Research Article

TfR mAb-Cross-Linked Rituximab/MTX-PEG-PLL-PLGA Drug-Loaded Nanoparticles Enhance Anticancer Action in B Lymphocytes

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Nanoparticles could enhance the drug targeted to the cancer cell by the enrichment of the drug levels, which leads to the improvement of the codelivery of both drugs for an antitumor effect. In the current study, we reported an efficient, local drug-loaded delivery strategy with a nanoparticle-loaded system. After the synthesis of Rituximab/MTX-PEG-PLL-PLGA, the transferrin receptor monoantibody (TfR mAb) was subsequently cross-linked to the nanoparticles. Compared to the traditional drug, the nanoparticle-loaded system can precisely and efficiently transport the Rituximab and Methotrexate (MTX) drug into SU-DHL-4 cells, a typical kind of B lymphocytes, which can significantly increase the cell apoptosis in the SU-DHL-4 cells. Thus, the multifunctional drug-loaded nanoparticles displayed the persistent stability and precise targeting properties, which enhanced the efficiency of anticancer efficiency in B lymphocytes.

1. Introduction

Although decades of efforts of research and billions of funding have enhanced our understanding of the underlying mechanisms of tumorigenesis, the death rate from cancer remains running at a high level nowadays [1]. Innovative strategies fighting cancer by inducing antitumor responses from the immune system are urgently needed [2, 3]. Chemotherapeutics from the class of the anthracyclines have been shown to induce the death of cancer cells with immunogenic features and triggering immunogenic antitumor responses [4]. But they also bring big damage to the patient's immune system by the unspecific action of these cytotoxic drugs in systemic chemotherapy [5].

Nanotechnology carries the characteristics of antitumor drugs, and the emergence of nanotechnology has made it possible for drugs to be effectively delivered to tumors [6, 7]. The single drug carrier can monitor the therapeutic effect in real time by combining radiographic imaging technology and antitumor intervention, and then the drug can be directly and efficiently reach the inside of the tumor, to

maximize the reversal or overcome the antitumor multidrug resistance. Nanomedicine offers unique advantages in treating human cancers which are reported by several studies previously [8–10].

At present, the mechanism of tumor resistance is relatively complicated, but it also has shown that if a higher blood concentration is to be achieved and maintained, it is necessary to use a targeted site to deliver drugs to effectively control and reverse the starvation resistance of most tumor cells [2, 11]. In order to better increase the accumulation of antitumor drugs in tumor sites, it is necessary to achieve nanocarrier preparation. There are three key points in the progress of nanocarrier preparation. Firstly, passive targeting of high permeability will allow a retention effect by enhancing the permeability [12]. Secondly, active tumor targeting of nanoparticles through the specific molecules on the surface of tumor cells can be an optimal strategy [13]. Thirdly, it should be damage-free or should give low toxicity to the surrounding of the normal cells. For the aim of having a damage-free tissue, it is necessary to apply responsive nanoparticles to achieve targets under the stimulation of

physicochemical factors [14]. Some previous studies have found that the dendritic molecules have unique chemical structure properties, which make them act on tumors for a short period of time, inefficient tumor accumulation, and limited drug loading [15, 16]. The novel nanodrug carrier shows excellent potentials in increasing the antitumor activity of the drug and reducing the side effects [17]. Although nanomedicine carriers are an important part of fighting cancer, cancer treatment drugs are also critical.

Rituximab was the first monoclonal antibody to be approved for the treatment of cancer, and it is estimated that more than 4 million people have been treated with Rituximab worldwide [18, 19]. In the meantime, Methotrexate (MTX) is also still used in the treatment of a variety of tumors, including acute lymphocytic leukemia, breast cancer, osteosarcoma, primary central nervous system lymphoma, and head and neck cancers [20, 21]. Combined MTX and Rituximab with the nanodrug carrier may improve the accumulation of anti-tumor drugs and enhance permeability and retention effect.

Here, we aimed to investigate the effect of TfR mAb-cross-linked Rituximab/MTX-PEG-PLL-PLGA drug-loaded nanoparticles, which served as a precise and efficient carrier for the Rituximab and Methotrexate (MTX) drug into SU-DHL-4 cells, a typical kind of B lymphocytes, which can significantly increase the cell apoptosis in the SU-DHL-4 cells. The novel multifunctional drug-loaded nanoparticles persistently and precisely targeted SU-DHL-4 cells, which enhanced the efficiency of anticancer efficiency in B lymphocytes.

2. Materials and Methods

2.1. Synthesis of Rituximab/MTX-PEG-PLL-PLGA. The amount of PEG-PLL-PLGA was dissolved in 3 mL of a mixed solvent of dichloromethane-acetone-DMSO as the oil phase, while MTX (80 mg) (Sigma-Aldrich, St. Louis, Mo.) and Rituximab (40 mg) (Selleck Chemicals Houston, TX) were dissolved in 6 mL of 0.9% physiological saline as the aqueous phase. Then, the water phase was divided into 2 equal parts, labeled as aqueous phase 1 and aqueous phase 2, following the next two steps: (1) Aqueous phase 1 was poured into the oil phase, ultrasonically forming an emulsion, (2) and aqueous phase 2 was successively added dropwise to the oil phase, and the ultrasonication continued to form an emulsion. Finally, the above emulsion was added to 3 mL PVA (3 mg/mL) in 0.9% physiological saline. After ultrasonication for 6 min, the solution was evaporated on a vacuum rotary evaporator to remove methylene chloride-acetone in the solution. The solution was centrifuged at 15000 r/min for 10 min twice in 3 mL 0.9% normal saline. The supernatant was collected and freeze-dried to solid. The product was obtained, weighed (427.1 mg), and stored at 2–8°C. In the preparation of PEG-PLL-PLGA nanoparticles, the procedure is the same as above using the 0.9% physiological saline without drugs.

To determine the content of Rituximab/MTX in micelles, a series of known amounts of Rituximab/MTX-loaded nanoparticles were separately dissolved in 3 mL PBS. A UV-Vis spectrophotometer (Cary 300, Agilent Technologies, USA)

was used to determine the concentration of Rituximab at 278 nm and MTX at 258 nm to obtain a standard curve. Based on the standard curve, the drug loading content (DLC) and drug loading efficiency (DLE) could be calculated by the following equations:

$$\text{DLC (\%)} = \frac{\text{weight of drug in nanoparticles}}{\text{weight of drug-loaded nanoparticle}} \times 100\%, \quad (1)$$

$$\text{DLE (\%)} = \frac{\text{weight of drug in nanoparticles}}{\text{weight of feeding drug}} \times 100\%. \quad (2)$$

2.2. Preparation of TfR mAb/Rituximab/MTX-PEG-PLL-PLGA. To prepare the TfR mAb-cross-linked Rituximab/MTX-PEG-PLL-PLGA, 30 mg Rituximab/MTX-PEG-PLL-PLGA was dissolved in 3 mL PBS and ultrasonicated. Then, 300 µg TfR mAb (Invitrogen) and 9 mg Genipin were mixed gently at room temperature for 2 hours and an additional 12 hours at 2–8°C. The solution was centrifuged at 15000 r/min for 10 min twice in 1 mL 0.9% normal saline. The product was obtained after lyophilization and stored at 2–8°C.

To determine the TfR mAb modification rate in Rituximab/MTX-PEG-PLL-PLGA, a series of known amounts of TfR mAb-cross-linked Rituximab/MTX-PEG-PLL-PLGA nanoparticles were separately dissolved in 3 mL PBS. A UV-Vis spectrophotometer was used to determine the concentration of TfR mAb at 279 nm. The modification rate (%) was calculated by the above equation (1).

2.3. Characterization. The morphology of PEG-PLL-PLGA and drug-loaded nanoparticles was analyzed using a transmission electron microscope (TEM; Hitachi S-4800, Japan) with a voltage of 10 kV. The size and size distribution of nanoparticles were determined using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK). A UV-Vis spectrophotometer (Cary 300, Agilent Technologies, USA) was used to determine the concentration of Rituximab, MTX, and TfR mAb.

2.4. In Vitro Drug Release. 300 mg of the Rituximab/MTX-loaded PEG-PLL-PLGA nanoparticles was dissolved in 5 mL 0.9% physiological saline. The diluted solutions were transferred into dialysis membrane tubes and immersed in 60 mL PBS (0.01 M) then shaken at 150 rpm at 37°C. At scheduled intervals, 3 mL of solution was withdrawn from the release medium and replaced with the same volume of fresh PBS. The Rituximab/MTX content in the withdrawn solution was analyzed by a UV-Vis spectrophotometer and calculated based on the standard curve.

2.5. Cell Culture. The human SU-DHL-4 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). The SU-DHL-4 cells were cultured in RPMI-1640 medium (Hyclone, UT, USA), supplied with 10% FBS (Gibco, CA, USA) and 1% penicillin-streptomycin liquid at 5% CO₂ in a humidified incubator at 37°C.

FIGURE 1: (a–c) The morphology of PEG-PLL-PLGA and drug-loaded nanoparticles was analyzed using TEM. Scale bar = 100 nm. (d–f) Particle size and size distribution of nanoparticles were determined using a Malvern Zetasizer Nano ZS instrument. The average diameters (d (nm)) of particles were calculated by intensity.

2.6. In Vitro Cytotoxicity Evaluation. Cytotoxicity was measured using a CCK8 assay (Dojindo, Japan) according to the manufacturer's suggested procedures. SU-DHL-4 cells were seeded into 96-well plates at a concentration of 2.5×10^4 cells, and different concentrations of nanoparticles were added to be cultured for 48 and 72 hours, respectively. Then, 10 μL of the CCK8 reagent was added to each well and incubated for 2 hours in the incubator. The OD value was measured at 450 nm with a microplate reader.

2.7. Analysis of Cell Cycle and Cell Apoptosis. SU-DHL-4 cells were inoculated into a 6-well plate at a concentration of 2×10^6 cells/well while the drug concentration was 175 $\mu\text{g/L}$ Rituximab, 300 $\mu\text{g/L}$ MTX, 175 $\mu\text{g/L}$ Rituximab plus 300 $\mu\text{g/L}$ MTX, and 2100 $\mu\text{g/L}$ TfR mAb-MTX-PEG-PLL-PLGA nanoparticles, and the medium treatment group was used as a control. After 48 hours of drug treatment, the cells were harvested and resuspended in 1 mL PBS. After centrifugation, the supernatant was discarded and the cells were

FIGURE 2: UVA sensitivity and stability of Rituximab- (a), MTX- (b), or TfR mAb- (c) loaded nanoparticles. (d) The drug loading content (DLC) and drug loading efficiency (DLE) of Rituximab and MTX and the modification rate of TfR mAb in TfR mAb/Rituximab/MTX-PEG-PLL-PLGA nanoparticles.

resuspended in 0.5 mL PBS and 5 mL of precooled 70% ethanol. After being mixed well, the cells were fixed at 4°C overnight and then washed with PBS. RNase A (50 µg/mL) was added to dissolve for 30 min in a water bath at 37°C, and then 5 µL of PI (50 µg/mL) (Solarbio, China) was added to stain for 15 min at 4°C in the dark. Finally, the samples were detected by flow cytometry (BD Biosciences).

To detect cell apoptosis, cells were resuspended in 400 µL of 1x Binding Buffer and supplied with 5 µL FITC-Annexin V and 5 µL PI (50 µg/mL) (Solarbio, China). The control cells were divided into the nonstained group, single-stained Annexin V group, single-stained PI group, and double-stained Annexin V plus PI group. The samples were gently bounced, mixed, and incubated for 15 min at room temperature in the dark. 400 µL of 1x Binding Buffer was added to each tube and mixed. Finally, the samples were detected by flow cytometry (BD Biosciences) within 1 hour.

2.8. Statistics. Pharmacokinetic data were analyzed using the noncompartmental WinNonlin method. Statistical analysis was performed using the SPSS 22.0 program. *P* values were obtained using the two-sample *t*-test. For cellular uptake, bidistribution, and antitumor activity data, statistical analyses were performed by ANOVA, with *P* < 0.05 considered to be statistically significant and *P* < 0.01 considered to be an extremely significant difference. Statistical analysis was performed using the GraphPad Prism 5 (GraphPad Software, San Diego, CA).

FIGURE 3: The sustained release percentage of MTX- and Rituximab-loaded nanoparticles *in vitro*.

3. Results and Discussion

Prior to carrying out efficacy studies, the Rituximab and MTX drug-loaded PEG-PLL-PLGA nanoparticles were synthesized, followed by cross-linking by TfR mAb. The particles of the drug-loaded nanoparticles were evaluated by transmission electron microscope (TEM) morphology, which showed uniform particle distribution in three kinds

FIGURE 4: *In vitro* cytotoxicity evaluation of SU-DHL-4 cells using CCK assay. The SU-DHL-4 cells were treated by different drugs for 48 hours. (a) PEG-PLL-PLGA, (b) Rituximab, (c) MTX, (d) Rituximab combined with MTX, and (e) TfR mAb/Rituximab/MTX-PEG-PLL-PLGA nanoparticles. Data are shown as the mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$.

of nanoparticles before and after drug loading: PEG-PLL-PLGA (Figure 1(a)), Rituximab/MTX-PEG-PLL-PLGA (Figure 1(b)), and TfR mAb Rituximab/MTX-PEG-PLL-PLGA (Figure 1(c)). The size and size distribution of nanoparticles were also determined. The average diameter of the kinds of particles ranged from 310.7 nm before drug loading (Figure 1(d)) to 354.4 nm after Rituximab/MTX loading (Figure 1(e)) and to 365.8 nm after TfR mAb cross-linking (Figure 1(f)).

Then, a UV-Vis spectrophotometer was used to determine the concentrations of Rituximab at 278 nm (Figure 2(a)), MTX at 258 nm (Figure 2(b)), and TfR mAb at 279 nm (Figure 2(c)) in the drug-loaded nanoparticles. Based on the standard curve, the drug loading content (DLC) and drug loading efficiency (DLE) were calculated. The percentages of DLC of Rituximab and MTX were 8.17% and 14.87% (Figure 2(d)), respectively. The percentages of DLC of Rituximab and MTX were 87.25% and 79.41% (Figure 2(d)), respectively. In addition, the TfR mAb modification rate is up to 70.90% (Figure 2(d)), suggesting that the rates of drug loading and encapsulation were comparable and efficient.

The drug release of the nanoparticles *in vitro* was further evaluated. The sustained release rates of Rituximab and MTX reached 49.9% and 54.12%, respectively (Figure 3). Until 168 hours (7 days), the sustained release efficiency was maintained to 77.6% and 81.81% (Figure 3), indicating that the

sustained release of the two drugs in the nanoparticles owned a long effect.

To assess the cytotoxicity of the drugs, we treated the B lymphocytes and SU-DHL-4 cells by different concentrations of drugs (PEG-PLL-PLGA, Rituximab, MTX, Rituximab +MTX, and TfR mAb/Rituximab/MTX-PEG-PLL-PLGA), respectively. The cell viability of SU-DHL-4 cells was not affected treated with PEG-PLL-PLGA for 48 hours (Figure 4(a)), while it was significantly reduced treated with 175 and 250 $\mu\text{g/L}$ Rituximab for 48 hours (Figure 4(b)), MTX (ranged from 20 to 450 $\mu\text{g/L}$) for 48 hours (Figure 4(c)), and Rituximab combined with MTX for 48 hours (Figure 4(d)), respectively. Moreover, the cell viability of SU-DHL-4 cells treated with a high concentration of TfR mAb/Rituximab/MTX-PEG-PLL-PLGA for 48 hours was significantly reduced, ranging from 0.59 to approximately 0.62 ($P < 0.01$ vs. mock control), which was commensurate with other groups (Figure 4(e)).

We selected drug concentrations in subsequent experiments for further evaluation as follows: 175 $\mu\text{g/L}$ Rituximab, 300 $\mu\text{g/L}$ MTX, 175 $\mu\text{g/L}$ Rituximab+300 $\mu\text{g/L}$ MTX, and 2100 $\mu\text{g/L}$ TfR mAb/Rituximab/MTX-PEG-PLL-PLGA for 48 hours of treatment. The cell cycle was detected by flow cytometry (Figure 5(a)). Compared with the control group, Rituximab, MTX, or coprocessing of Rituximab plus MTX treatment resulted in a decrease in the proportion of S phase

FIGURE 5: (a) Cell cycle analysis of SU-DHL-4 cells treated by different kinds of drug for 48 hours using flow cytometry. (b) The percentages of phases in the cell cycle are shown as the mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$.

FIGURE 6: Cell apoptosis analysis of SU-DHL-4 cells treated by different kinds of drug for 48 hours using flow cytometry with PI and FITC-Annexin V staining.

cells while there is an increase in the proportion of G0-G1 (Figure 5(b)). However, TfR mAb/Rituximab/MTX-PEG-PLL-PLGA treatment did not have a significant effect on the cell cycle (Figure 5(b)).

To further identify the cytotoxicity of the drugs, cell apoptosis affected by drugs was determinate by forward scatter

(FSC) and side scatter (SSC) in flow cytometry. Being measured for cell size and granularity in cell apoptosis, the cell shrinks and releases apoptotic blebs as reflected by decreasing FSC and increasing SSC compared to normal cells. Exposed phosphatidylserine can be detected in the presence of calcium using FITC-Annexin V. After SU-DHL-4 cells

were treated with Rituximab, MTX, Rituximab+MTX, or TfR mAb/Rituximab/MTX-PEG-PLL-PLGA, the cell apoptosis rates of all treated groups were significantly higher than that of the control group (Figure 6). These results demonstrated the effect of cytotoxicity by TfR mAb/Rituximab/MTX-PEG-PLL-PLGA drug-loaded nanoparticles which relied on cell apoptosis.

4. Conclusions

In summary, we showed novel anticancer effects of TfR mAb cross-linked Rituximab and MTX drug-loaded nanoparticles on B lymphocytes and preliminarily studied the interference mechanism involved in cell cycle and cell apoptosis. The multifunctional single drug-loaded nanoparticles displayed maintained stability and precise targeting, which enhanced the anticancer efficiency of cell death for B lymphocytes. This enhanced cancer treatment provides a potential strategy in clinical anticancer therapy and deeper insights into the physiological and pathophysiological variability among individual patients. These findings also guide the design of precise nanomedicine towards personalized cancer treatments.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Supplementary Materials

A precise and efficient carrier for the Rituximab and Methotrexate (MTX) was designed as nanomedicine for anticancer action in B lymphocytes. After the synthesis of Rituximab/MTX-PEG-PLL-PLGA, the TfR mAb was subsequently cross-linked to the nanoparticles. The nanoparticle-loaded system can precisely and efficiently transport the Rituximab and MTX drug with long-term sustained release of into SU-DHL-4 cells. The TfR mAb/Rituximab/MTX-PEG-PLL-PLGA nanoparticle increased the cell apoptosis in the SU-DHL-4 cells, resulting in a notable cytotoxicity in B lymphocytes. (*Supplementary Materials*)

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