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Therapeutic administration of Y-40138, a multiple cytokine modulator, inhibits concanavalin A-induced hepatitis in mice

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Abstract

Concanavalin A-induced hepatitis is often used as a model of liver injury. In this model, plasma tumor necrosis factor-α (TNF-α) level increased in concanavalin A-injected mice. Prophylactic treatment with Y-40138, *N*-[1-(4-[4-(pyrimidin-2-yl)piperazin-1-yl]methyl phenyl) cyclopropyl] acetamide·HCl, significantly suppressed the increase in plasma TNF-α level. In this study, we compared the effect of Y-40138 with those of pentoxifylline and anti-TNF-α antibody on concanavalin A-induced hepatitis. Prophylactic treatment with pentoxifylline, anti-TNF-α antibody and Y-40138 reduced plasma alanine aminotransferase level. Therapeutic treatment with Y-40138 significantly reduced plasma alanine aminotransferase level, but pentoxifylline and anti-TNF-α antibody did not. Therapeutic treatment with Y-40138 significantly reduced plasma interferon-γ (IFN-γ) and monokine induced by interferon-γ levels. From these results, Y-40138 may be expected as a new class of therapeutic drug for treatment of TNF-α, IFN-γ and/or chemokine-related liver diseases such as alcoholic liver disease.

Keywords: Y-40138; TNF-α; IFN-γ; IL-4; Chemokine; Pentoxifylline; Anti-TNF-α antibody; Concanavalin A; Hepatitis

1. Introduction

In many liver diseases, including viral hepatitis, autoimmune hepatitis and alcoholic liver disease, activated T lymphocytes and macrophages appear to play important roles. Alcohol-related liver disease is a major cause of morbidity and mortality worldwide, and the clinical syndrome of alcoholic hepatitis carries a particularly poor prognosis. Many investigators have focused on cytokines such as tumor necrosis factor-α (TNF-α) in acute alcoholic liver disease because TNF-α can induce metabolic abnormalities and can cause liver injury. Treatment with pentoxifylline, an inhibitor of TNF-α production, significantly improves short-term survival in patients with severe acute alcoholic liver disease (Akriviadis et al., 2000). But this increase in survival rate is less satisfying in clinical study. And anti-TNF-α antibody, infliximab, warrants investigation since in preliminary trials of severe acute alcoholic liver disease it improves survival (Tilg et al., 2003). Therefore, synthetic TNF-α blocker may be an effective therapeutic drug for severe alcoholic liver disease.

As a model of liver injury, concanavalin A-induced mouse hepatitis is widely used (Tiegs et al., 1992). Among various cytokines released during concanavalin A-induced hepatitis, TNF-α and interferon-γ (IFN-γ) play critical roles in the development of hepatocellular apoptosis and necrosis (Gantner et al., 1995, Küsters et al., 1996). Previously, we reported that a novel synthetic compound, *N*-[1-(4-[4-(pyrimidin-2-yl)piperazin-1-yl]methyl phenyl)cyclopropyl] acetamide (free base of Y-40138), suppresses lipopolysaccharide-induced TNF-α production in mice (Fukuda et al., 2000). Therefore, Y-40138 is expected to show a therapeutic effect on concanavalin A-induced hepatitis through TNF-α suppression.

In concanavalin A-induced hepatitis, natural killer T (NKT) cells play critical roles in the induction of hepatic injury by cooperating with conventional T cells and macrophages, and IFN-γ, interleukin(IL)-4 and/or TNF-α mediated system (Ogasawara et al., 1993; Kondo et al., 1997; Seino et al., 1997; Tagawa et al., 1998). There is a significant increase in the number of macrophages and neutrophils in the liver

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parenchyma of concanavalin A-induced hepatitis (Miyazawa et al., 1998; Bonder et al., 2004). Chemokines are considered to play an important role in leukocyte accumulation in sites of hepatic inflammation. Macrophage inflammatory protein-2 (MIP-2) induces hepatic neutrophil accumulation and liver injury in concanavalin A-induced hepatitis (Lentsch et al., 1998). Monokine induced by interferon-γ (MIG) and interferon gamma-inducible protein-10 (IP-10) are chemoattractants for T lymphocytes and monocytes/macrophages in inflamed liver tissues, respectively, and may play important roles in the development of hepatitis (Tamaru et al., 2000; Zlotnik and Yoshie, 2000; Rossi and Zlotnik, 2000).

In this study we showed that prophylactic treatment with Y-40138, anti-TNF-α antibody and pentoxifylline protected against concanavalin A-induced hepatitis. Unlike anti-TNF-α antibody and pentoxifylline, Y-40138 showed a therapeutic effect in this model. Y-40138 may be a new class of therapeutic drug for treatment of cytokine and chemokine-mediated liver diseases such as alcoholic liver disease.

2. Materials and methods

2.1. Animals

Female specific pathogen-free BALB/c mice (5–6 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). Mice were housed under conditions of controlled temperature (23±3 °C) and illumination (7:00–19:00) for at least 5 days before experiments. All experiments were approved by the Animal Ethical Committee of Mitsubishi Pharma Co. and performed in accordance with guidelines of the Japanese Pharmacological Society.

2.2. Compounds

Y-40138 was synthesized at Mitsubishi Pharma Co. and dissolved in pyrogen-free saline. Pentoxifylline was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in pyrogen-free saline. Anti-mouse TNF-α antibody was purchased from Genzyme-Techne Co. (Minneapolis, MN) and dissolved in phosphate-buffered saline (PBS).

2.3. Induction of hepatitis

Concanavalin A (type IV and V, Sigma Chemical Co.) dissolved in pyrogen-free saline was injected at doses of 18–20 mg/kg into mice via the tail vein. Y-40138 and anti-TNF-α antibody were administered intravenously on indicated administration schedules to concanavalin A-injected mice. Pentoxifylline was administered intraperitoneally on indicated administration schedules to concanavalin A-injected mice.

2.4. Measurement of aminotransferase and cytokine levels

Blood was collected from abdominal artery with heparin under ether anesthesia at the indicated times. Plasma was obtained by centrifugation at 2000×*g* and stored at −30 °C. Plasma alanine aminotransferase levels were measured by the multiple film analytical element Drichem (Fujifilm Medical Co., Tokyo, Japan) at various time points after concanavalin A injection. Plasma TNF-α, IFN-γ, MIP-2, IL-4, IP-10, and MIG levels were determined at various time points after concanavalin A injection by enzyme-linked immunosorbent assay (ELISA), using a commercially available kit (TNF-α, MIP-2, IP-10, and MIG, Genzyme-Techne Co.; IFN-γ and IL-4, BioSource International, Inc., Camarillo, CA).

2.5. Statistical analysis

The data are presented as the mean±S.E.M. Statistical significance of alanine aminotransferase and cytokine levels was determined using Wilcoxon test. Differences were assessed with two-sided test with alpha level of 0.05.

3. Results

3.1. Effect of Y-40138 on plasma transaminase

In non-stimulated mice treated with and without Y-40138 (10 mg/kg, i.v.) plasma alanine aminotransferase levels were 21.8±3.4 and 24.2±0.9 U/L, respectively. Plasma alanine aminotransferase levels increased in response to concanavalin A injection in mice and reached over 3000 U/L 8 h after concanavalin A injection. Y-40138 protected against alanine aminotransferase level elevation when Y-40138 was administered to mice 15 min prior to concanavalin A injection (Fig. 1). Y-40138 suppressed the elevated alanine aminotransferase levels when it was administered to mice 3 h after concanavalin A injection (Fig. 2A). Y-40138, at doses of 1 and

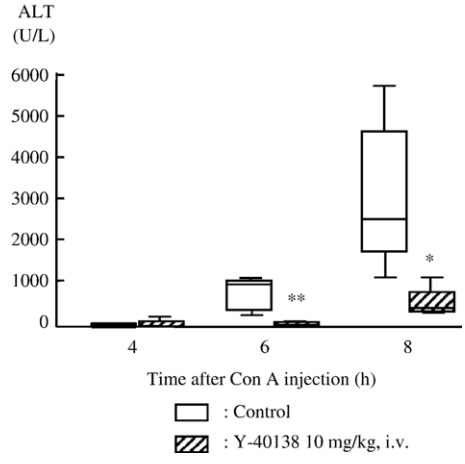

Fig. 1. Prophylactic treatment of Y-40138 inhibited concanavalin A-induced increase in alanine aminotransferase levels in BALB/c mice. Y-40138 was administered intravenously 0.25 h prior to concanavalin A injection (18 mg/kg, i.v.). Alanine aminotransferase levels in the plasma were measured at 4, 6, and 8 h. Results are expressed as the mean±SEM ($N=5$). *$P<0.05$, **$P<0.01$ significantly different from control (Wilcoxon test).

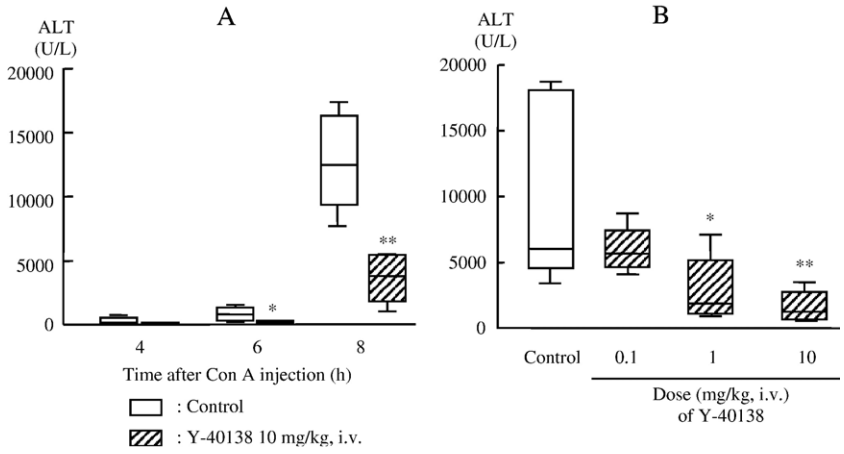

Fig. 2. Therapeutic treatment of Y-40138 inhibited concanavalin A-induced increase in alanine aminotransferase levels in BALB/c mice. Y-40138 was administered intravenously 3 h after concanavalin A injection (20 mg/kg, i.v.). A) Alanine aminotransferase levels in the plasma were measured at 4, 6, and 8 h. Results are expressed as the mean±SEM ($N=6$). *$P<0.05$, **$P<0.01$ significantly different from control (Wilcoxon test). B) Alanine aminotransferase levels in the plasma were measured at 8 h. Results are expressed as the mean±SEM ($N=5$). *$P<0.05$, **$P<0.01$ significantly different from control (Wilcoxon test).

10 mg/kg, significantly suppressed elevated plasma alanine aminotransferase levels at 8 h after concanavalin A injection (Fig. 2B).

3.2. Effect of pentoxifylline and anti-TNF-α Ab on plasma transaminase

Prophylactic treatment with 100 mg/kg, i.p. of pentoxifylline (−2 h) and 250 μg/mouse i.v. of anti-TNF-α antibody (−15 min) decreased in plasma TNF-α level (data not shown). Prophylactic treatment with pentoxifylline and anti-TNF-α antibody suppressed the increase in alanine aminotransferase concentration to the levels of controls, 64% and 62%, respectively (Fig. 3A, B). However, pentoxifylline and anti-TNF-α antibody did not suppress the increase in alanine aminotransferase level when administered 3 h after concanavalin A injection.

3.3. Effect of Y-40138 on cytokine production

3.3.1. Prophylactic treatment with Y-40138

Y-40138 was administered to mice 15 min prior to concanavalin A injection. Plasma TNF-α levels reached the maximal level within 1.5 h after concanavalin A injection and decreased gradually until 8 h (Fig. 4A). Y-40138 significantly inhibited the increase in TNF-α levels at 1.5 and 3 h. Plasma MIP-2 level reached the maximal level at 3 h after concanavalin A injection (Fig. 4B). Y-40138 significantly inhibited the increase in MIP-2 level at 3 h. Plasma IL-4 level reached the maximal level within 1.5 h (Fig. 4C). Y-40138

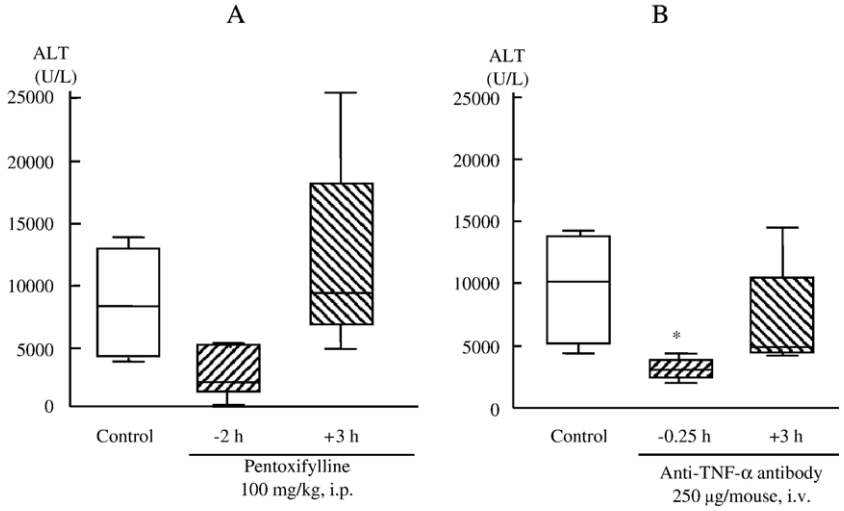

Fig. 3. Prophylactic and therapeutic effects of pentoxifylline and anti-TNF-α antibody on concanavalin A-induced increase in alanine aminotransferase level in BALB/c mice. Pentxifylline was administered peritoneally 2 h prior to or 3 h after concanavalin A injection (18 mg/kg, i.v.). Anti-TNF-α antibody was administered intravenously 0.25 h prior to or 3 h after concanavalin A injection. Alanine aminotransferase levels in the plasma were measured at 8 h. Results are expressed as the mean±SEM ($N=5$). *$P<0.05$ significantly different from control (Wilcoxon test).

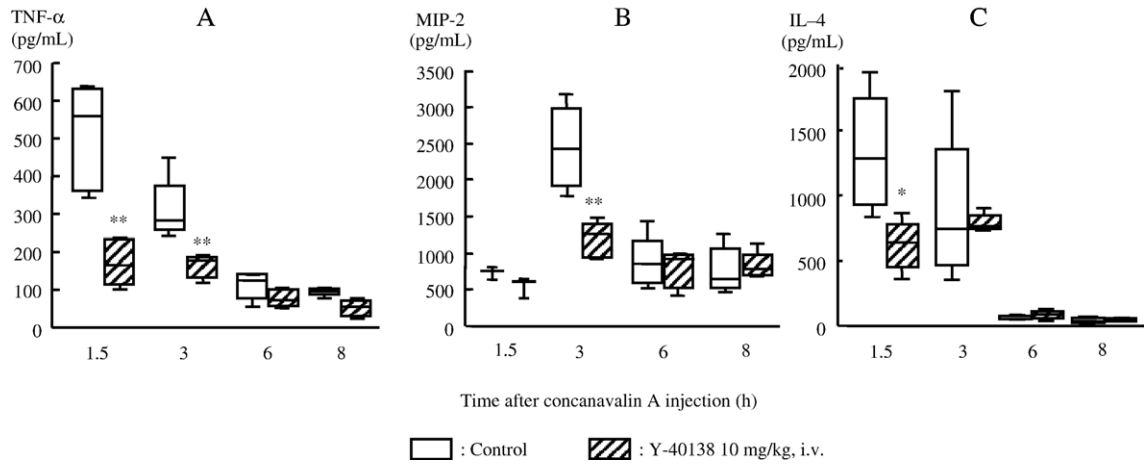

Fig. 4. Prophylactic effect of Y-40138 on concanavalin A-induced productions of TNF-α, MIP-2, and IL-4 levels in BALB/c mice. Y-40138 was administered intravenously 0.25 h prior to concanavalin A injection (18 mg/kg, i.v.). Amount of cytokines in the plasma was measured at 1.5, 3, 6, and 8 h. Results are expressed as the mean±SEM (N=3–5). *P<0.05, **P<0.01 significantly different from control (Wilcoxon test).

significantly inhibited the production of IL-4 at 1.5 h. These plasma cytokine levels were not detected in non-stimulated mice.

3.3.2. Therapeutic treatment with Y-40138

Y-40138 was intravenously administered to mice 3 h after concanavalin A injection, and plasma levels of TNF-α, IFN-γ, IP-10, and MIG were measured 4, 6, and 8 h after the concanavalin A injection. Although Y-40138 did not reduce the plasma TNF-α and IP-10 levels (Fig. 5A, B), it significantly decreased IFN-γ and MIG levels at 8 h (Fig. 5C, D).

4. Discussion

Concanavalin A-induced hepatitis is one of the commonly used animal models of non-viral hepatitis. TNF-α greatly participates in progress of concanavalin A-induced hepatitis. In fact, pentoxifylline, TNF-α inhibitor, prevents concanavalin A-induced hepatitis by reducing TNF-α levels (Shirin et al., 1998). It has been suggested that MIP-2, IL-4 and IFN-γ, in addition to TNF-α, play a central role in concanavalin A-induced hepatitis (Küsters et al., 1996; Schumann et al., 2000; Nakamura et al., 2001; Sass et al., 2002). MIP-2, which is released from TNF-α-

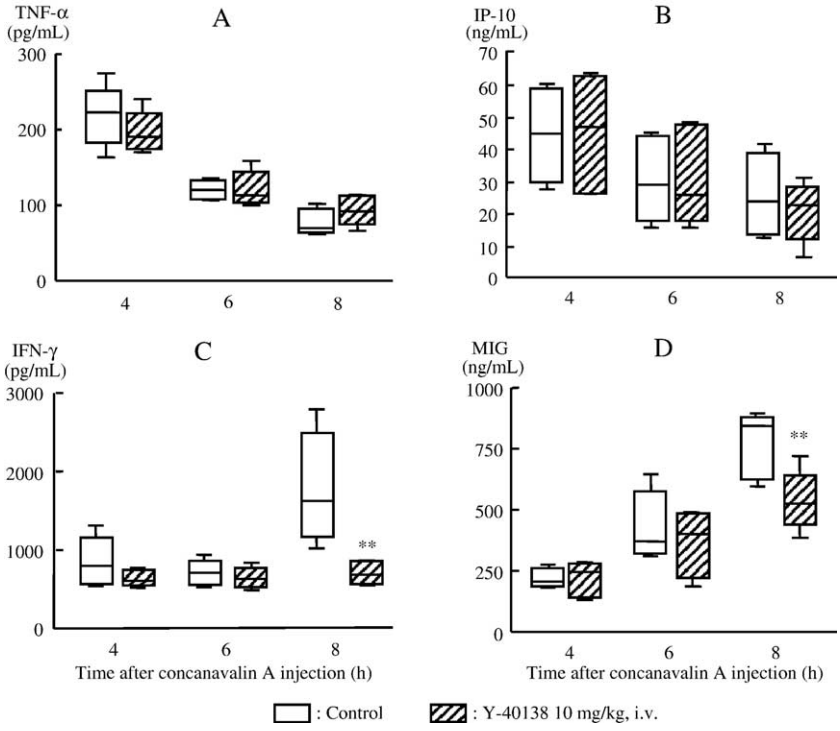

Fig. 5. Therapeutic effect of Y-40138 on concanavalin A-induced productions of TNF-α, IFN-γ, MIG, and IP-10 in BALB/c mice. Y-40138 was administered intravenously 3 h after concanavalin A injection (20 mg/kg, i.v.). Amount of cytokines in the plasma was measured at 4, 6 and 8 h. Results are expressed as the mean±SEM (N=6). **P<0.01 significantly different from control (Wilcoxon test).

activated liver cells including kupffer cells, sinusoidal endothelial cells and hepatocytes, activates neutrophils and enhances production of inflammatory mediators by neutrophils (Lentsch et al., 1998; Bajt et al., 2001). Anti-MIP-2 antibodies significantly suppressed plasma alanine transaminase levels in the concanavalin A-induced hepatitis model suggesting that MIP-2 is involved in the pathology (Bajt et al., 2001). In concanavalin A-induced hepatitis, IL-4 is produced from activated T cells and Vα14 NKT cells. NKT cells are activated by direct concanavalin A stimulation. The progress of hepatitis is involved in increase of cell surface expression of FasL and granzyme B, which is induced by activation of Vα14 NKT cells (Toyabe et al., 1997; Kaneko et al., 2000).

Histological evidence of hepatitis is observed 8–24 h after concanavalin A injection, as shown by plasma transaminase level (Tiegs et al., 1992). We measured plasma transaminase level of mice at 8 h after concanavalin A injection. In concanavalin A-injected mice, prophylactic treatment with pentoxifylline and anti-TNF-α antibody decreased alanine aminotransferase levels at doses which suppress TNF-α production. Prophylactic treatment with Y-40138 also reduced alanine aminotransferase and TNF-α levels in plasma. These data suggest that Y-40138, pentoxifylline and anti-TNF-α antibody protect hepatitis through reducing TNF-α levels. And it has been reported that at the early stage of concanavalin A-induced hepatitis, MIP-2 and IL-4, in addition to TNF-α, are produced at maximal levels at 1–3 h (Nakamura et al., 2001; Sass et al., 2002). Y-40138 suppressed MIP-2 and IL-4 production in plasma. MIP-2 is released from TNF-α-activated liver cells (Lentsch et al., 1998), so Y-40138 suppresses MIP-2 production perhaps via suppression of TNF-α production, thereby suppressing progress of hepatic injury. On the other hand, prophylactic administration of Y-40138 suppressed IL-4 production. The action mechanism of Y-40138 on IL-4 production is unknown, but we suppose that Y-40138 may suppress activation of T cells and/or NKT cells. It is suggested that Y-40138 has a hepatoprotective activity through suppression of TNF-α, MIP-2 and IL-4 production.

While administration of pentoxifylline or anti-TNF-α antibodies 3 h after concanavalin A injection had no effect, Y-40138 reduced plasma alanine aminotransferase levels. Under this condition, Y-40138 did not suppress TNF-α production, nor did it suppress MIP-2 or IL-4 (data not shown). These plasma cytokine levels reached the maximum level within 3 h after concanavalin A injection. These results mean that Y-40138 may also protect progress in liver injury induced by factors other than TNF-α, MIP-2 and IL-4. In the next study, when Y-40138 was administered 3 h after concanavalin A injection, changes in cytokine and chemokine levels other than TNF-α, MIP-2 and IL-4 were investigated. At a later stage of the inflammatory reactions of concanavalin A-induced hepatitis, IFN-γ and MIG are produced (Ito et al., 2001). IFN-γ, produced in activated T cells and NKT cells, increases hepatic inflammation by the upregulation of Fas/Fas ligand and perforin expression in the liver cells (Roth and Pircher, 2004). MIG is one of the chemokines for activated lymphocytes that shares its receptor and most of its activity with IP-10. It is produced in IFN-γ-activated monocytic cells and induces lymphocyte migration (Loetscher et al., 1996; Farber, 1997). When administered 3 h after concanavalin A injection, Y-40138 did not suppress IP-10 production (maximal at 4 h), which is produced earlier than MIG. Unexpectedly, Y-40138 significantly suppressed production of IFN-γ and MIG at 8 h after concanavalin A injection. Based on the observation that both were simultaneously suppressed, we consider that Y-40138 may have a protective activity on hepatitis through suppression of IFN-γ and MIG production. It is expected that Y-40138 may have a broader range of efficacy against severe alcoholic liver diseases than pentoxifylline or anti-TNF-α antibody.

Y-40138 inhibits TNF-α production and augments IL-10 production in lipopolysaccharide-injected mice (Fukuda et al., 2000). IL-10 potently inhibits TNF-α and IFN-γ production and prevents concanavalin A-induced hepatitis (Di Marco et al., 1999). Unexpectedly, prophylactic and therapeutic administration of Y-41038 did not increase plasma IL-10 levels in concanavalin A-injected mice (data not shown). This result means that the protective effect of Y-40138 is not mediated through the enhancement of IL-10 production on concanavalin A-induced hepatitis. These finding suggest that prophylactic and therapeutic treatment with Y-40138 protect the progress of concanavalin A-induced hepatitis through suppression of TNF-α, MIP-2, IL-4, IFN-γ and MIG production even under the condition with no influence in IL-10 production. The cellular and molecular mechanisms of therapeutic activities of Y-40138 remain unclear. Further investigations will be required on its actions against neutrophil infiltration and apoptosis in the liver.

In summary, unlike anti TNF-α antibody and pentoxifylline, therapeutic administration as well as prophylactic administration of Y-40138 significantly suppressed the development of concanavalin A-induced hepatitis. Therefore, Y-40138 may contribute to suppressing progress of hepatitis and prolonging life in patients with liver damage such as alcoholic hepatitis in which cytokines and/or chemokines are involved.

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