

The effects of TPL-PEI-CyD on suppressing performance of MCF-7 stem cells

Yimin Zhu and Fuying Xu*

The First Affiliated Hospital of Lishui University / College of Medicine and Health, Lishui University, Lishui, China

Abstract: Triptolide, an ingredient of *Tripterygium wilfordii*, has been demonstrated to possess many biological activities such as immunomodulatory, antitumor activity in experiment. The purpose of this study was to survey the toxicity of TPL-PEI-CyD on renal cells and its effects on breast carcinoma stem cells. The cytotoxicity of TPL-PEI-CyD and TPL on HK-2 was comparatively assessed by CCK-8. After incubation and culturing with TGF- β 1, the MCF-7 cells were assessed by flow cytometry for the proportion of CD₄₄⁺ CD₂₄⁻ cells; then the CD₄₄⁺ CD₂₄⁻ cells were sorted by immunomagnetic beads as MCF-7 stem cells. To assess the effect of TPL-PEI-CyD on MCF-7 stem cells, Western Blot was used to detect the expression of Oct-4 and ALDH1 in MCF-7 stem cells after being dosed with TPL- PEI-CyD. Results showed that, compared with TPL, the toxicity of TPL-PEI-CyD on HK-2 cells was significantly reduced ($P < 0.05$). Breast carcinoma stem cells can be enriched by TGF- β 1 and isolated from MCF-7 cells by immunomagnetic sorting. TPL- PEI-CyD can even more significantly suppress the expression of Oct-4 and ALDH1 in MCF-7 stem cells than TPL ($P < 0.05$). In conclusion, after coupling TPL and PEI-CyD, TPL-PEI-CyD showed characteristics of effective suppression to breast carcinoma stem cell and decrease of cytotoxicity. It presented the unique effect of traditional Chinese medicine as an efficient and low toxic drug carrier complex for breast carcinoma treatment.

Keywords: Triptolide, breast carcinoma, tumor stem cell.

INTRODUCTION

Triptolide (TPL) is an effective active ingredient of Chinese herbal medicine (*Tripterygium wilfordii*) with the power of anti-inflammatory, immunosuppressive and multi-target anti-tumor (Chen *et al.*, 2019; Liang *et al.*, 2019; Liu *et al.*, 2015). TPL has become a focus of research as a natural anti-tumor drug (Liu *et al.*, 2015; Sun *et al.*, 2019). In clinical application, however, TPL has acute multi-organ toxicity including cytotoxicity to kidney, which could cause necrosis of renal tubular epithelial cells and even renal failure (Lin *et al.*, 2017).

In recent years, researchers had developed a drug carrier and release system, trying to reduce the toxicity of TPL (Liu *et al.*, 2017; Wu *et al.*, 2017). In our previous study, the triptolide-polyethyleneimide-cyclodextrin (TPL-PEI-CyD) complex was prepared by coupling PEI-CyD and TPL. It was found that TPL-PEI-CyD could inhibit the proliferation and induce apoptosis of human breast carcinoma cell line MCF-7/Taxol and reverse the chemotherapy resistance of MCF-7/Taxol. Breast carcinoma stem cell with the molecular surface markers CD₄₄⁺ CD₂₄⁻, played an important role in chemotherapy resistance and the occurrence, recurrence of breast carcinoma. The aim of this study was to investigate the effect of TPL-PEI-CyD on breast carcinoma stem cell and try to find a breakthrough of clinical treatment for breast carcinoma.

*Corresponding author: e-mail: zym330422@163.com

MATERIALS AND METHODS

Basic method of cell culture

Human breast carcinoma cell line MCF-7 and renal tubular epithelial cell line HK-2 were obtained from Shanghai Oulu Biotechnology Company and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with fetal bovine serum at a final concentration of 10%. All the cells grew in a humidified incubator at 37°C with 5% CO₂.

Assessing cytotoxicity of TPL-PEI-CyD by CCK-8

1.0×10⁴ cells / well of HK-2 cells were seeded in a 96-well plate and cultured for 24h. TPL-PEI-CyD was constructed and provided by the Institute of Chemical Biology and Medicine of Zhejiang University ; its structure of synthesis was shown in fig. 1. Detected by NMR spectroscopy, TPL was the 10% coupled ingredient in the composition of TPL-PEI-CyD. TPL-PEI-CyD was formulated into a concentration gradient (1, 2, 5, 10, 20μg/mL) solution with RPMI1640 (Hyclone Company). Since TPL accounted for 10% quality of TPL-PEI-CyD, TPL monomer was formulated into a concentration gradient (0.1, 0.2, 0.5, 1, 2μg/mL) with RPMI1640 as the controls. Different concentrations of 0.2ml drug-containing culture medium were added to 5 wells of different groups for incubation period of 4h. Then, after further incubating in culture medium for 24h, 20μl of CCK-8 (Dojindo, Japan) was added to each well and the incubation continued for 2h. The absorbance value of each well was then detected using a microplate reader at a

wavelength of 450 nm. Cell viability = (A value of the experimental / A value of the control) × 100%. All the experiments were in triplicate.

Enrichment of breast carcinoma stem cell

A 2mL MCF-7 cell suspension (2×10^5 cells) was added to each well of the 6-well cell culture plate and incubated overnight in the incubator, then the supernatant was discarded and fresh culture medium with 20ng/mL TGF- β 1(Life Technologies) was added and incubated for 48h. MCF-7 cells added fresh culture medium without TGF- β 1 was set as the blank control. The experiments were triplicated.

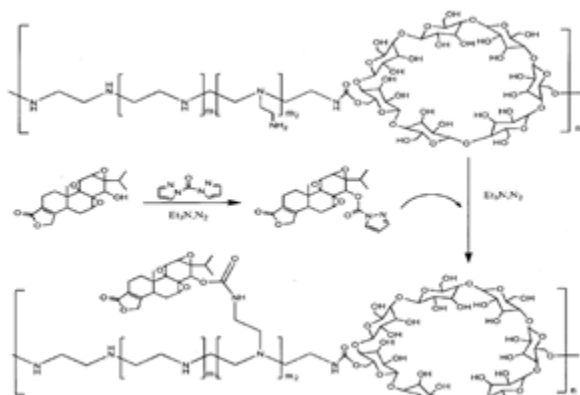


Fig. 1: The structure of synthesis for TP-PEI-CyD

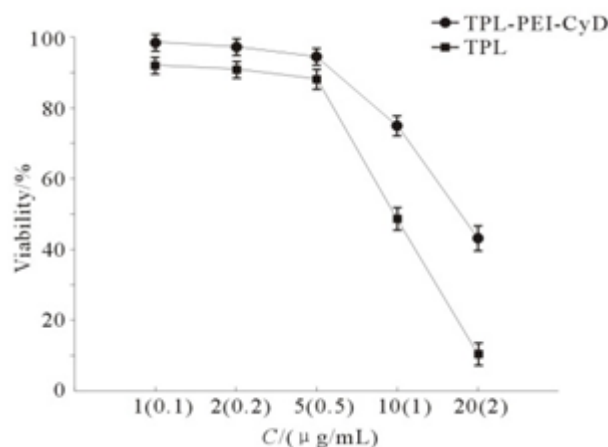


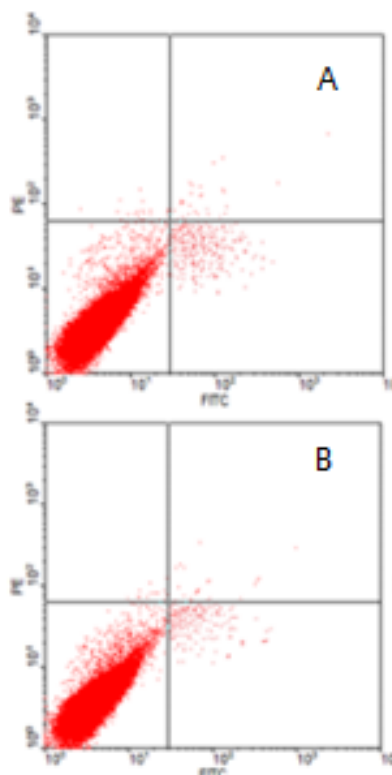
Fig. 2: Compared with TPL, the toxicity of TPL-PEI-CyD on HK-2 cells was significantly reduced ($P < 0.05$).

Flow cytometry (fcm) detecting enrichment of breast carcinoma stem cell

After MCF-7 was incubated with TGF- β 1, 0.1mL (1×10^6 /mL) cell suspension was added with 5µL of CD44-FITC and CD24-PE respectively and incubated in the dark at 4°C for 15min. Then the cells were added 1mL of PBS and centrifuged for 5 min (1000 rpm). The supernatant was discarded and the cells were washed with PBS once again. 20000 cells were counted by FCM detection with standard procedure, mercury excitation wavelength of 488nm. The experiments were repeated thrice.

CD44⁺ CD24⁻ Cells sorted by immunomagnetic beads

1×10^7 cell suspension of MCF-7 cells treated by TGF- β 1 was prepared and the volume was adjusted to 100µL. 10 µl of CD24 antibody (Biolegend) was well mixed to the cell suspension. After being incubated for 15min, it was added to 50µL of IgG immunomagnetic beads for 30 min. The cell suspension was placed in a magnetic separator for 5min and then the cell supernatant (CD24⁻) was collected. The resuspended suspension was added 10µL of CD44 antibody (Biolegend) and incubated at 4°C for 30 min. Then, the suspension was added 50µL of IgG magnetic beads (Beijing 4A Biotech Company) for 30 min and placed in a magnetic separator (Germany Miltenyi Biotech) for 5min. The supernatant was discarded, and the remaining cells were CD44⁺ CD24⁻ cells (Zai *et al.*, 2016).



A. 20ng/mL TGF- β 1 B. The blank control

Fig. 3: The proportion of CD44⁺CD24⁻ cells after incubation by TGF- β 1 obviously increased than The blank control ($P < 0.05$).

Detecting proportion of CD44⁺ CD24⁻ Cell by FCM

The CD44⁺ CD24⁻ cells sorted by immunomagnetic beads were washed with PBS and became single-cell suspension. The suspension was put in 5µl of anti-human CD24-FITC and 0.625µl rabbit anti-human CD44-PE, and incubated for 0.5 h. The proportion of CD44⁺ CD24⁻ cell was detected by FCM. All experiments were repeated three times.

Effect of TPL-PEI-CyD on the expression of Oct-4 and ALDH1 in MCF-7 stem cells

In this experimental phase, there were 4 groups consisting of TPL-PEI-CyD group, PEI-CyD group, TPL group and the blank control group. MCF-7 stem cells were seeded into 24-well plates at 1×10^5 cells/ well incubating overnight. In the TPL-PEI-CyD group, each well had 1 mL of fresh culture solution containing TPL-PEI-CyD (concentration $5 \mu\text{g/mL}$) added and incubated for 48h. While in the PEI-CyD group, TPL group and the blank control group, the same amount of PEI-CyD, TPL or culture solution were added and incubated respectively. The expression of Oct-4 and ALDH1 in each group were detected by Western Blot. The experiment was repeated thrice. According to the instructions of the Protein Extraction Kit, protein lysate was added, and the total proteins were extracted by homogenization. The same amount of sample in each group was taken for SDS-PAGE gel electrophoresis, and proteins were blotted onto PVDF membranes. The membranes were hybridized with a monoclonal antibodies Anti-Oct-4, anti-ALDH1 (Boster Company) at 4°C overnight followed by incubation with a secondary antibody for 1 hour at room temperature. GAPDH was used as a loading control. Protein bands were detected using ECL detection system. The optical density of blank control group was set as 1.

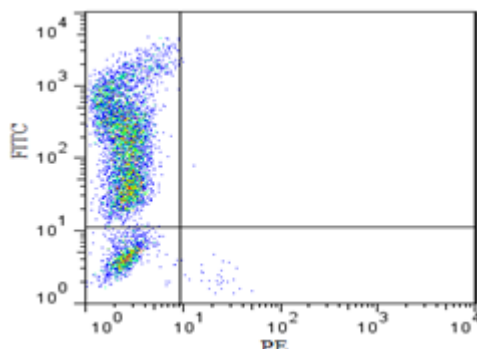
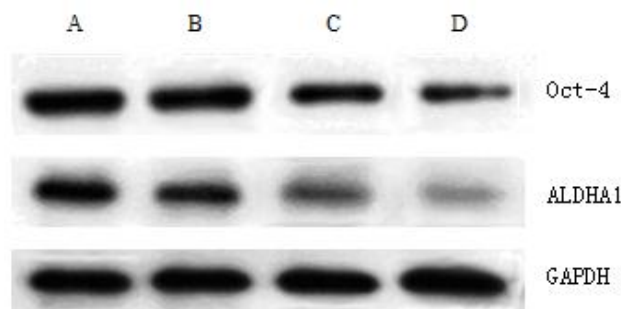


Fig. 4: After sorting by magnetic beads, the proportion of $\text{CD}_{44}^+\text{CD}_{24}^-$ cells was assessed by FCM



A The blank control B PEI-CyD group C TPL group D TPL-PEI-CyD group

Fig. 5: Results of Western Blots showed that the expression of Oct-4 and ALDH1 were significantly lower in TPL-PEI-CyD group and TPL group than in the

blank control group and PEI-CyD group ($P < 0.05$), while there were significant differences between the TPL-PEI-CyD group and TPL group ($P < 0.05$).

STATISTICAL ANALYSIS

The data were analyzed by SPSS 22.0 statistical software and the results expressed as the mean \pm standard deviation ($\bar{x} \pm s$). Independent *t*-test and One-Way ANOVA were used in comparison among the groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

The cytotoxicity of TPL-PEI-CyD

The cytotoxicity of TPL-PEI-CyD and TPL to the renal epithelial cells were assessed by cell counting kit. The experimental results were plotted as shown in fig. 2. TPL inhibited cell proliferation indicating its cytotoxicity, which showed a significant dose-dependence. While TPL attached to PEI-CyD, the toxicity to renal cells was significantly declined.

IC_{50} of TPL-PEI-CyD after incubating HK-2 for 4 hours was $17.50 \pm 0.61 \mu\text{g/mL}$, while the IC_{50} of TPL was $0.86 \pm 0.04 \mu\text{g/mL}$. There was a significant difference ($P < 0.05$).

Enrichment of breast carcinoma stem cells

After culturing with TGF- β 1, the percentage of $\text{CD}_{44}^+\text{CD}_{24}^-$ cells in MCF-7 was up to $(4.92 \pm 0.51) \%$ by FCM assessment; while in the blank control it was $(1.78 \pm 0.18) \%$ ($P < 0.05$). As shown in fig. 3.

Screening for $\text{CD}_{44}^+\text{CD}_{24}^-$ Cells by FCM after magnetic beads sorting

The cells sorted by magnetic beads were then screened by FCM and the proportion of $\text{CD}_{44}^+\text{CD}_{24}^-$ labeled cells was $(89.11 \pm 1.50) \%$ (fig. 4), so that the acquired cells could meet the demand of subsequent experiments.

To determine the expression of Oct-4 and ALDH1 by western blot

Results of Western Blot were shown in fig. 5. The relative expression of Oct-4 and ALDH1 in TPL-PEI-CyD group and TPL group were (0.34 ± 0.02) , (0.27 ± 0.01) and (0.40 ± 0.01) , (0.31 ± 0.01) ; while in the blank control group and PEI-CyD group were (0.72 ± 0.05) , (0.63 ± 0.04) and (0.72 ± 0.04) , (0.62 ± 0.05) respectively. The expression of Oct-4 and ALDH1 in the TPL-PEI-CyD group and TPL group were significantly lower than in blank control group and PEI-CyD group ($P < 0.05$). However, there were significant differences between the TPL-PEI-CyD group and TPL group ($P < 0.05$). TPL-PEI-CyD could effectively reduce stem cell marker proteins in MCF-7 stem cells.

DISCUSSION

The polymer nanocarrier was able to reduce the toxicity of drugs by increasing its solubility, enhancing stability and bioavailability, and then providing sustained release (Chen *et al.*, 2017; Gadde, 2015; Li *et al.*, 2019). The positively charged nanospheres adhere to the surface of negatively charged cell membranes, facilitating endocytosis or ligand binding into the cell. While the drug was decorated with the polymer carrier material, it formed a sustained release preparation, which prevents the acute toxicity of the drug. Cationic nanocarriers have a unique proton buffering effect that protects the contained drug from attack or degradation by lysosomes during intracellular migration. TPL decorated with cationic nanocarriers obtained obvious advantages over TPL monomers. With low toxicity, TPL-PEI-CyD can efficiently penetrate into cells and display high activity.

Due to the extremely low amount of stem cells in the tumor tissue, it is difficult to be sorted directly. We incubated MCF-7 with TGF- β 1 and then got a high proportion of CD44⁺CD24⁻ cell subtype, so that the breast carcinoma stem cells are enriched. The performance of immunomagnetic bead was moderate, by which the cells were selected kept good viability. In the present study, CD44⁺CD24⁻ cells subsets were successfully isolated by magnetic separation. The results of FCM showed that the obtained cells had high purity, large amount and good activity.

Oct-4 and ALDH1A1 had been considered the common markers for some tumor stem cells. The expression of Oct-4 and ALDH1 in MCF-7 stem cells was reduced after incubation with TPL-PEI-CyD medium, which verified that the performance of MCF-7 stem cells could be suppressed by TPL-PEI-CyD.

Since this experiment was only an *in vitro* assay in single cell line, we will further study the effects of TPL-PEI-CyD on the performance of multiple cell lines. If the follow-up results can be achieved as expected, then TP-PEI-CyD can be proceeded to preclinical trials. In view of the safety and efficiency of TPL-PEI-CyD, it would play a superior anti-tumor effect as a fine praeparatum of traditional Chinese medicine to treat patients of advanced, recurrent or metastases breast carcinoma.

CONCLUSION

This study demonstrated that after coupling TPL and PEI-CyD, TPL-PEI-CyD showed characteristics of effective suppression to breast carcinoma stem cell and decrease of cytotoxicity. It presented the unique effect of traditional Chinese medicine as an efficient and low toxic drug carrier complex for breast carcinoma treatment.

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