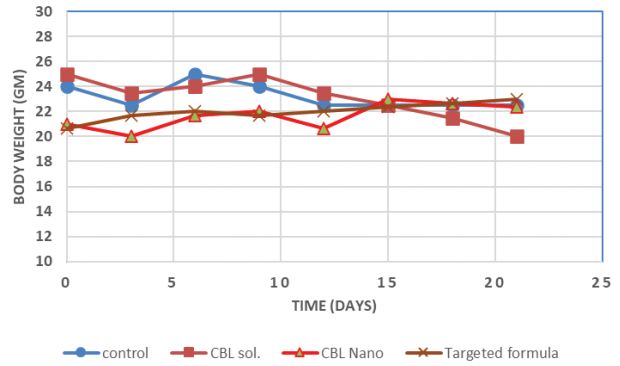

Figure 3. Body weight changes of the tumor bearing mice over all the duration of the experiment (21 days) after i.v. injection of control (NS), CBL solution, CBL loaded non-targeted formulation, and CBL-bearing targeted formulation.

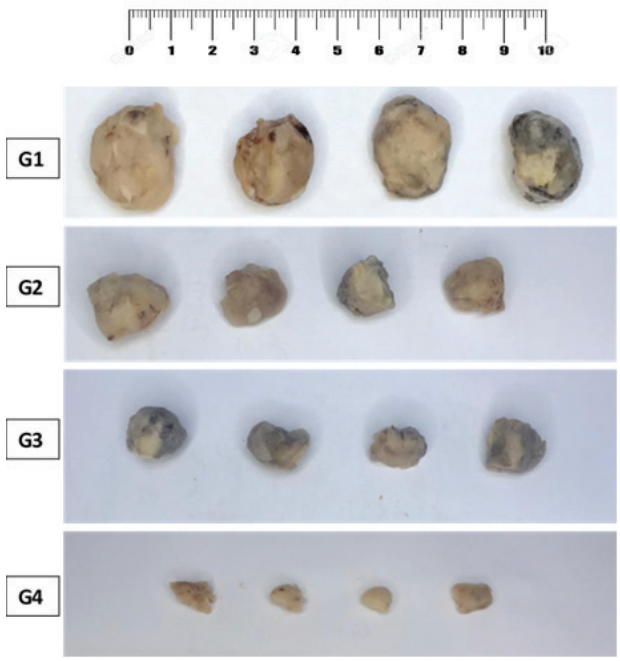

Figure 4. The excised tumors at the experimental end with different formulations, normal saline (G1), CBL solution (G2), non-targeted (G3), and targeted (G4) formulations.

Table 1. Tumor Volume Doubling Time (Dt) and tumor Growth Inhibition Rate (IR%) Parameters After Injection of Normal Saline, CBL Solution, Targeted, and Nontargeted Formulations to the 4T1 Cells Tumor Bearing Mice.

Treatment group	Tumor volume doubling time, Dt (days)	Tumor growth inhibition rate, IR (%)
Control	4.65±0.18	-
CBL sol	5.90±0.04	52.17±4.11
CBL-DPF	7.38±0.49	64.62±2.73
CBL-FPF	9.77±0.98	76.47±1.99

role in its higher activity as an anticancer therapy. These outcomes explained that the antitumor efficacy depends on the dose and exposure time (Bekaii-Saab and Villalona-Calero 2005).

Furthermore, to evaluate the adverse effects and the systemic toxicity of all these tested formulations, mice's body weight was recorded continuously throughout the experiment time, as shown in Fig. 3. There was no loss in body weight with a steady state pattern for targeted formulation and fluctuation in body weight for control and non-targeted formulations; whereas, about a 20% reduction in the body weight for the CBL solution injected mice.

As a result of the increased systemic toxicity of the CBL solution injected into mice, the mice lost body weight throughout the experiment. These findings showed that when delivered intravenously, the targeted formulation had stronger in vivo anticancer efficacy and less systemic toxicity than the CBL solution, making it more suitable for future clinical applications. The images of the tumor masses from each treated mice group excised on day 21 were illustrated in Fig. 4.

Conclusion

In this study, there are two main surface grafted formulations, PEGylated and folated formulations, were prepared. A lipid types DSPE (as nanolipid core), DSPE-PEG2000 (nanolipid shell as circulation stabilizer), and DSPE-PEG-folate (as targeting moiety) were utilized for nanocarrier preparation. Nanolipid carriers with suitable sizes for systemic circulation and high entrapment efficiency were prepared by homogenization and ultrasonication technique. Four main formulations; folate-targeted and non-targeted nano-formulations, CBL solution, and normal saline; were injected into the tumor-induced mice through the tail vein for antitumor activity study. This study continued for three weeks and the mice's weight and tumor size were measured continuously. The results observed more effectiveness of the targeted and non-targeted formulated nanocarriers (CBL-DSPE-PEG2000 and CBL-DSPE-PEG-folate) compared to CBL solution with lower systemic toxicity. Moreover, the targeted formulation exhibited superior activity to the non-targeted one, confirming the effective targeting affinity of the folate towered folic acid receptor.

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