

Synthesis, Characterization and *In Vitro* Evaluation of Triptolide-lysozyme Conjugate for Renal Targeting Delivery of Triptolide

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(Received April 20, 2005)

A triptolide-lysozyme (TP-LZM) conjugate was synthesized to achieve renal specific delivery and to reduce the side effects of triptolide. Triptolide was coupled to lysozyme through succinic *via* an ester bond with an average coupling degree of 1 mol triptolide per 1 mol lysozyme. The lysozyme can specifically accumulate in the proximal tubular cells of the kidney, making it a potential carrier for targeting drugs to the kidney. The structure of triptolide succinate (TPS) was confirmed by IR, ¹H-NMR, MS and UV. The concentrations of triptolide in various samples were determined by reversed-phase high-performance liquid chromatography (HPLC). In this study, the physicochemical and stability profiles of TP-LZM under various conditions were investigated. The stability and releasing profiles of triptolide-lysozyme (TP-LZM) under various conditions. *In vitro* release trails showed triptolide-lysozyme was relatively stable in plasma (less than 30 % of free triptolide released) and could release triptolide quickly in lysosome (more than 80 % of free triptolide released) at 37°C for 24 h. In addition, the biological activities of the conjugate on normal rat kidney proximal tubular cells (NRK52E) were also tested. The conjugate can effectively reduce NO production in the medium of NRK52E induced by lipopolysaccharide (LPS) but with much lower toxicity. These studies suggest the possibility to promote curative effect and reduce its extra-renal toxicity of triptolide by TP-LZM conjugate.

Key words: Triptolide, Renal drug targeting, Prodrug, Targeted drug delivery system

INTRODUCTION

Renal tubular epithelial cell is one of the major cell kinds in tubulointerstitium, and always thought to be the "victim" of tubulointerstitial injuries. Recent works suggested that renal tubular epithelial cells played an important role in renal inflammatory damages. Renal tubular epithelial cells have been proved to up-regulate class II major histocompatibility complex (class II MHC) molecules in inflammatory renal diseases when stimulated by IFN- γ and lipopolysaccharide (LPS) (Banu *et al.*, 1999). In addition, *in vitro* and *in vivo* studies demonstrated that renal tubular epithelial cells could be induced to express costimulatory molecules, such as B7-1 and ICAM-1, under different experimental conditions (Jevnikar *et al.*, 1990). The relevance of these cell-surface markers to immune-mediated events in the

kidney has been further supported by their increased expression on renal tubular epithelial cells *in vivo*, in settings such as chronic-graft-versus-host disease, allograft rejection, and lupus nephritis. These results suggest that renal tubular epithelial cells may play an important role in initiating or propagating renal damage through interactions with T lymphocytes.

Tripterygium wilfordii Hook f (TWHf), a member of Celastraceae plant family, has been used as an anti-inflammatory agent in traditional Chinese medicine for centuries. It is reported that the extract of the herb was proved to have potent immunosuppressive activity *in vivo* and *in vitro* (Tao *et al.*, 1991; Gu *et al.*, 1992). Studies have shown that TWHf extract is effective in the therapy of rheumatoid arthritis, systemic lupus erythematosus, glomerular nephritis, and acute rejection after transplantation (Yang *et al.*, 1992). Hundreds of patients who suffered from inflammatory renal diseases had taken the TWHf extract and showed obvious improvement in China (Li *et al.*, 2002). Among the large number of components that have been isolated from the extract of TWHf, triptolide is

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one of the major active components with the most potent immunosuppressive activity (Gu *et al.*, 1995; Yang *et al.*, 1998). Triptolide inhibited the over-expression of class II MHC, B7-1, and B7-2 molecules in a concentration-dependent manner.

However, its clinical use has been limited mainly due to scarce water solubility and toxic effects. The incidence of adverse drug reactions (ADRs) was significantly higher than that of other drugs. The ADRs of triptolide include gastrointestinal, genital, cardiovascular, blood circulatory system, bone marrow as well as hypersusceptibility of skin. Therefore, the utilization of novel drug delivery systems holds great promise to improve therapeutic effects and clinical application of triptolide. Recently, the controlled release delivery systems such as solid lipid nanoparticle (SLN) and microemulsion have been developed for topical delivery of triptolide (Mei *et al.*, 2003).

It was previously demonstrated that low-molecular-weight-proteins (LMWPs), such as lysozyme, can significantly accumulate in the kidney and coupling of drug with lysozyme dose not alter its *in vitro* behavior (Franssen *et al.*, 1993; Haverdings *et al.*, 2002). After glomerular filtration, these conjugates are reabsorbed by the proximal tubular cells and degraded from the lysosomal compartment, and the activated drug can diffuse to other parts of the kidney. Lysosome (MW 14 400 and isoelectric point of 11) contains 149 amino acids and seven free amino groups. These groups are available for drug derivatization (Qiang *et al.*, 2005). Therefore, we hypothesized that after its systemic administration, triptolide conjugated to lysozyme would increase its solubility, specifically accumulate in the kidney and gradually release the active drug, resulting in sustained effects and fewer side effects in kidney. Tubular epithelial cells are a critical cell type in the regulation of renal inflammatory processes. Most chronic human kidney diseases are characterized by a final common pathway consisting of interstitial inflammation and ultimately leading to interstitial fibrosis. Within this process, tubular epithelial cells (TECs) play a critical role. Both *in vitro* and *in vivo* it has been demonstrated that TECs are an important source of various cytokines, chemokines, growth factors, adhesion molecules and extracellular matrix components (Kooten *et al.*, 1999). Moreover, the conjugate is supposed to be delivered to tubular epithelial cells. Therefore, normal rat kidney epithelial (NRK52E) cells were chosen to evaluate the biological activity of the conjugate *in vitro*.

With the aim of using innovative ways to deliver triptolide, possibly overcoming or alleviating the solubility and toxicity problems, a conjugate of triptolide-lysozyme was synthesized and characterized. The biological activities of the conjugate on normal rat kidney proximal tubular cells (NRK52E) were also tested. The conjugate can effectively reduce NO production in the medium of NRK52E induced

by lipopolysaccharide (LPS) but with much lower toxicity.

MATERIALS AND METHODS

Materials

Triptolide was purchased from Chengdu Xijiang Company (Chengdu, China). Lysozyme, new born calf serum and lipopolysaccharide (LPS) were supplied by Boao Biochemical Company (Shanghai, China). N-hydroxybenzotriazole (HOBT) was purchased from GL Biochem (Shanghai) Ltd. (Shanghai, P.R. China); 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride(EDC), trimethylamine and 4-dimethylaminopyridine (DMAP) and Sephadex G-25 were obtained from Sigma Chemical CO.(St Louis, MO). All other chemicals were obtained from commercial suppliers, and used without further purification. Normal rat kidney epithelial-type (NRK52E) cell stocks (ATCC CRL 1571) were acquired from the American Type Culture Collection (Rockville, MD). Adult male Sprague–Dawley rats (200–225 g) were provided by the Laboratory Animal Center of Sichuan University.

Preparation of triptolide succinate (TPS)

Triptolide (100 mg, 0.27 mmol) and succinic anhydride (150 mg, 1.5 mmol) were dissolved in 3 mL anhydrous methylene chloride (CH₂Cl₂), followed by the addition of appropriate amount of trimethylamine and 4-dimethylaminopyridine (DMAP). The reaction mixture was stirred at room temperature for 24 h under a nitrogen purging. The reaction was monitored by TLC with the solvent system of chloroform-methanol (20:1, v/v), and the spot were detected by charring with 2% 3,5-dinitrobenzene in ethanol and 10% potassium hydroxide in ethanol. The organic layer was separated and washed thoroughly with distilled water. After drying and concentrating under vacuum, the obtained crude TPS was further purified by column chromatography over a silica gel eluting with chloroform-methanol (20:1, v/v) isocratic system, to afford 117.6 mg (91.5%) TPS as a pale yellow solid. The structure of the product was confirmed by ¹H-NMR, IR, MS, UV, respectively. IR data were recorded on a FT-IR spectrophotometer (200SXV, Nicolet, U.S.A.). ¹H-NMR spectra were run at 400 MHz on a NMR spectrometer (Unity Inova-400, varian, USA) and the chemical shifts were in ppm downfield from tetramethylsilane. MS spectra were obtained with a Mass Spectrometer (LCQ^{data}, Finnigan, U.S.A.). Ultraviolet spectral measurements were performed on a spectrophotometer (UV-2201, Shimadzu, Japan) with 1 cm quartz cells.

Preparation of triptolide succinate-lysozyme conjugate (TP-LZM)

Lysozyme (100 mg, 7.0 μmol) was dissolved in 0.1 mol/L borate buffer at a concentration of 20 mg/mL TPS (100

mg, 0.2 mmol). EDC (100 mg, 0.74 mmol) and HOBt (50 mg, 0.26 mmol) were dissolved in 0.5 mL acetonitrile and added dropwise to the lysozyme solution. Subsequently, the solution was stirred at 0°C for 24 h with magnetic stirrer in refrigerator. After concentration (2000 rpm, 10 min), insoluble products, uncoupled TPS and other low molecular residues were removed by using a Sephadex G-25 column eluted with dioxane/water 80/20 (v/v) eluents. The protein fraction of the eluents was dissolved in distilled water, followed by dialysis against distilled water using a dialysis membrane (molecular weight cut off: 1000) for 3 days to further remove lower molecular substances. TP-LZM was obtained by freeze-drying (Modulyod-230, Thermosavant, U.S.A.) of the dialyzed solution with a 41% yield.

Stability of TP-LZM conjugate in plasma

For renal drug targeting system, it is crucial for the conjugate to remain stable in blood circulation before reaching the kidney, where it is hydrolyzed by enzymes secreted by lysosomes to release free drug. Therefore, it is necessary to investigate the *in vitro* stability of TP-LZM in plasma to lay the foundations for further studies *in vivo*. Blood was obtained from adult male Sprague-Dawley rats by cardiac puncture. Approximately 4 IU of heparin was added to each microliter of blood to prevent coagulation. Immediately after the collection of plasma, TP-LZM was added to produce blood concentrations of 20 µg/mL (triptolide equivalent) ($n=3$), and the samples were incubated at 37°C in a shaking water bath for 24 h. At predetermined time points, a sample (1 mL) was taken for the concentration assay of free triptolide. For control, samples were also prepared in isotonic phosphate buffer (5 mg/mL, pH=7.4), instead of blood, and subjected to identical procedures for the blood samples.

In vitro evaluation of triptolide release from TP-LZM in renal lysosome

After reaching the kidney, TP-LZM is supposed to be hydrolyzed by enzymes secreted by lysosomes to release free triptolide. So it is very important to investigate the hydrolysis of TP-LZM in kidney lysosomes. Lysosomes were isolated from adult male Sprague-Dawley rats according to previous methods with minor modifications (Mehvar *et al.*, 2000). Briefly, the kidneys were removed and then homogenized in 0.25 mol/L sucrose and subjected to differential centrifugation which resulted in collection of a lysosomal fraction. The potency of the lysosomal fraction was tested by the activity of acid phosphatase which is the marker for the lysosomes. The specific activity of the enzyme was calculated, in both the kidney homogenate and the lysosomal fraction, as the ratio of the enzyme activity per mg of protein. The activity

of acid phosphatase was measured using a colorimetric assay. In agreement with previous studies, the specific enzyme activity in the lysosomal fraction was 30-fold higher than that in the homogenate. The lysosomal fraction was stored at -40°C and used within one week of preparation. For lysosomal hydrolysis studies ($n=3$), a solution of TP-LZM was added to the lysosomal fraction to produce final concentrations of 20 µg/mL (triptolide equivalent), and the samples were incubated at 37°C in a shaking water bath for 24 h. At a predetermined time interval, 1 mL of the solution was removed, and the concentration of triptolide was analyzed by HPLC. For controls, similar samples were prepared by addition of drug solutions to 0.25 M sucrose (instead of lysosomal fraction), and subjected to the procedures identical to those for the lysosome samples.

Biological evaluation

Normal rat kidney epithelial-type (NRK52E) cell stocks (ATCC CRL 1571) were acquired from the American Type Culture Collection (Rockville, MD) and were propagated in Dulbecco's modified Eagle's medium (DMEM) with high glucose, pyruvate, and L-glutamate supplemented with 10% heat-inactivated newborn calf serum plus 100 units/mL penicillin and 100 units/mL streptomycin. Incubation conditions were 5% CO₂, 95% relative humidity, and 37°C temperature. For experimental studies, cells were seeded in 6-well (1×10⁶ cell/well) and stimulated with LPS in the presence or absence of TP-LZM for 24 h. NO₂⁻ accumulation in the medium was used as an indicator of NO production as previously described (Kim *et al.*, 2004). Briefly, 100 µL aliquots of the culture supernatants were incubated with same volume of the solution containing 1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 2.5% phosphoric acid at room temperature. After 10 min, absorbance was measured at 540 nm using a spectrometer, and nitrite concentrations were calculated based on the standard curve generated with NaNO₂.

Cytotoxicity of TP-LZM was assayed using MTT assay. Briefly, NRK52E cells were seeded in 96 well plates at 1×10⁴ cells/well and incubated for 48 h, and various concentrations of TP-LZM and triptolide solutions were then added to the well. After incubating for 48 h, 25 µL of MTT stock solution in pH 7.4 PBS (5 mg/mL) was added into each well with a final concentration of 0.5 mg/mL MTT. The plate was then incubated at 37°C in 5% CO₂ for 4 h. The medium was removed and 150 µL of DMSO was added to dissolve the formazan crystals. The plate was read spectrophotometrically at 550 nm in a Microplate Reader (Bio-Rad, U.S.A.). The relative cell viability was calculated as $([Abs]_{\text{sample}} - [Abs]_{\text{blank}}) / ([Abs]_{\text{control}} - [Abs]_{\text{blank}})$.

Determination of triptolide by HPLC

The concentrations of free triptolide in the samples were

determined by a reversed-phase HPLC method described before (Mao *et al.*, 1999). The HPLC system (Shimadzu, Japan) consisted of an SPD-10A variable UV-VIS detector and a set of Model LC-10AT liquid chromatography including two pumps, a manometric module and a dynamic mixer. The mobile phase consisted of methanol/water (50/50) solution, which was filtered through 0.45 μm membrane filter before use. A Shimpack ODS column (150 \times 4.6 mm, 5 μm) was eluted with the mobile phase at a flow rate of 1.0 mL/min. The eluate was monitored by measuring the absorbance at 218 nm at 35°C. The Class VP V5.0 software was employed for the data analysis. Triptolide was determined after an extraction procedure. The conjugate was added to 1.0 mL NaOH (6 N). The extraction was performed after hydrolysis for 0.4 h at 30°C to release triptolide from the conjugate. The samples were subsequently acidified with 1.0 mL of HCl (6 N). After this, 1.0 mL of dichloromethane was added and mixed for one minute and was analyzed by HPLC. Ion spray mass-spectrometry was also employed to qualify the amount of triptolide per mole of lysozyme.

RESULTS AND DISCUSSION

Preparation of TP-LZM

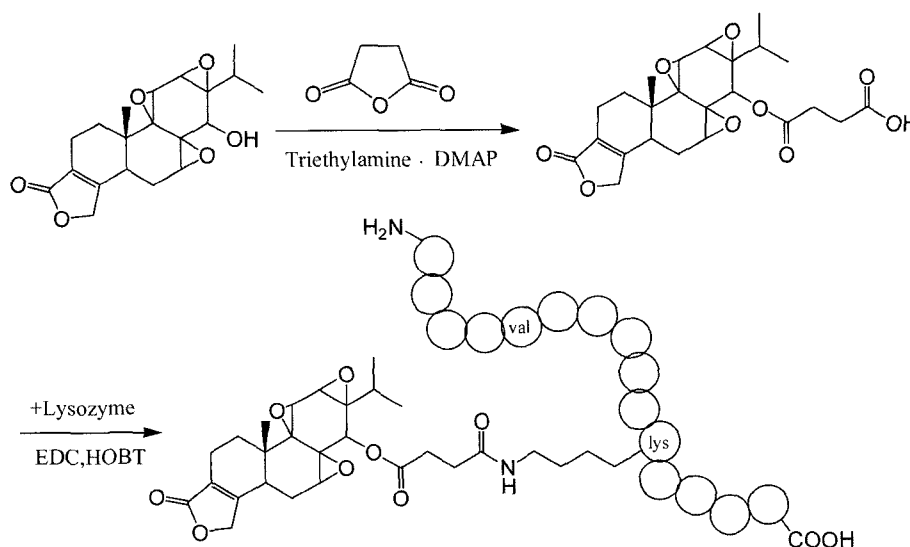
Triptolide has hydroxyl group at 14 position, which can be conjugated to lysozyme *via* a spacer. In this experiment, we chose succinic anhydride as a spacer. Since it seems difficult to couple triptolide with succinic anhydride under moderate condition, some catalysts including DMAP and triethylamine were used. The schematic synthetic route was shown in Scheme 1. The first step was to synthesize TPS as the intermediate and then the product was obtained: mp 109~111°C; IR (KBr) 3463 (-COOH) cm^{-1} ; MS m/z 459 (M-H)⁺; ¹H-NMR (400 MHz, CDCl_3): 5.06 (1H, s, -14CH),

4.67 (2H, s, 19-CH₂), 3.82 (1H, d, 11-CH), 3.50 (1H, d, 12-CH), 3.43 (1H, d, 7-CH), 2.75 (5H, m, CH₂CH₂,5-CH), 2.30 (1H, d-m, 15-CH), 2.15 (2H, m, 6-CH_a,2-CH_a), 1.88 (2H, m, 2-CH_b, 6-CH_b), 1.55 (1H, m, 1-CH_b), 1.20 (1H, m, 1-CH_a), 1.05 (3H, s, 20-CH₃), 0.95 (3H, d, 16-CH₃), 0.83 (3H, d, 17-CH₃); UV: λ_{nm} :217.5 nm. These data proved TPS was synthesized successfully.

As egg-white lysozyme (MW 14 400 and isoelectric point 11) contains seven free amino groups, which are available for drug derivatization. Two methods were used to characterize the conjugate. The average coupling degree of triptolide to lysozyme was determined by measuring the protein concentration in combination with triptolide analysis after hydrolysis of the conjugate in the analytical section. Ion spray mass-spectrometry was also employed to determine the amount of triptolide per mole of lysozyme. In agreement with hydrolysis study, it is found that the molar ratio of triptolide to lysozyme within the conjugate was approximately 1:1. As shown in Fig. 1, the TP-LZM has a molecular weight of 15,000. The measured molecular weights correlated well with the hydrolysis values by HPLC.

Stability of TP-LZM conjugate in plasma

For tissue-specific drug delivery, one of the problems associated with ester prodrugs is rapid *in vivo* hydrolysis of the prodrug in blood before the achievement of appropriate concentrations of the prodrug at target tissue(s). In this case, the conjugate was supposed to be taken by proximal tubular cells and then enter cell through phagocytosis, where it is hydrolyzed by enzymes secreted by lysosomes to release triptolide. It was shown in Fig. 2 that the conjugate was stable in mouse plasma solution at 37°C within 24 h with less than 30% TP-LZM hydrolysis.



Scheme 1. Synthesis procedure of STP-LZM conjugate

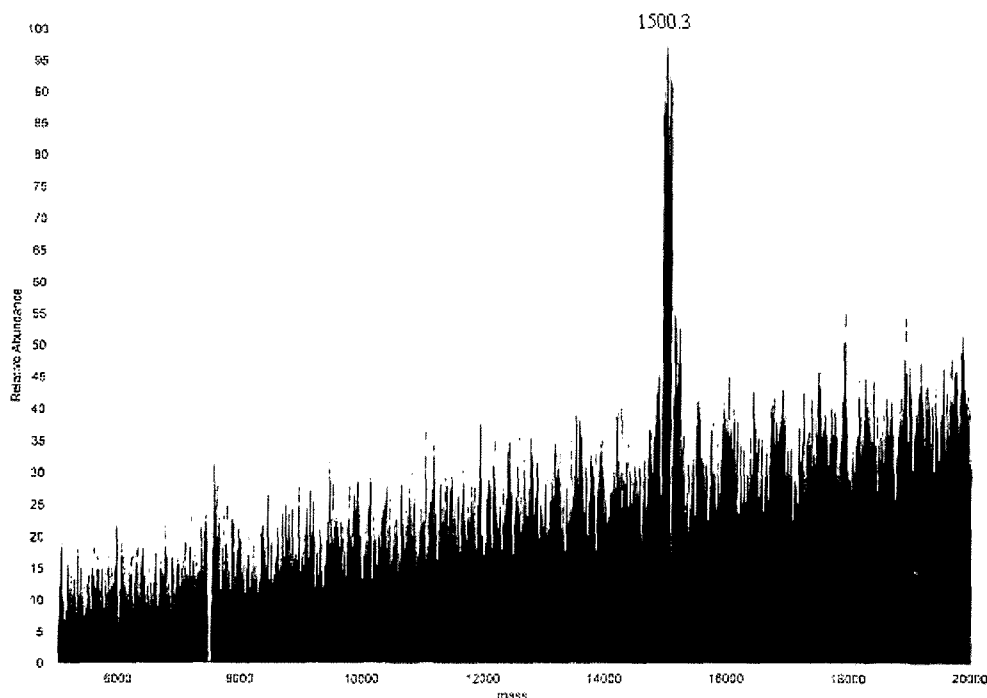


Fig. 1. A qualitative measurement of triptolide molecules covalently coupled to lysozyme in the conjugate by I on spray mass-spectrometry

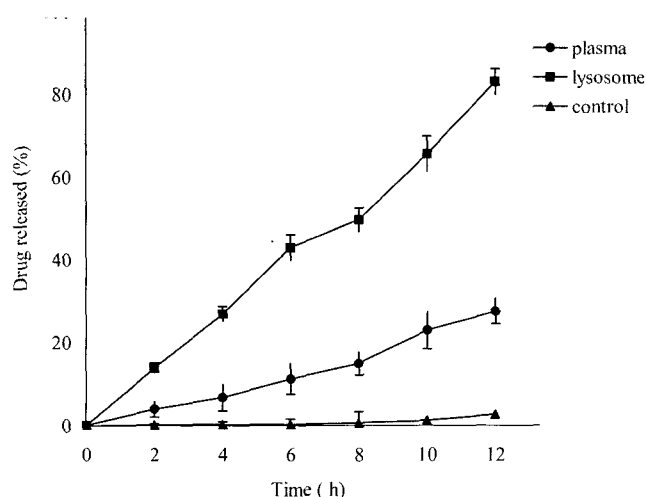


Fig. 2. Stability and release profiles of triptolide from TP-LZM conjugate at 37°C. Data values are mean \pm S.D.(n=3).

As native lysozyme, lysozyme was predominantly and rapidly (within 20 min) taken up by the kidney (Marrijke Haas *et al.*, 1997). The relatively slow hydrolysis of TP-LZM conjugate in blood should allow distribution of TP-LZM to its target sites (kidney) after its systemic administration. A disadvantage of using ester bounds for drug targeting conjugations might be their relative instability in the transfer to the target site due to the presence of plasma esterase. The present study shows that, at least for the lysozyme used here, this is not a major problem.

After intravenous injection, lysozyme is rapidly cleared from the blood stream by glomerular filtration and almost completely reabsorbed and catabolized by renal lysosomes without reentering the bloodstream. The observation that ester bond is rather stable in plasma is in agreement with that of Franssen *et al.* who demonstrated that naproxen coupled to lysozyme *via* ester bond is stable in plasma. Therefore we conclude that, at least in the case of TP-LZM conjugate, ester bond is indeed sufficiently stable in the bloodstream to transport of the lysozyme-conjugated triptolide to the kidneys. These data suggested the good stability of the conjugate *in vitro* and indicated its suitability for further studies *in vivo*.

***In vitro* evaluation of triptolide release from TP-LZM in renal lysosomes**

For the synthesis of a suitable kidney specific drug-lysozyme conjugate, it is essential that the bond which links drugs and carrier will be degradable otherwise the active parent drug will not be generated. The lysosomal medium offers in principle several possibilities to cleave such bonds, either enzymatically or chemically. The lysosomes contain many different hydrolytic enzymes such as proteinases and esterases. The pH of the lysosomes is 4-5. Similar to blood, the rate constants of hydrolysis of TP-LZM conjugate to triptolide in the lysosomal fractions were significantly different from those in the control samples, suggesting that the hydrolysis of TP-LZM conjugate in lysosomes is through enzymatic, rather than chemical,

hydrolysis. Overall, the rate of hydrolysis of TP-LZM in the lysosomes was much faster than that in blood. This is mostly, not completely, due to difference in enzyme activity. However, the *in vitro* hydrolysis of TP-LZM conjugate in the lysosomal fraction appears to be very slow with a half-life of more than 8 hours. In addition to esterases and other enzymes, the kidney lysosomes also contain proteases which are expected to reduce the molecular weight of lysozyme. A reduction in molecular weight of lysozyme carrier in the lysosomes is then expected to make the ester bond more susceptible to the action of esterases in this compartment. However, it should be noted that, in contrast to the lysosomal proteases, the esterases play an important role in the hydrolysis of the conjugate. However, these *in vitro* results should be extrapolated to *in vivo* situation. Therefore, future studies, investigating the *in vivo* release and effects of triptolide after the administration of TP-LZM are needed to be determined whether the conjugate releases triptolide in the kidney in a sufficient and slow manner to produce a sustained immunosuppressive hydrolysis effect in this organ.

Biological evaluation

NRK52E cells were pretreated for 30 min with various concentrations of TP-LZM and subsequently treated with 50 ng/mL LPS. As shown in Fig. 3, NO was barely detectable in unstimulated cells, but markedly increased 24 h after the treatment of 50 ng/mL LPS. Cells pretreated with TP-LZM showed a dose-dependent inhibition of NO production following LPS stimulation. NRK52E cells that were not treated with TP-LZM expressed high level of NO when stimulated with 50 ng/mL LPS for 24 h. Addition of TP-LZM inhibited this LPS-stimulated NO production in a dose-dependent manner. In untreated cells, 50 ng/mL LPS evoked a 10-fold induction of nitrite production versus the unstimulated control; this induction was inhibited by TP-LZM treatment in a dose dependent manner. The concentration and duration of TP-LZM treatment used in these studies had no significant effect on the viability of NRK52E. Similar to the TP-LZM, the amounts of NO in the medium were decreased at 24 h after the addition of the 0.1 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ of triptolide to the LPS pretreated cells. As shown in Fig. 3, the decrease in the amount of NO in the medium suggests a immunosuppressive potential of the TP-LZM on the renal tubular epithelial cell. Renal tubular epithelial cells constitute the first line of defense against toxicant-induced injury to the kidney and play a critical role in the process of inflammation (Woods *et al.*, 1999). NO is an important host defense and microbial and tumor cell killing agent, as well as a regulator of proinflammatory genes *in vivo*. Indeed, it is reported that the inhibitory effect of triptolide on iNOS gene expression and modulation of iNOS expression could potentially

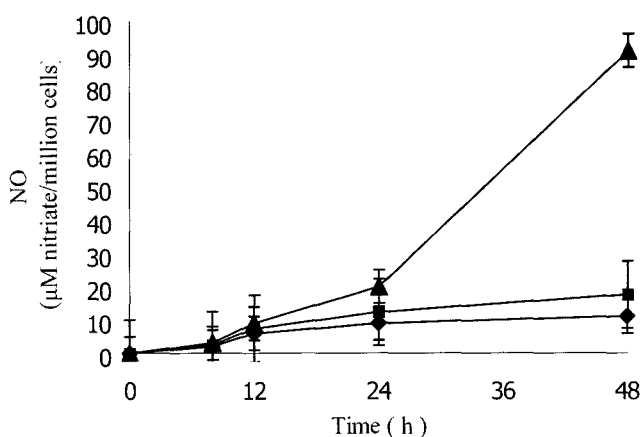


Fig. 3. Release of NO in the supernatant of 1×10^6 NRK52E (triptolide equivalent). Data values are mean \pm S.D. (n=3).

control chronic and acute inflammatory diseases. Here, we demonstrated that TP-LZM inhibits NO production and decrease the toxicity in LPS-stimulated cultured renal tubular epithelial cells, and that these effects are maintained through the hydrolysis of TP-LZM.

The cytotoxicity profile of TP-LZM on the NRK52E cells was measured using a MTT assay. Cells were incubated with increasing amounts of TP-LZM and triptolide ranging from 20 to 1000 $\mu\text{g/mL}$ (triptolide equivalent) as described in experimental section. After incubation of NRK52E cells for 24 h in the presence of 100 $\mu\text{g/mL}$ TP-LZM, the cells did not show a decrease in population, indicating minimal toxicity of TP-LZM. In contrast, triptolide exhibited higher toxicity than TP-LZM. Triptolide, at the dose of 20 $\mu\text{g/mL}$ showed significant toxicity to NRK52E in culture. At higher

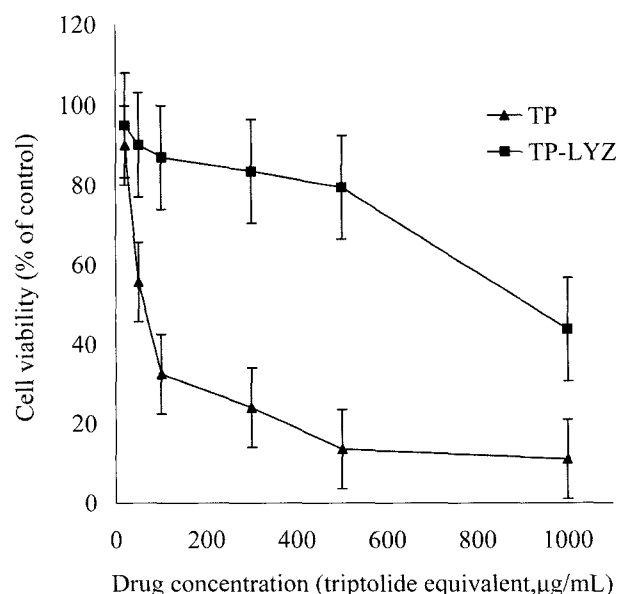


Fig. 4. Cytotoxicity of triptolide and TP-LZM conjugate against NRK52E. Data values are mean \pm S.D. (n=3).

concentrations of TP-LZM, up to 50 µg/mL, there was still no significant change in cell morphology and proliferation relative to controls. Fig. 4 shows the percentage of the live cell in the presence of varying concentrations of the conjugate relative to control. TP-LZM showed a decreased cytotoxicity *in vitro*. The effective dose (IC₅₀) of the TP-LZM was found to be ~800 µg/mL (triptolide equivalent) in 24 h.

In conclusion, we have designed and prepared a potential renal specific TP-LZM conjugate to achieve renal specific targeting and to reduce the side effects of triptolide. *In vitro* studies showed that the conjugate was stable and less cytotoxic with biological activity remained. These data suggested the good potential of the conjugate *in vitro* and indicated its suitability for further studies *in vivo*.

ACKNOWLEDGEMENTS

This work was funded by the National Natural Science Fund (No. 30572264) of People's Republic of China.

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