TP-58, a Novel Thienopyridine Derivative, Protects Mice from ConcanavalinA-Induced Hepatitis by Suppressing Inflammation

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Introduction

Hepatic injury, caused by misdirected immune stimulation or infection of virus, is an acute inflammatory injury characterized by infiltration of inflammatory cells into liver and the production of proinflammatory cytokines...
by these cells [1]. Therapies of hepatic injury have been wildly studied. However, effective drugs with limited side effects are still lacking and the precise mechanisms are not fully understood. Concanavalin A (ConA)-induced hepatitis is the mouse model of immune-mediated liver injury resembling viral and autoimmune hepatitis in humans [2]. It is characterized by markedly increased plasma levels of transaminase and infiltration of the liver with T cells, neutrophils, and macrophages, followed by apoptosis and necrosis of the hepatocytes [2, 3]. In this model, activities of T cells and macrophages (kuffer cells) induced by ConA have shown to be specifically important and various proinflammatory cytokines produced by the cells, including IFN-γ, TNF-α, IL-1β, IL-6, and Nitro Oxide (NO), play essential roles for liver injury [3-5].

TP-58 is a novel thienopyridine derivative (shown in Fig. 1A) from the small molecular compound library of our own synthesis laboratory, has previously been shown an apparently anti-tumor activity in vitro [6]. In previous screening of anti-inflammatory agents, TP-58 presented the inhibitory effects on production of NO and TNF-α in the supernatant of LPS-stimulated murine macrophage cell line, RAW264.7 cells, suggesting a potential anti-inflammatory activity.

The aim of this study was to analyze whether TP-58 is able to ameliorate liver injury in the murine fulminant hepatitis model induced by ConA and explore its possible mechanism.

Materials and Methods

Reagent
TP-58 was synthesized with purity more than 99.5% in our lab, and dissolved in saline containing 10% Tween-80 (Sigma-Aldrich, St. Louis, USA). The structure and purity were identified by high performance liquid chromatography, a Q-TOF Premier Mass Spectrometer (Waters Micromass, Milford, USA) and Nuclear Magnetic Resonance (Bruker Avance 400 NMR system).

Establishment of a Murine Model with Hepatitis
Six to eight week-old female BALB/c mice were obtained from Western China Experimental Animal Center, Sichuan University, maintained under controlled conditions, and treated with humane care according to National Institutes of Health Guidelines of China. ConA is dissolved in saline and administered in a total volume of 200 µl per mouse for i.v. injections. Female BALB/c mice were weighed and randomized into treatment groups of 10 animals and normal control group of 6 animals. The acute hepatitis in mice was induced by a single intravenous injection of 15 mg/kg of ConA [7]. For drug treatment, two concentrations of TP-58, 50 and 250 mg/kg, respectively were administered by gavage after 0.5 hour of ConA i.v. injection. 500 μg/kg dexamethasone (Sigma, USA) was administered intraperitonially 1 hour before ConA administration as a positive control [7]. Mice were sacrificed 20 hours post injection of ConA, then liver and serum samples were collected. To examine survival rate, mice were challenged with a 30 mg/kg of lethal dose of ConA and pretreated with 250 mg/kg TP-58 at 1 hour prior to ConA injection. Then mice were monitored every 2 hours for the survival.

Analysis of liver enzymes and quantification of cytokine concentrations in plasma samples or cells supernatants
Liver injury was measured by serum enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by the Chemical Lab in the National Chengdu Center for Safety Evaluation of Drugs. The concentrations of TNF-α and IL-6 in plasma samples or RAW264.7 cells supernatants were analyzed by ELISA using commercially available kits (JingMei, China) according to the manufacturer’s instruction.

Histology
Liver sections were fixed in 10% formalin, embedded in paraffin, cut into 4-µm sections, stained with hematoxylin-eosin [8] to evaluate liver injury under optical microscope.

RNA Isolation and RT-PCR
Total RNA was isolated from liver tissue using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. The concentration of RNA was determined and cDNA was generated using total RNA with the Reverse Transcriptase kit (TaKaRa, Japan). For amplification of the desired cDNA, the specific primers (Table 1) were used as described previously [9]. RT-PCR products were visualized through 1% agarose gels containing ethidium bromide by electrophoresis.

EMSA
After nuclear extracts were prepared according to the method of Hentze [10], electrophoretic mobility shift assays (EMSA) were performed as previously described [11]. The oligonucleotide probes used for this experiment were sense, 5’-AGT TGA GGC GAC TTT CCC AGG C-3’, anti-sense, 5’-GCC TGG GAA AGT CGC CTC AAC T-3’. The oligonucleotides were synthesized by Shanghai Sangon Biotechnology Co., Ltd.

Western Blot
Twenty micrograms of protein from murine liver tissue or RAW264.7 cells was mixed with an equal volume of 2×SDS sample buffer boiled for 5 min and then separated by 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to PVDF membrane. Membranes were incubated with an anti-IκB antibody or an anti-phosphorylated IκB antibody (Cell Signaling,USA) or an anti-GAPDH antibody (Sigma, USA). An anti-p-AKT (Ser-473) antibody (Cell Signaling,USA) was used for p-AKT determination, an anti-AKT (Cell Signaling) and an anti-GAPDH antibody (Sigma, USA) were used respectively for determination of the total

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AKT and GAPDH. The protein signal was quantified by scanning densitometry using an image analysis system (Bio-Rad).

**Cell culture and Nitrite assay**

Raw264.7, a mouse macrophage-like cell line was obtained from the American Type Culture Collection (Cryosite, Lane Cove, NSW, Australia). These cells were grown in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 95% air, 5% CO2 humidified atmosphere at 37 °C. Raw 264.7 cells were plated at 2×10⁴ cells/well in 96-well plates, and then incubated in the culture medium for 24 hours. The cells were then pre-treated with various concentrations of TP-58 (1.25 µM, 5 µM, 10 µM) for 1 hour before stimulation with LPS (1 µg/ml). The nitrite accumulation in the supernatant was assessed by the Griess reaction after 24 hours of LPS stimulation [12]. Each 50 µl of culture supernatant was mixed with an equal volume of Griess reagent [0.1% N-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid] and incubated at room temperature for 10 minutes. The absorbance at 540 nm was measured in an automated microplate reader, and a series of known concentrations of sodium nitrite was used as a standard.

**Statistical analysis**

Statistical analysis was performed with the SPSS software system (SPSS for Windows, version 13.0; SPSS Inc, Chicago, IL). Parametric data were statistically analyzed by the Student’s t test or one-way ANOVA followed by post hoc tests when appropriate. Differences in Non-parametric data were evaluated

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequence</th>
<th>Product (bp)</th>
<th>No. of cycles</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>R 5’-TGCCCTGCTTCCACCACTTCCTTCT-3’</td>
<td>529</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>F 5’-AGGCCGGTGCTGAGTATGTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>R 5’-ACCTGCCCGGACTCCGAA-3’</td>
<td>559</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>F 5’-GTCCTATGGTCGACCCCTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>R 5’-CTC GGAGGTCTGATGTCAG-3’</td>
<td>382</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>F 5’-GCAACTGTTCCTGAACCTCA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>R 5’-TGCTCATTTGTAATGCTTG-3’</td>
<td>342</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>F 5’-AAGGCTACACACTGCTAC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>R 5’-TGTAATCTTCTGGAAGACT-3’</td>
<td>532</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>F 5’-TTTCTTCTTCAAGAAGACT-3’</td>
<td></td>
<td></td>
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<tr>
<td>iNOS</td>
<td>R5’-CATGGCTTTGCCTGCCAAGTTTCTTCTAAG-3’</td>
<td>754</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>F5’-GCACGATCCCCTGATGGTGCCATGC-3’</td>
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**Table 1.** Sequences of gene-specific primers used for PCR amplification of cytokine and iNOS. mRNA, product size predicted and number of cycles. Primer sequences were obtained from previous reports by Williams et al. [9].

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ALT units/ml</th>
<th>AST</th>
</tr>
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<tr>
<td>normal control</td>
<td>6</td>
<td>33.4 ± 6.2</td>
<td>182.8 ± 47</td>
</tr>
<tr>
<td>ConA</td>
<td>10</td>
<td>5134 ± 848.9</td>
<td>9165.3 ± 916</td>
</tr>
<tr>
<td>ConA + Dexamethasone</td>
<td>10</td>
<td>31.23 ± 5.4</td>
<td>219.4 ± 24</td>
</tr>
<tr>
<td>ConA + TP-58 50 mg/kg</td>
<td>10</td>
<td>1444.1 ± 202.46*</td>
<td>1536.5 ± 281.9*</td>
</tr>
<tr>
<td>ConA + TP-58 250 mg/kg</td>
<td>10</td>
<td>736.16 ± 146.1*</td>
<td>791.63 ± 138.19*</td>
</tr>
<tr>
<td>TP-58 250 mg/kg</td>
<td>10</td>
<td>11.6 ± 2.61</td>
<td>119.96 ± 13.55</td>
</tr>
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**Table 2.** ConA-induced liver injury was attenuated by TP-58 as determined by ALT/AST. Values represent mean ± S.D, n = 10 in each group except normal control group which n = 6. Significantly different: *p < 0.05 vs. ConA group; †p < 0.01 vs. ConA group.

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by the Mann-Whitney U test. Survival curves were statistically analyzed using Kaplan-Meier test. Data were expressed as means ± SD. A significant difference was defined as $p < 0.05$.

Results

Therapeutic effects of TP-58 on the ConA-induced acute hepatic injury model

ConA injection causes hepatic injury, including hepatocytic necrosis, steatosis and inflammatory infiltration. To estimate the efficacy of TP-58 in acute hepatic injury, we treated mice with 50 mg/kg and 250 mg/kg TP-58 respectively after 0.5 hour of intravenous injection of ConA and sacrificed mice at 20 hours. Treatment of TP-58 markedly alleviated ConA-caused liver injury in mice, as indicated by the reduction of serum aminotransferase (Table 2). Higher dose of TP-58 (250 mg/kg) inhibited the ConA-induced transaminase more significantly than the lower dose of 50 mg/kg. Routine histopathology confirmed that ConA-induced hepatic injury consisted of degenerative hepatocellular swelling and cytoplasmic vacuolization, centrilobular hepatic necrosis and slight to mild periportal
infiltrates of mononuclear inflammatory cells (Fig. 1B). Mice treated with either 50 mg/kg or 250 mg/kg of TP-58 significantly reduced the severity of hepatic necrosis and vacuolization compared with ConA-treated animals as shown in Fig. 1B. Histological scores of centrilobular necrosis for individual animals showed that ConA induced a higher score of centrilobular necrosis (3.09±0.59) and with the 50 mg/kg or 250 mg/kg of TP-58 treatment, the scores of centrilobular necrosis significantly and respectively decreased to 2.33±0.41 and 1.42±0.73 (Fig. 1C).

As reduced liver damage correlated with enhanced survival, we also studied whether TP-58 pretreatment elevated the survival rate of mice which were challenged with 30mg/kg of ConA, a lethal dose reported by previous research [13]. Mice were observed every 2 hours for survival after ConA injection and the results showed that animals pretreated for 1 hour with TP-58 produced a

**Fig. 2.** Dose-effect influence of TP-58 on IL-1β, TNF-α, IL-6, IFN-γ and NO levels in vivo and in vitro. A. RT-PCR analysis for IL-1β, TNF-α, IL-6, IFN-γ and iNOS in the liver tissue. B and C. Protein levels of TNF-α (B) and IL-6 (C) in the serum from mice given ConA, ConA plus TP-58 50 mg/kg, ConA plus TP-58 250 mg/kg or TP-58 250 mg/kg alone were determined by ELISA (n=5 per group). D and E. Effects of the compounds TP-58 on TNF-α (D) and IL-6 (E) secretion in vitro. RAW264.7 macrophage cells were treated with increasing TP-58 concentrations (from 1.25 µM to 40 µM) for 1 hour followed by LPS (1 µg/ml) for 30min. F. Effects of TP-58 on Nitrite production of RAW264.7 macrophage cells stimulated by LPS; Representative results were from 3 independent experiments. Data are means ± SD; * p<0.05 vs. ConA.
statistically significant increased survival rate (Fig. 1D). Thirty percent of the animals pretreated with the TP-58 survived greater than 1 week compared with 0% for animals only with ConA administration.

These results indicated that TP-58 was effective on protecting mice from hepatic injury induced by ConA.

**TP-58 inhibits the production of proinflammatory cytokines**

Proinflammatory cytokines play an important role in both human and animal model of hepatitis. These factors are not only indispensable for the onset of hepatitis by their cooperative signaling but also directly damage hepatocytes [14]. We analyzed the effects of TP-58 on proinflammatory cytokines. As shown in Fig. 2A, ConA challenge led to elevation of the expressions of mRNA of the IFN-γ, TNF-α, IL-1β, IL-6 and iNOS at 2 hours after ConA injection, compared with normal control group. Administration of TP-58 into ConA injected mice resulted in significant suppression of the mRNA levels of TNF-α, IL-1β, IL-6 and iNOS, but not IFN-γ (Fig. 2A). We also measured the levels of TNF-α and IL-6 in the serum of ConA-induced mice. As shown in Fig. 2B and C, TP-58 treatment significantly reduced the levels of TNF-α (Fig. 2B) and IL-6 (Fig. 2C) in serum of ConA-induced mice.

We further investigated the effect of TP-58 in LPS-stimulated RAW 264.7 cells. With LPS stimulation, the levels of TNF-α and IL-6 in the supernatant of RAW 264.7 cells were raised, while TP-58 significantly decreased the levels of TNF-α (Fig. 2D) and IL-6 (Fig. 2E) in the supernatant of LPS-stimulated RAW 264.7 cells. We also determined the production of NO in the supernatant of LPS-stimulated RAW 264.7 cells by measuring the amount of nitrite, a stable metabolite of NO. During the 18 hours incubation, RAW264.7 macrophages in the resting state produced 6.54±0.48 µM nitrite. When LPS (1 µg/ml) was added, the nitrite was dramatically increased to 18.22±1.69 µM (Fig. 2F). Pretreatment with TP-58 inhibited LPS induced NO production in a concentration-dependent manner (Fig. 2F). These results indicated that TP-58 suppressed production of cytokines during the inflammation, which might protect the liver from injury.

**TP-58 suppresses NF-κB activation**

As NF-κB plays a key role in regulating inflammatory responses, to further explore the mechanism of TP-58 in anti-inflammation, we analyzed the activation of NF-κB through electrophoretic mobility shift assay (EMSA) in the ConA-induced acute hepatic injury mice with 50 mg/kg or 250 mg/kg of TP-58 treatment. As shown in Fig. 3A, a specific NF-κB DNA binding activity was induced in mouse liver at 1 hour of ConA injection, whereas TP-58 pretreatment significantly inhibited the activation of NF-κB (Fig. 3A). The specificity of the
NF-κB/probe interaction was confirmed by addition of an excess of unlabeled “cold” probe [15]. The inhibitory activation of NF-κB by TP-58 was also identified in LPS stimulation in raw264.7 macrophage cells (Fig. 3B). As shown in Fig. 3B, LPS inducing activation of NF-κB DNA binding was most pronounced at 30 minutes after administration of LPS, which was significantly inhibited by TP-58 pre-exposure (Fig. 3B). These results indicated that TP-58 interfering the process of inflammation by inhibiting the NF-κB pathways.

TP-58 inhibits IκB Phosphorylation

Phosphorylation of the inhibitory protein IκB enables nuclear translocation and DNA binding of NF-κB [16]. To gain further insight into the mechanism of TP-58 mediated regulation of NF-κB, we studied the change of IκB phosphorylation. As shown in Fig. 4A and Fig. 4B, TP-58 significantly inhibited IκB phosphorylation of both in the murine hepatitis model induced by ConA (Fig. 4A) and in the supernatant of LPS-stimulated RAW 264.7 cells (Fig. 4B).

TP-58 does not inhibit AKT Phosphorylation

AKT signaling is an important pathway related with cell survival/proliferation, and is also relative to migration of immunocyte [17]. We observed whether the suppressed liver injury upon TP-58 was also associated with AKT signaling. As shown in Fig. 4C, ConA actually enhanced AKT phosphorylation of mouse liver, but which was not inhibited by TP-58 administration. Instead, we found a

Fig. 4. Effects of TP-58 on levels of phosphorylation-IκB and phosphorylation-AKT. A. Western blot of hepatic phosphor-IκB. Mice were injected with ConA 1 hour after TP-58 50 mg/kg and TP-58 250 mg/kg administration then were sacrificed 20 hours later. B. The phosphorylation state of IκB in vitro was determined by Western blot. RAW264.7 macrophage cells were treated with increasing TP-58 concentrations (from 1.25 µM to 10 µM) for 1 hour followed by LPS (1 µg/ml) for 30 min. C. Western blot of hepatic phospho-AKT. Mice were injected with ConA 1 hour after TP-58 50 mg/kg and TP-58 250 mg/kg administration then were sacrificed 20 hours later. Representative results were from 3 independent experiments. Data are means ± SD;* p<0.05 vs ConA.
slight increase of AKT phosphorylation with the high dose (250 mg/kg) of TP-58 treatment (Fig. 4C).

**Toxicity of TP-58**

To examine the toxicity of TP-58, mice were sacrificed 4 weeks after consecutively oral administration of 250 mg/kg TP-58. Then histopathological analysis of several organs and serum biochemical parameters were analyzed. As shown in Fig. 5, we found no significant difference of histopathology and serum transaminase between control group and TP-58 group (Fig. 5).

**Discussion**

In our study, TP-58 markedly suppressed ConA-caused liver injury and increased survival ratio of mice challenged with a lethal dose of ConA. The administration of TP-58 significantly alleviated ConA-caused liver injury in mice by the reduction of serum aminotransferases and liver necrosis. The analysis of proinflammatory cytokines revealed that TP-58 decreased both hepatic mRNA expressions and serum protein levels of TNF-α and IL-6. And the result from LPS-stimulated RAW 264.7 cells showed TP-58 suppressed the production of TNF-α, IL-6, and NO in the supernatant of LPS-stimulated RAW 264.7 cells. TP-58 also inhibited the activation of NF-κB both in the murine hepatitis model induced by ConA and in LPS-stimulated RAW 264.7 cells. The inhibitory effect was also accompanied by a parallel reduction of IκB phosphorylation.

Thienopyridine derivatives have been used as the inhibitor of the platelet adenosine diphosphate receptor in cardiovascular diseases [18] and also recently reported to be effective on antitumor and inhibiting inflammation [6, 19]. Our current study identified the role of TP-58, the novel thienopyridine derivative, which protected mice from ConA-induced fulminate hepatitis by suppressing inflammation.

Proinflammatory cytokines play critical roles in the process of inflammations and the increased production of IFN-γ, TNF-α, IL-1β, IL-6 and NO has been reported...
to be associated with both human hepatitis and ConA-induced liver injury [20-24]. In current study, ConA injection caused massively intrahepatic increases of mRNAs levels of INF-γ, TNF-α, IL-1β, IL-6 and iNOS in 2 hours of ConA administration, which was consistent with previous reports [6, 25-27]. TP-58 clearly inhibited the serum protein levels of TNF-α and IL-6 in LPS-stimulated RAW264.7 macrophages. 

In the in vitro experiments further identified TP-58 suppressed production of NO, TNF-α, IL-1β, IL-6 and iNOS, but not INF-γ, a T cell-produced cytokine in ConA-induced mice. The in vitro experiments further transcription factor NF-κB, which has been known to mainly depend on the activation of NF-κB both in the murine hepatitis model induced by ConA and in LPS-stimulated RAW 264.7 cells. These results supported TP-58 protecting mice from ConA-induced liver injury by inhibiting NF-κB pathways. Although we found that ConA-induced liver injury also presented an enhanced phosphorylation of AKT (Fig. 4C), which is associated with the cellular survival [33], administration of TP-58 led to little change of phosphorylation of AKT, indicating TP-58 ameliorated ConA-induced liver injury mainly by interfering the NF-κB pathways, but not AKT signaling. Tina Morwick et al. have reported that thienopyridine derivatives are the potent IKK-β inhibitors, which reduce the activation of NF-κB by inhibition of IKKβ [34]. So it is worthy to identify if TP-58 also reduces NF-κB by inhibition of IKKβ in the future study.

In conclusion, this study firstly reports the effects and mechanisms of TP-58 on ameliorating liver injury by suppressing inflammation, suggesting that TP-58 might define an anti-inflammatory role in immunologically mediated diseases and might even set the new stage for therapy of inflammatory liver diseases.

Acknowledgements

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References


