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JKB-122 is effective, alone or in combination with prednisolone in Con A-induced hepatitis

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ABSTRACT

Con A-induced hepatitis in mice is an established model of autoimmune hepatitis (AIH). JKB-122, a toll-like receptor 4 (TLR4) antagonist, was tested for hepatotprotectant activity. Within several hours of Con A challenge (15 mg/kg *iv*), increased production of proinflammatory cytokines with inflammatory infiltrate occurred in the liver. The severity of tissue necrosis and the amount of circulating liver enzymes peak at 24 h post Con A challenge. JKB-122 was given 24 and 16 h before, then concurrently, and 4 and 8 h (× 5 doses) after challenge with Con A. Serum and liver were harvested at 3, 9 and 24 h post Con A challenge. JKB-122 at 20 and 50 mg/kg *po* prevented the increase of serum liver enzymes by 47% and 95% respectively *vs* vehicle control 24 h post Con A. JKB-122 significantly inhibited Con A-induced pathological lesions in the liver and the amount of IFN-γ IL-1β, IL-4, IL-5, IL-6, IL-17A and TNF-α starting as early as 3 h post Con A. Moreover, JKB-122 given concurrently (× 3 doses) with Con A showed similar effect. Finally, JKB-122 enhanced the therapeutic effects of submaximal dose of prednisolone with improved lesion score. It is concluded that JKB-122 at 20 and 50 mg/kg *po* caused dose-dependent inhibition of elevated liver enzymes in Con A-induced hepatitis in mice, indicating hepatoprotectant activity. The results suggest that JKB-122 as monotherapy or in combination with prednisolone may offer a viable approach to the treatment of AIH.

1. Introduction

T cell-mediated hepatitis is a life-threatening event that can be caused by viral infection, hepatotoxin, autoimmunity and other etiologies (Heymann and Tacke, 2016). Concanavalin A (Con A), a mitogen for T cells, induces acute inflammatory cell infiltrate and tissue injury in the liver (Tiegs et al., 1992). Apart from T cells, the inflammatory infiltrate also contains NKT cells, eosinophils, and neutrophils (Takeda et al., 2000; Louis et al., 2002; Hatada et al., 2005). Research has shown that IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-17A and TNF-α produced by activated immune cells and hepatic sinusoidal endothelial cells contribute to tissue damage in Con A hepatitis (Tagawa et al., 1997; Trautwein et al., 1998; Louis et al., 2002; Xu et al., 2011; Wang et al., 2012). Neutralization of the proinflammatory cytokines inhibits Con A-induced liver injuries. Similar to autoimmune hepatitis (AIH) in humans, Con A hepatitis is responsive to intervention with immunosuppressant and anti-inflammatory agents with reduced production of proinflammatory cytokines and associated liver injuries (Tiegs et al., 1992; Lohse et al., 1998; Kwon et al., 2014).

Recently, the role of Toll-like receptor 4 (TLR4) in liver diseases has been demonstrated in humans and in animals (Pradere et al., 2010; Gao et al., 2011; Yang and Seki, 2012; Szabo and Petrasek, 2015). TLR4 is a cell surface receptor that can be activated by endogenous and exogenous ligands such as lipopolysaccharide (LPS), High mobility group box 1, hyaluronic acid, and fatty acids (Pradere et al., 2010). Upon ligand binding, TLR4 initiates intracellular signaling pathways that involve NF-kB and MAPK, and upregulates expression of proinflammatory cytokines IL-1β, IL-6 and TNF-α (Pradere et al., 2010; Yang and Seki, 2012). TLR4 is highly expressed by cells of the monocyte lineage, while in the liver TLR4 can be found on resident Kupffer cells, hepatocytes, and mesenchymal cells (Yang and Seki, 2012). TLR4 signaling-deficient mice have diminished severity of Con A hepatitis as manifested in reduced serum liver enzymes, liver lesions and hepatocyte apoptosis (Ojiro et al., 2010; Lin et al., 2012). Furthermore, depletion of endotoxin (LPS) by antibiotics prevented Con A-induced liver injury and production of proinflammatory cytokines in wild type mice (Lin et al., 2012).

JKB-122, a TLR4 antagonist (Hutchinson et al., 2008; Wang et al., 2016), is known to have anti-inflammatory and hepatoprotectant properties. Pretreatment with JKB-122 in rats prevented serum TNF-α and liver enzyme elevation in a LPS-induced sepsis model (Lin et al., 2005). JKB-122's hepatoprotectant activity was further demonstrated in the LPS/D-galactosamine-induced hepatitis mouse model, where JKB-122 reduced

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serum liver enzymes, liver superoxide and liver inflammatory cell infiltrate (Wang et al., 2008). In addition, JKB-122 suppressed serum liver alanine transaminase (ALT) increase, liver injury and fibrosis in a chronic cholestasis model in rats (Payabvash et al., 2007; Ebrahimkhani et al., 2006). The anti-inflammatory effect of JKB-122 is presumably mediated by TLR4 antagonism with subsequent reduction in cytokine production (Hutchinson et al., 2008; Wang et al., 2016). JKB-122 is currently being evaluated clinically for treating chronic liver inflammation in viral-induced hepatitis and AIH (NCT02293941 and NCT02556372).

In the current study, the hepatoprotectant activity of JKB-122 was explored in Con A-induced hepatitis in mice, a translational model for human AIH. We show that JKB-122 treatment ameliorates liver injury and proinflammatory cytokines induced by Con A. The combined effect of JKB-122 with prednisolone was also evaluated in Con A-induced hepatitis.

2. Materials and methods

2.1. Animals

Male in-bred BALB/c mice (weighing 24 ± 2 g) were purchased from BioLasco, Taiwan, and maintained under IACUC-approved and ethical animal care conditions in an AAALAC-accredited laboratory animal facility throughout the study. The mice were housed in IVC, 36 Mini Isolator systems under controlled temperature (20–24 °C), humidity (30–70%) and 12 h (h) light/dark cycle, and given free access to sterilized food and water.

2.2. Experimental design and hepatitis induction

Experimental design is described in Fig. 1. JKB-122 (manufactured by SALARS S.p.A., Italy) was dissolved in 1% Tween80 (vehicle), and administered to mice by prophylactic dosing (Fig. 1A), concurrent dosing (Fig. 1B) or combined dosing (Fig. 1C). Experimental T-cell mediated hepatitis was induced by one *iv* bolus injection of Con A (15 or 20 mg/kg) (Sigma, USA). Blood and liver were harvested at designated time-points after Con A challenge. Serum were collected from whole blood, and stored at − 80 °C until analysis. Liver tissue blocks were cut and kept in appropriate conditions for cytokine (protein) and histopathology analysis.

2.3. Liver enzymes assessment

Serum ALT and aspartate aminotransferase (AST) were measured by an optimized UV method using commercial kits (Denka Seikan, Japan) on an automated analyzer (TBA-120FR, Toshiba).

2.4. Histopathology

Liver tissue was fixed in 10% formalin for two days and transferred to 70% ethanol before finally being embedded in paraffin wax. 4-micrometer sections were made and stained with Hematoxylin-Eosin (H & E). The histopathology lesions were scored from 1 to 4 according to criteria described by Shackelford et al. (2002).

2.5. Cytokine quantification

Serum and liver cytokine content was analyzed by a commercial kit (Procataplex Multiplex Immunoassay, eBioscience) according to the distributor's manual, and the signals were detected by Luminex® 100 (R & D Systems, Minneapolis, MN, USA). Total liver tissue lysate was extracted by Procarta Lysis Buffer following the manual's instructions.

2.6. Statistical analysis

Data were expressed as means ± standard error of means (S.E.M.). The significance of differences between vehicle and naïve groups was analyzed by Student's *t*-test. The significance of differences between vehicle and test groups was analyzed by one-way ANOVA with Bonferroni post-hoc test or Student's *t*-test. A P-value of < 0.05 is considered statistically significant.

3. Results

3.1. JKB-122 given prophylactically improves Con A-induced liver enzymes and liver lesions in a dose-dependent manner

JKB-122 was orally administered at − 24, − 16, − 0.5, 4, and 8 h post Con A challenge, and the effects were examined at 24 h after Con A (Fig. 1A). Con A induced a significant elevation of both ALT and AST in the serum as compared with naïve mice (ALT, 3617 ± 392 *vs* 83 ± 30 U/L; AST, 3417 ± 614 *vs* 109 ± 9 U/L) ($P < 0.05$ vs naïve mice; Student's *t*-test) (Fig. 2A). JKB-122 at 20 and 50 mg/kg x 5 significantly reduced Con A-induced serum ALT by 46% (to 1900 ± 348 U/L) and 95% (to 195 ± 44 U/L) ($P < 0.05$, vs vehicle; one-way ANOVA with Bonferroni's post-hoc test) (Fig. 2A). Similarly, JKB-122 at 50 mg/kg × 5 significantly reduced Con A-induced serum AST by 92% (to 272 ± 45 U/L) ($P < 0.05$ vs vehicle; one-way ANOVA with Bonferroni's post-hoc test), while JKB-122 at 20 mg/kg × 5 reduced AST by 31% (to 2347 ± 641 U/L) ($P > 0.05$, vs vehicle control) (Fig. 2A). Dexamethasone (Dex), a glucocorticoid with immunosuppressant activity, has been shown to reduce hepatic injury in Con A-induced hepatitis in mice and is used as a positive treatment control (Tiegs et al., 1992). Dex at 0.1 mg/kg was given at − 0.5, 4 and 8 h post Con A challenge (Fig. 1A), and significantly reduced ALT and AST elevation by 84% and 85%, respectively ($P < 0.05$, vs vehicle; one-way ANOVA with Bonferroni's post-hoc test) (Fig. 2A).

Con A hepatitis was associated with tissue necrosis and inflammatory infiltrate at 24 h post challenge (Fig. 2 B and C). JKB-122 at 20 or 50 mg/kg × 5 tended to mitigate severity of tissue lesions ($P > 0.05$, vs vehicle), while Dex significantly improved the tissue lesions ($P < 0.05$, vs vehicle; one-way ANOVA with Bonferroni's post-hoc test) (Fig. 2C).

3.2. JKB-122 given concurrently with challenge improves Con A-induced hepatitis

In order to ascertain the therapeutic effect of JKB-122, JKB-122 was administered at 50 mg/kg concurrently with (− 0.5 h), and 4 and 8 h post Con A challenge in mice (Fig. 1B). Serum and liver samples were harvested at 3, 9 and 24 h post Con A to provide an evaluation of its hepatoprotectant effect over time. Serum ALT and AST were significantly increased from 9 h post Con A challenge compared with naïve mice (Fig. 3A). The histopathology of associated liver lesions was performed at corresponding time-points (Fig. 3B and Supp 1).

JKB-122 significantly suppressed Con A-induced ALT and AST by 44% and 39% at 9 h post challenge, and by 60% and 63% at 24 h post challenge ($P < 0.05$, vs vehicle; Student's *t*-test) (Fig. 3A). Correspondingly, the liver lesions as graded by histopathological scores were also improved by JKB-122 at 9 and 24 h post challenge (Fig. 3B and Supp 1). The results demonstrated significant therapeutic effect of JKB-122 on Con A-induced hepatitis.

3.3. JKB-122 augments the hepatoprotectant effect of submaximal dose of prednisolone

To illustrate the translational value of JKB-122, we tested the combination of JKB-122 and prednisolone, a corticosteroid used in the clinical setting as standard of care for treating AIH, in an oral therapeutic regimen. Prednisolone (0.1 and 0.3 mg/kg *po*) and JKB-122 (10 and 20 mg/kg *po*) were tested in combination for hepatoprotectant effect. JKB-122 and prednisolone were given alone or in combination in designated doses at − 0.5, 4 and 8 h post Con A