Enhanced Selective Cytotoxicity in Pancreatic Cancer Cells Using EGF-Conjugated Liposome-Encapsulated Curcumin

U.M. Le, D. Ngo, T.M. Nguyen, Q.T. Nguyen, and J. Ton

Abstract

Pancreatic cancer is one of the most lethal cancers. Overexpression of epidermal growth factor receptor (EGFR) on the cell surface significantly enhances the tumor resistance, which makes the treatment even more challenging. Curcumin, a polyphenol found in turmeric (*Curcumin longa*), has been demonstrated to inhibit the growth, metastasis, and invasion of cancer cells via the EGFR signaling pathways. Our purpose is to develop an epidermal growth factor (EGF)-conjugated liposome-encapsulated curcumin (EGF-LP-Cur) to target the EGFR. As a result, the prepared liposomal EGF-LP-Cur had spherical vesicles with diameters of around 120 nm and contained high concentrations of curcumin and controlled concentrations of EGF. It was demonstrated to be stable in term of size and zeta potential within 21 days. The targeted formulation produced a significant increase of cytotoxicity on EGFR-overexpressed human pancreatic cancer cells as compared to the non-targeted one ($p < 0.05$). This is a promising candidate for the drug development in the treatment of pancreatic cancer, which remains challenging.

Keywords

Liposomes • EGF • Curcumin • Conjugated • Target • Pancreatic cancer

1 Introduction

Curcumin (diferuloylmethane) is derived from the rhizome of the *Curcuma longa* which is a member of botanicals that is native to Southeast Asia. Curcumin is considered pharmacologically safe on normal organs [1]. Furthermore, curcumin has been shown to have antioxidant, anti-inflammatory, and especially anti-cancer activity [2, 3]. For anti-cancer activity, curcumin has been demonstrated to suppress carcinogenesis, inhibit both NF-κB and AP-1 activation that involves in the cellular pathways leading to tumorigenesis, selectively induce apoptosis in tumor cells, induce autophagic cell death, and inhibit angiogenesis and tumor metastasis [4]. The major drawback of curcumin for cancer treatment application is its poor bioavailability. Curcumin is insoluble in water but readily soluble in organic solvents. An emerging technology to overcome this disadvantage is incorporating curcumin into nanocarriers. In addition, along with the line of searching for improved therapies for cancers, curcumin was cancer-targeted using various ligands, such as anti-P-glycoprotein trapped PLGA nanoparticles on multidrug-resistant cervical cancer cells [5], folate decorated dual drug loaded nanoparticles in colon cancer cells [6], folate-linked liposomes o KB and Hela cells [7], and hyaluronic acid conjugated gold nanoparticles on HeLa cells, glyoma cells and Caco 2 cells [8].

In this project, we propose to conjugate epidermal growth factor (EGF) to curcumin-containing liposomes to increase the delivery of liposomes into tumor cells, and thus improve the resultant anti-tumor activity. Epidermal growth factor receptor (EGFR, a 170 kD endogenous cell surface glycoprotein) has been known to tie with cell proliferation, survival, adhesion, migration, and differentiation. An over-expression of EGFR was reported in various tumor

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cells, such as human head and neck cancer cells, human breast cancer cells, and human pancreatic cancer cells [9–11]. EGFR targeting agents have been developed for cancer therapy and showed promising clinical efficacy [12]. In this study, the over-expressed EGFR is exploited as a target to specifically deliver curcumin into pancreatic cancer cells.

2 Materials

Curcumin was purchased from Sigma-Aldrich (St. Louis, Missouri). EGF was obtained from PeproTech (Rocky hill, NJ). Phospholipids and PEG-2000 were from Avanti Polar Lipids (Alabaster, AL). CBQCA kit was from ThermoFish Scientific (Waltham, MA). All pancreatic cancer cells and media were purchased from ATCC (Manassas, VA). LDH cytotoxicity detection kit was from Clontech (Mountain View, CA).

3 Methods

Preparation and characterization of EGFR-targeted curcumin liposomes (EGF-LP-Cur). Curcumin-containing liposomes (Cur-LP) is prepared by incorporating curcumin into 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-maleimide(polyethylene glycol) (PEG-DSPE)-coated phospholipids using thin film hydration method subsequently with freeze-thaw, sonication, and extrusion [13]. EGF was conjugated onto Cur-LP via a covalent conjugation with the maleimide groups at the termini of PEG-DSPE chains [14]. The formulation was purified using gel permeation chromatography on Sephadex^{TM} G-25 column and then Sepharose 4B column. The concentration of EGF in the liposomes is determined using CBQCA Protein Quantitation Kit. The concentration of curcumin in the final liposome preparation is determined using a Biotek synergy microplate reader 450 nm [15]. The particle size and zeta potential of liposomes are determined using Malvern Zetasizer. The formulation morphology was characterized using a transmission electron microscope (TEM) [16].

In vitro *cytotoxicity study*: Different human pancreatic cancer cells (BxPC-3, Panc-1, and MIA PaCa-2) were seeded in 96-well plate and incubated overnight at 37 °C, 5% CO_2. Cells were then incubated in the presence of different concentrations of EGF-LP-Cur, LP-Cur, and mix of EGF +LP-Cur for 24, 48, and 72 h. The cytotoxicity was determined using an LDH cytotoxicity detection kit [17].

Statistical analysis: All statistical analyses are completed using ANOVA followed by pair-wise comparisons using the Fisher's protected least significant different procedure. A p-value of <0.05 was considered to be statistically significant.

4 Results and Discussions

4.1 Formulation and Characterization of EGF-LP-Cur

The EGFR-targeted EGF-LP-Cur was prepared by conjugating murine thiolated EGF onto freshly prepared LP-Cur via the distal end maleimide groups. The particle size, zeta potential, percentage of curcumin release, EGF, and curcumin concentrations are shown in Table 1.

The prepared EGF-LP-cur was negatively stained with uranyl acetate on a Formvar coated 200 mesh copper grid. The grid was examined in a Phillips CM-10 transmission electron microscope at 80 kV. Liposome images are shown in Fig. 1.

4.2 Stability of EGF-LP-Cur

In a 4-week stability study, liposomes were stored in ambient condition in an aqueous suspension. Particle size and zeta potential were quantified periodically. In general, these parameters were not remarkably different within 3 weeks (Table 2).

Table 1 Characterization of EGF-LP-Cur

Size (nm)	Zeta potential (mV)	[Cur] (μg/mL)	[EGF] (ng/mL)	% Curcumin released after 24 h
123 ± 1.3	−56.9 ± 0.47	286.8 ± 4.7	795.7 ± 12	1.5 ± 0.2

Data shown are mean ± SD ($n \geq 3$)

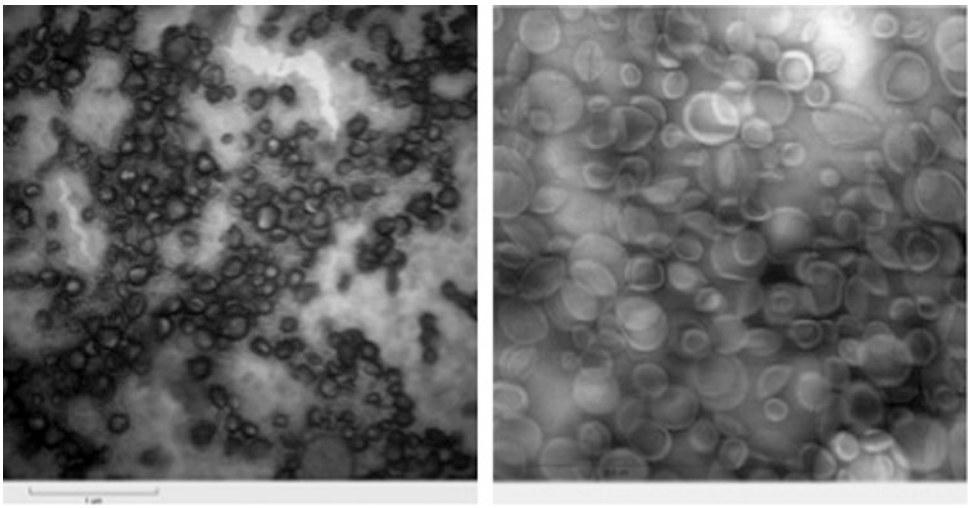

Fig. 1 TEM images of EGF-LP-Cur (resolutions of 35000× (*left*) and 72000× (*right*))

Table 2 Stability of EGF-LP-Cur

Time	Particle size (nm)	Zeta potential
0 week	123.6 ± 1.3	−56 ± 0.47
1 week	122.3 ± 1.0	−56 ± 0.52
2 weeks	130.7 ± 7.1	−57.3 ± 1.56
3 weeks	132.3 ± 6.0	−57.6 ± 1.36
4 weeks	163.5 ± 2.2	−67.3 ± 0.7

Data shown are mean ± SD ($n \geq 3$)

4.3 Cytotoxicity of EGF-LP-Cur on Human Pancreatic Cancer Cells

The cytotoxicities of the EGF-LP-Cur in BxPC3, Panc-1, and Mia Paca-2 cells were evaluated and compared. The EGF-LP-Cur was more cytotoxic at higher concentrations and after longer period of incubation. Furthermore, it appeared that the extent to which the EGF-LP-Cur was more toxic dependent on the density of the EGFR on the cells (Figs. 1, 2 and 3). In another word, the cytotoxicity of EGF-LP-Cur on BxPC3 and Panc-1 were higher than those on Mia Paca-2. It could be explained by the fact that BxPC3 and Panc-1 are overexpressed with EGFR while Mia Paca-2 cells have low EGFR expression [18]. However, it appeared that BxPC3 cells were more sensitive to EGF-LP-Cur than Panc-1. The selective cytotoxicity was more apparent when EGF was used to block EGFR on the cell surface. With BxPC-3 and Panc-1, most of groups blocked with EGF for 1 h prior to being treated with EGF-LP-Cur (EGF+EGF-LP-Cur) demonstrated reduced cytotoxicity. Interestingly, the EGF blocking made their cytotoxicity even lower than those from LP-Cur groups. No cytotoxic difference was shown among EGFR-targeted versus non-targeted groups on Mia Paca-2 cells, which have low-expression of EGFR (see Fig. 4).

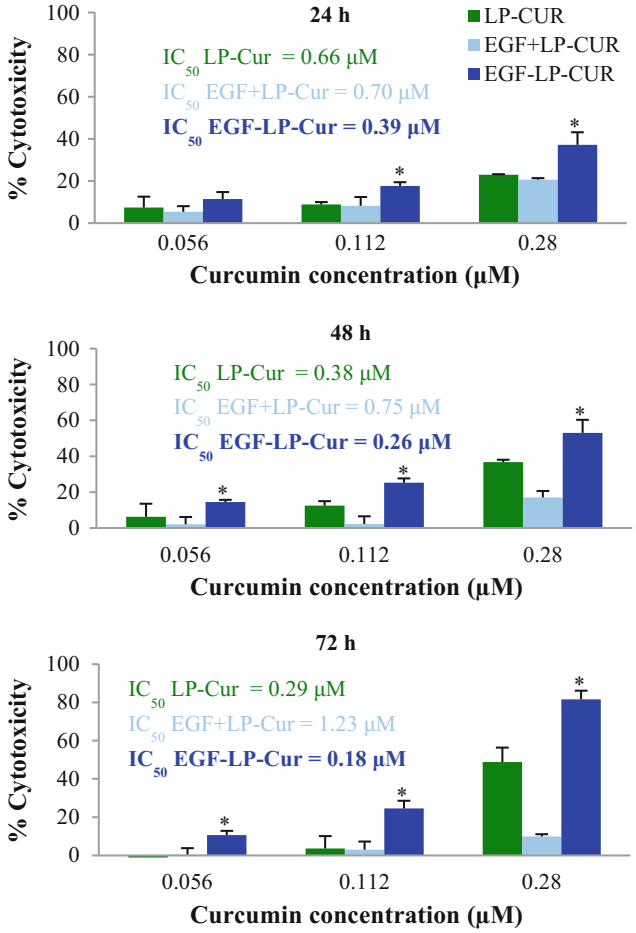

Fig. 2 Cytotoxicity of EGF-LP-Cur on BxPC3 cells (4000 cells/well) at 24 h, 48 h, and 7 s of incubation time. Data shown are mean ± SD ($n \geq 3$). *Asterisk* inidated significance difference ($p < 0.05$) of % cytotoxicity from the EGF-LP-Cur as compared with each other

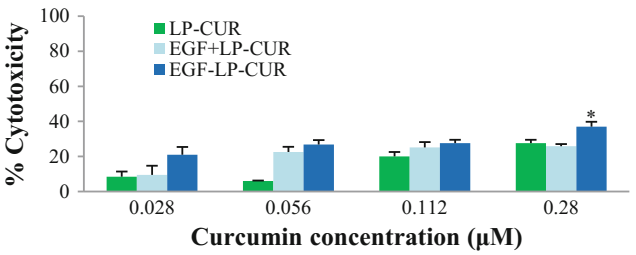

Fig. 3 Cytotoxicity of EGF-LP-Cur on Panc-1 cells (4000 cells/well) at 72 h of incubation. Data shown are mean ± SD ($n \geq 3$). *Asterisk* inidated significance difference ($p < 0.05$) of % cytotoxicity from the EGF-LP-Cur as compared with each other

5 Conclusions

The data demonstrated that the proposed EGFR-targeted liposome-encapsulated curcumin selectively kills the human pancreatic cancer cells in vitro more effectively than the non-targeted ones. Further investigations on the internalization and apoptosis are needed to confirm and clarify the mechanisms of action of the formulations.

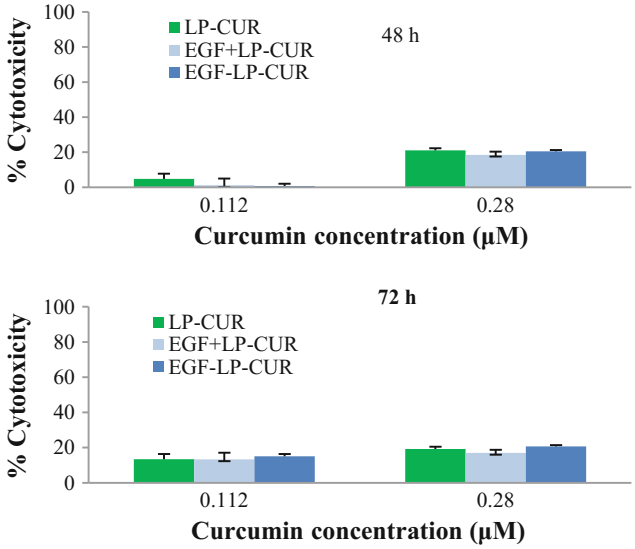

Fig. 4 Cytotoxicity of EGF-LP-Cur on Mia Paca-2 cells (4000 cells/well) at 48 and 72 h of incubation. Data shown are mean ± SD ($n \geq 3$)

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