Efficacy of pegylated liposomal etoposide nanoparticles on breast cancer cell lines

Mohammadreza MEHRABI[1], Parisa ESMAEILPOUR[2], Azim AKBARZADEH[1], Zahra SAFFARI[1],
Maryam FARAHNAK[1], Ali FARHANGI[1], Mohsen CHIANI[1,*]

[1]Department of Pilot Nanobiotechnology, Pasteur Institute of Iran, Tehran, Iran
[2]Department of Chemical Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran

Background/aim: This study aimed to investigate the efficacy of pegylated liposomal etoposide nanoparticles (NPs) against T-47D and MCF-7 breast cancer cell lines.

Materials and methods: Pegylated liposomal etoposide NPs were prepared by reverse phase evaporation method. The size, size distribution, and zeta potential of the NPs was measured by a Zetasizer instrument. The cytotoxicity of NPs was inspected by methyl thiazol tetrazolium assay. The release pattern of the drug from the vesicles was studied by the dialysis method. Drug loading and encapsulation efficiency (EE) were also measured.

Results: The mean size, size distribution, and zeta potential of pegylated liposomal etoposide NPs were 491 ± 15.5 nm, 0.504 ± 0.14, and –35.8 ± 2.5 mV, respectively. Drug loading and EE were 10.3 ± 1.6% and 99.1 ± 2.8%, respectively. The etoposide release in the formulation was estimated at about 3.48% after 48 h. The cytotoxicity effect of etoposide NPs on T-47D and MCF-7 cell lines of breast cancer showed higher antitumor activity as compared with those of the free drug.

Conclusion: Liposome-based NPs may hold great potential as a drug delivery system.

Key words: Etoposide, pegylated liposomal NPs, breast cancer, cytotoxicity

1. Introduction

Cancer is a major public health problem in the world. There were 14.1 million new cancer cases and 8.2 million cancer deaths in 2012 worldwide. If these rates do not change, the global cancer burden is expected to nearly double to 21.4 million cases and 13.5 million deaths by 2030. Breast cancer is the most common cancer among women worldwide, with nearly 1.7 million new cases diagnosed in 2012 (the second most common cancer). This represents about 12% of all new cancer cases and 25% of all cancers in women (http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.).

Topoisomerase inhibitors, such as etoposide, are the first line of chemotherapeutic agents that are used in the treatment of many types of cancer. Etoposide acts by forming a ternary complex with topoisomerase II and DNA, causing DNA breaks and cell death (1). In addition to the many side effects related to the drug (2–4), the administration of etoposide is limited by its low solubility in aqueous solutions (5,6). Therefore, finding an effective

approach to facilitate the transport of drugs and to improve the bioavailability of therapeutics is necessary.

Liposomes, closed vesicular structures consisting of one or more lipid bilayers, are widely used as drug delivery vehicles. In particular, they have been investigated for their ability to improve the delivery of chemotherapeutic agents to tumors in efforts to increase therapeutic efficacy and decrease toxicity to normal cells (7).

Many molecules have been encapsulated into liposomes and released to the market, and new drugs are added to the list every year. The main goal of this technology is to increase the therapeutic index of the drug while minimizing its side effects.

Solid lipid nanoparticles such as liposomes afford an interesting drug delivery system because they are biodegradable, biocompatible, and nontoxic at therapeutic doses. These drug carriers increase the half-life of the drugs in circulation and at the tumor site (8,9).

In the present study the efficacy of pegylated liposomal etoposide nanoparticles (NPs) against T-47D and MCF-7 breast cancer cell lines was evaluated.

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* Correspondence: chiani110@yahoo.com

2. Materials and methods

2.1. Materials
Etoposide was purchased from Koçak Farma (Turkey). Phosphatidylcholine, polysorbate 80, cholesterol, polyethylene glycol 2000 (PEG-2000), and methyl thiazol tetrazolium (MTT) (0.5 mg/mL) were obtained from Sigma (USA). The RPMI-1640 culture medium was purchased from Invitrogen (USA). T-47D, MCF-7, and MCF-10A cell lines were supplied by Pasteur Institute of Iran (Iran).

2.2. Preparation of NPs
The preparation of NPs was carried out by the reverse phase evaporation technique. PEG-2000, phosphatidylcholine, polysorbate 80, cholesterol, and etoposide (5:50:5:30:10 molar ratio) were dissolved in 40 mL of 98% ethanol (at 300 rpm, for 24 h, at room temperature) to obtain a transparent yellow suspension. Then the solvent phase of the obtained solution was removed using a rotary evaporator (Heidolph, Germany) at 90 rpm, for 2 h, at 50 °C. Afterwards, 20 mL of phosphate buffer (pH 7.4, 20 mM) was added to the resulting precipitate and was stirred (at 300 rpm for 24 h) at room temperature. A control formulation was also prepared without etoposide. Finally, the formulation was homogenized (Bandelin Sonorex Digitec, 60 Hz, Germany) for 5 min to reduce the size of liposomes and for the enhancement of their homogeneity (10).

2.3. Characterization of NPs
The mean size, size distribution, and zeta potential of particles were determined by Zetasizer (Nano ZS3600, Malvern Instruments, UK). The morphology of NPs was studied by a scanning electron microscope. For this purpose, the sample was lyophilized and placed on aluminum stubs and the surface was coated with a layer of gold particles using a sputter coater. The shape of the NPs was determined by scanning electron microscopy (SEM) (XL30, Philips, the Netherlands) at 15 kV and 750 mA.

2.4. In vitro study of drug release
Drug release was determined by the dialysis technique. Two milliliters of each formulation (pegylated nanoliposomal etoposide, its control, and the free drug) were poured into dialysis bags (with a cutoff of 12,000 Da, Sigma) and the bags were submerged in phosphate buffer (pH 7.4, 20 mM) and stirred (100 rpm, 48 h, 37 °C). At predetermined time intervals 2 mL of the phosphate buffer were taken and replaced by fresh phosphate buffer. Finally, the amounts of released etoposide in the phosphate buffer were measured using the spectrophotometric method at 284 nm and the rate of etoposide release was obtained using the standard curve.

2.5. Determining the drug loading and encapsulation
Encapsulation efficiency (EE) is the ratio of the weight of the drug entrapped into a carrier system to the total drug added. Drug loading is the ratio of the weight of the drug to the weight of the total carrier system (all excipients taken together). In order to determine drug loading and

EE, two formulas were used (11). For this purpose, 1.5 mL of the pegylated nanoliposomal drug (containing 15 mg of etoposide) and its control were centrifuged at 14,000 rpm for 50 min at 4 °C. Then the optical density of the upper phase of the formulation was measured at 284 nm by a spectrophotometer (UV-160IPC, Shimadzu, Japan).

$$EE\ (\%) = \frac{(Amount\ of\ drug\ in\ carrier)}{(Amount\ of\ drug\ fed\ initially)} \times 100 \qquad (1)$$

$$Loading\ efficiency\ (\%) = \frac{(Amount\ of\ drug\ in\ nanoparticle\ (mg))}{(Weight\ of\ nanoparticle\ (mg))} \times 100 \qquad (2)$$

To obtain a standard curve, different concentrations of etoposide were prepared and their optical density was measured at 284 nm.

2.6. In vitro cytotoxicity of nanoliposomal etoposide
The cytotoxicity of pegylated nanoliposomal etoposide was determined by MTT assay on T-47D, MCF-7, and MCF-10A cell lines. The MCF-10A cell line was used as normal cells. Cells were seeded in a 96-well plate at a density of 1×10^4 and cultivated with 5% CO_2 at 37 °C in RPMI-1640 culture medium (containing 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics); they were incubated for 24 h. After removing the supernatant, cells were treated with free etoposide and pegylated nanoliposomal etoposide in concentrations of 5 to 100 µg/mL. Cytotoxicity was evaluated after 48 h and 72 h of incubation. Absorbance was measured at 570 nm by Elisa reader (BioTek Instruments, USA). The half maximal inhibitory concentration (IC50) was determined by Pharm-PCS software (Springer Verlag, USA).

2.7. Statistical analysis
The results were expressed as means ± standard deviation (SD, n = 3). The data were statistically analyzed by one-way ANOVA using SPSS version 19, and the statistical significance was set at $P < 0.05$.

3. Results

3.1. Characterization of NPs
The size, size distribution, and zeta potential of blank pegylated liposomal NPs were 392.6 ± 5.4 nm, 0.511 ± 0.12, and –33.3 ± 1.2 mV respectively; for pegylated nanoliposomal etoposide NPs they were 491 ± 15.5 nm, 0.504 ± 0.14, and –35.8 ± 2.5 mV, respectively. SEM indicated that the nanoparticles had a smooth surface and a monodispersed pattern (Figure 1).

3.2. In vitro study of drug release
The release of etoposide from pegylated liposomal NPs in phosphate buffer (pH 7.4, 20 mM), was determined in 2, 4, 6, 8, 28, 31, and 48 h intervals (Figure 2). Our findings indicated that 3.84% of the drug in the pegylated

Table. IC 50 values (µg/mL) for free etoposide and NPs containing the drug in different cell lines during 48 h and 72 h of incubation at 37 °C under 5% CO_2. The cytotoxicity was tested by MTT assay at different concentrations of the formulations.

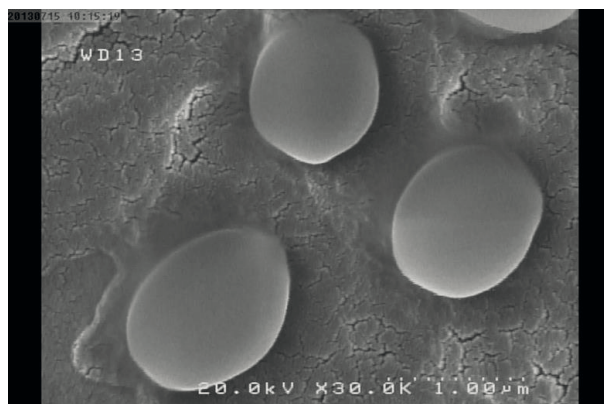| Formulations	Cell line type		
	T-47D	MCF-7	MCF-10A
48 h of incubation			
Free etoposide	57.23 ± 6.82	62.31 ± 6.23	76.38 ± 4.80
NPs containing the etoposide	14.35 ± 2.41	19.23 ± 3.20	45.42 ± 3.80
72 h of incubation			
Free etoposide	45.52 ± 5.24	53.45 ± 5.55	63.35 ± 2.62
NPs containing the etoposide	8.15 ± 1.82	15.41 ± 2.52	36.33 ± 3.31

All data are presented as means ± SD (n = 3).

Figure 1. Scanning electron microscopy image of pegylated liposomal NPs of etoposide. The NPs had a smooth surface and a monodispersed pattern.

Figure 2. In vitro release profile of free etoposide and pegylated nanoliposomal etoposide formulations in phosphate buffer saline (pH 7.4, 20 mM) at 37 °C (n = 3) at predetermined time intervals. The experiment was performed by the dialysis method. Approximately 4% of the drug was released from the carrier within 48 h. The rate of release was lower than that of the free drug during the same period (48 h).

nanoliposomal formulation was released into the phosphate buffer.

3.3. Determination of drug loading and encapsulation

The EE is defined as the ratio of the drug amount entrapped in NPs to that of the total drug added to the formulation. The encapsulation percentage was calculated according to the standard curve for drug formulation. Using the two formulas, the percentages of EE and loading efficiency were 99.1 ± 2.8% and 10.3 ± 1.6%, respectively.

3.4. In vitro cytotoxicity and cell viability of etoposide NPs

A safe concentration was obtained after several experiments and the optimum dose of free NPs was determined. Different concentrations of the NPs containing the drug were tested in MTT assay and the experiment was repeated three times, each time in triplicate, to enhance the accuracy of the results.
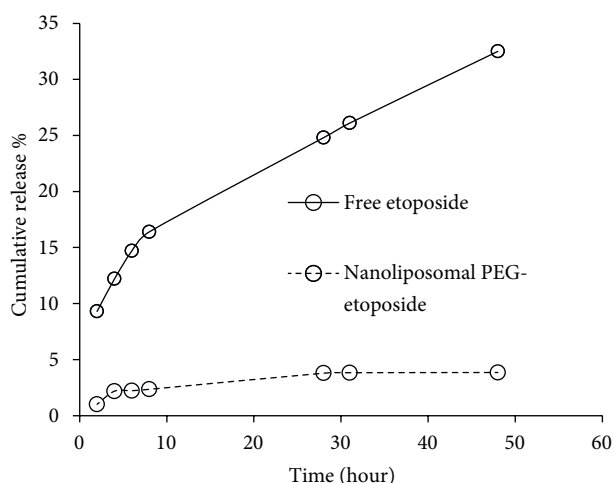
The in vitro cytotoxic activity and viability of the pegylated liposomal etoposide NPs and the free drug were assessed by MTT assay in T-47D, MCF-7, and MCF-10A cells. The IC50 values of the pegylated liposomal etoposide NPs and free etoposide for the mentioned cells are illustrated in the Table. The results of the viability tests are presented in Figures 3 and 4. It was shown that both the free drug and the pegylated nanoliposomal etoposide exhibited clear dose-dependent cytotoxicity against the cell lines. However, blank NPs had no effects on cell viability and showed similar result as the nontreated
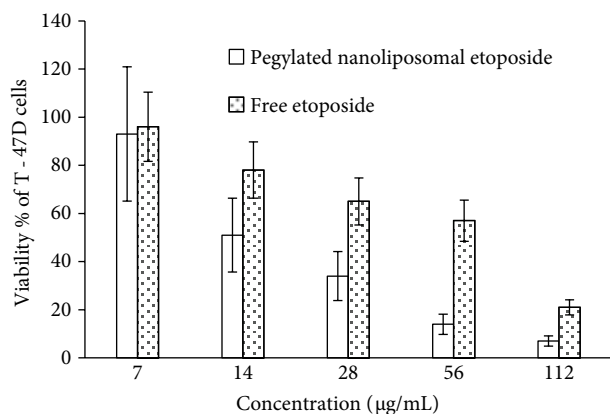
Figure 3. Cell viability of pegylated nanoliposomal and free etoposide in the T-47D cell line during 48 h of incubation at 37 °C under 5% CO2. It was tested by MTT assay at different concentrations of the formulations. All data are presented as means ± SD (n = 3).

Figure 4. Cell viability of pegylated nanoliposomal and free etoposide in the MCF-7 cell line during 48 h of incubation at 37 °C under 5% CO2. It was tested by MTT assay at different concentrations of the formulations. All data are presented as means ± SD (n = 3).

cells (P > 0.5). Because the blank NPs were selected in a safe concentration with no toxic effect on the cells, the cytotoxicity of pegylated nanoliposomal etoposide could be attributed to the toxic effects of etoposide. By increasing the concentration, the toxicity was enhanced, which suggested that drug concentration plays a major role in the in vitro cytotoxicity of etoposide. These results indicated that the cytotoxicity of drug-loaded nanoparticles against tumor cells was significantly (P < 0.05) higher than that of the free drug.

4. Discussion

Lipid nanoparticles such as liposomes have received increasing attention in the last years for the improved delivery of a vast variety of agents, such as anticancer agents, imaging agents, vitamins, minerals, antigens, and genetic materials. In the present study we succeeded in preparing pegylated liposomal NPs with high EE and loading efficiency, confirming reverse phase evaporation as an appropriate method. The experiments were performed in triplicate. The results of the particle diameter measurements (using Zetasizer) confirmed the nanoscale of the particle size (12). Figure 2 shows the cumulative release rate of etoposide from pegylated liposomal etoposide NPs. Compared with the rate of the free drug, the release rate of the drug from NPs was very low. It was shown that NPs had a high drug retention capability. Only 3.84% (W/W) of the drug was released from NPs after 48 h of incubation, whereas about 32% of the free drug was released in the same period. The release study showed that the release process included two different phases, a quick and a slow diffusion. In the first 4 h of evaluation, a burst release of the drug was observed. The release rate decreased with time (only 0.7% of the drug was released). The presence of PEG probably led to the low level of
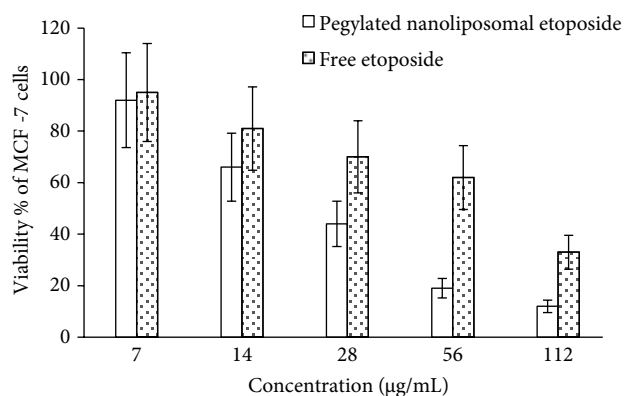
release (13). SEM results indicated that the nanoparticles had a smooth surface and a monodispersed pattern, which confirmed the slow release of the drug. Moreover, size was also an important factor that determined the release rate, and the nanoparticles prepared by this method had an appropriate size (14). The EE and loading efficiency of the formulation were calculated with respect to the standard curve, which showed acceptable values.

The cytotoxic effects of pegylated nanoliposomal etoposide formulations were studied by MTT assay, which showed that the formulation without the drug (the control) did not have any cytotoxic effects on the T-47D cell line. The IC50 of pegylated nanoliposomal etoposide was less than that of free etoposide. This might be due to the stabilizing effect of PEG in the pegylated nanoliposomal formulation slowing the rate of drug release. Moreover, PEG increased the drug solubility and its collision with the targeted cells. The effects of PEG on increasing the stability and antitumor efficacy of anticancer drugs were confirmed by Maitani et al. (15) and Reshetov et al. (16) in 2012. In another work Yang et al. (17) showed that pegylated liposomal paclitaxel had more antitumor activity than the conventional liposomal formulation. Their results indicated that pegylated liposomal paclitaxel had better antitumor efficacy against human breast cancer.

We prepared a new etoposide formulation by incorporating the drug into a liposome-based nanoparticle carrier. Our results suggest that the pegylated liposomal formulation of etoposide has a remarkable antitumor activity and may become a promising novel formulation for the treatment of human breast cancer.

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