Inhibitory Effect of TJN-101 ((+)-(6S,7S,R-Biar)-5,6,7,8-
Tetrahydro-1,2,3,12-Tetramethoxy-6,7-Dimethyl-10,11-
Methylenedioxy-6-Dibenzo[a,c]cyclooctenol) on
Immunologically Induced Liver Injuries

Yasufumi OHKURA, Yasuhiro MIZOGUCHI, Yoshihide SAKAGAMI,
Kenzo KOBAYASHI, Sukeo YAMAMOTO, Seiji MORISAWA*,
Shigefumi TAKEDA** and Masaki ABURADA**

The Third Department of Internal Medicine and *The First Department of Biochemistry,
Osaka City University Medical School, 5-7, 1-chome, Asahimachi, Abeno-ku, Osaka 545, Japan              **Tsumura Research Institute for Pharmacology, 3586 Yoshiwara, Ami-machi,
Inashiki-gun, Ibaraki 300-11, Japan

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Abstract—TJN-101, which is a lignan component isolated from schisandra fruits, inhibits hepatotoxic chemicals-induced liver injuries. In this study, effects of TJN-101 on immunologically induced liver injuries were investigated in vivo and in vitro. When a small dose of lipopolysaccharide was injected into mice previously injected with heat-killed Propionibacterium acnes, most of the animals died with acute hepatic failure which was produced by cytotoxic factors from activated adherent cells, and liver cells were injured by antibody-dependent cell-mediated cytotoxic (ADCC) reaction or activated macrophages in vitro. TJN-101 reduced the mortality of the mice with acute hepatic failure dose-dependently. Histologically, necrosis was suppressed by the treatment of TJN-101, but infiltration of non-specific inflammatory cells was not. TJN-101 inhibited the isolated liver cell injuries induced by ADCC reaction or activated macrophages in vitro. These results suggest that TJN-101 can be markedly protective against immunological liver injuries.
totoxicity (11–15). Activated macrophages may exert their cytotoxic actions on isolated liver cells through either cell-to-cell contact between effector and target cells or production of cytotoxic factors which have an effect on isolated liver cells (12, 16–18).

In this study, effects of TJN-101 on the mortality of the mice with acute hepatic failure and on liver cell injury induced by the ADCC reaction or activated macrophages were investigated.

Materials and Methods

Materials

Chemical structure of TJN-101 is shown in Fig. 1. This component was isolated from schisandra fruits according to the method of Ikeya et al. (1).

Methods

1. Biological and histological changes and mortality

Male Balb/c strain mice, weighing 20 to 25 g, were used. Acute hepatic failure was induced by the injection of 1 µg/mouse of LPS from Staphylococcus enteritidis (Difco) intravenously to mice that had been injected with 1 mg/mouse of heat-killed P. acnes (Department of Bacteriology, Osaka City University Medical School) intravenously 7 days before. TJN-101 (5, 10 or 50 mg/kg), suspended in 1% Tween 80 solution, was administered orally 0, 2 or 4 hr after the injection of LPS, and the mortality of the mice was periodically examined until 24 hr after.

Twenty-four hr after the injection of LPS, the surviving mice were killed by cervical dislocation, blood samples were collected from the abdominal aorta and livers were removed. Serum GOT and GPT activities were measured according to the method of Reitmann and Frankel (19). Liver tissues were fixed in 10% formalin and routinely processed for histological analysis. Preparations were stained with hematoxilin and eosin, and histological changes of liver tissues were examined microscopically.

2. Hepatocytotoxicities on isolated liver cells

1) Preparation of culture supernatant of ADCC reaction mixture: Liver specific lipoprotein was fractionated from rat liver according to the method of Meyer zum Buschenfelde (20). The liver was removed from a normal rat (male Wistar strain, weighing about 200 g) and homogenized with 0.25M sucrose solution at 4°C. Liver specific lipoprotein was separated from the supernatant of liver homogenate by a series of gel filtrations using a Sephadex G-100 column, a Sephadex G-200 column and a Sepharose 6B column through which liver specific lipoprotein was passed. Male rabbits, weighing about 3.5 kg, were immunized by the injection of liver specific lipoprotein (10 mg as protein) emulsified in an equal volume of Freund's complete adjuvant (Difco) 4 times at one week intervals. Seven days after the last injection, the immunized animals were sacrificed and sera was separated. Complement activities of the sera were inactivated by heating at 56°C for 30 min to obtain the antisera. These antisera were stored at −70°C until use.

Lymphocytes as the source of effector cells were obtained from the peripheral blood of healthy humans by Ficoll-Conray density gradient centrifugation, and the liver cell suspension as the target cell was prepared from a normal rat (male Wistar strain, weighing about 150 g) according to the method of Bellemann et al. (21). The cell concentration of each suspension was adjusted to 1 x 10^6 cells/ml by adding Eagle's MEM solution (Nissui) containing 10% fetal calf serum (Gibco). The antisera (0.01 ml/ml) were added to the cell suspension and incubated at 37°C for 3 hr. After liver cells were coated with the antisera, 100 ml of the effector cell suspension (1 x 10^7 cells/ml), prepared as described above, was added to the antisera-coated liver cell suspension (10 ml) and incubated at 37°C for 2 hr. After the incubation, the culture superna-
tant was separated by centrifugation at 1000 x g for 10 min for assay of the cytotoxic activity.

2) Preparation of culture supernatant of activated macrophages: Male Hartley strain guinea pigs, weighing about 400 g, were used. Twenty ml of sterilized Marcol 52 (Esso Oil Co.) was injected into the peritoneal cavity. Peritoneal exudate cells were collected 4 days after the injection of Marcol 52 by perfusing the peritoneal cavity with 200 ml of Hank's solution (Nissui). After the oil phase was removed, the aqueous phase was centrifugated at 800 x g for 10 min at 4 °C. The pellets were washed three times with Hank's solution and suspended in Eagle's MEM solution containing 10% fetal calf serum, 100 units/ml of penicillin (Gibco) and 100 μg/ml of streptomycin (Gibco); and the cell concentration of each suspension was adjusted to 2.5 x 10⁶ cells/ml. LPS (50 μg/ml) was added to the suspension and incubated at 37°C for 48 hr. After the incubation, the culture supernatant was separated by centrifugation at 800 x g for 10 min.

3) Assay of hepatotoxicity: The liver cell suspensions were prepared from rats according to the method of Bellemann et al. (21). TJN-101 was dissolved in ethanol, and 10 μl of each concentration of TJN-101 solution was added to 1 ml of liver cell suspension. After incubation for 6 hr, the same volume of the culture supernatant of the ADCC reaction mixture or activated macrophages reaction mixture, prepared as described above, was added to the liver cell suspension and incubated at 37°C for 24 hr. (3H)-L-leucine (1.0 μCi; specific activity, 47 Ci/mmol, Amersham Japan) was added to the liver cell suspension, and incubation was continued for another 24 hr. Hepatocytotoxicity was evaluated by the protein synthesis that was calculated by comparing the radio-activities incorporated into the acid-insoluble fraction in the TJN-101-treated groups with that in the control group.

Results

1. Mortality: The survival curves and mortalities of the mice with acute hepatic failure are shown in Fig. 2 and Fig. 3, respectively. When LPS was injected to the mice

![Fig. 2. Effect of TJN-101 on the survival curve after the injection of LPS in mice previously treated with heat-killed P. acnes. ———: Control, —○—: TJN-101, 5 mg/kg, —△—: TJN-101, 10 mg/kg, —□—: TJN-101, 50 mg/kg.](image-url)
that had been injected with heat-killed *P. acnes* 7 days before, the first mouse died 1 hr after the injection of LPS, and the mortality of the mice was increased to 89.5% at 24 hr after. The time to death and mortality at 24 hr after were markedly improved by TJN-101 that was administered immediately after the injection of LPS. On the other hand, when TJN-101 was administered 2 or 4 hr after the injection of LPS, an improvement against the mortality at 24 hr after was slight.

2. **Histological changes:** As shown in Fig 4, massive necrosis and infiltration of non-specific inflammatory cells were observed in the liver induced acute hepatic failure. TJN-101 suppressed massive necrosis, but not infiltration of mononuclear cells. The liver tissue of the mouse that was administered 50 mg/kg of TJN-101 immediately after the injection of LPS is shown in Fig 5.

3. **Serum GOT and GPT activities:** Serum GOT and GPT activities are shown in Fig 6. Serum GOT and GPT activities of normal
mice were 80±20 U/I (mean±S.D.) and 50±30 U/I, respectively. When acute hepatic failure was induced, the activities were elevated to 9920±2250 U/I and 3210±1070 U/I, respectively. These elevations of serum transaminase activities were not observed by the single injection of *P. acnes* or LPS.

The elevations of serum GOT and GPT activities were inhibited to 549±913 U/I and 118±204 U/I, respectively, by 50 mg/kg of TJN-101 administered immediately after the injection of LPS.

4. ADCC reaction: Incorporation of (3H)-L-leucine into liver cells is shown in Fig. 7. When the culture supernatant of the ADCC reaction mixture was added to the liver cell suspension, protein synthesis was significantly reduced to 68.9±2.0% as compared to the non-treated group. TJN-101 inhibits the reduction of protein synthesis and this effect of TJN-101 was significant at dosages from 0.05 to 0.5 μg/ml.

5. Activated macrophages reaction: As shown in Fig. 8, protein synthesis was markedly reduced to 66.0±10.1% as compared to the non-treated group when the culture supernatant of activated macrophages reaction mixture was added to the liver cell suspension. This reduction of protein synthesis was inhibited by the pre-incubation of the liver cells with TJN-101. This effect of TJN-101 was significant at dosages from 0.5 to 1.0 μg/ml.

**Discussion**

TJN-101 inhibits the hepatotoxic chemicals from inducing liver injury (2–5). It was thought that the antihapatotoxic effect of TJN-101 may have partly resulted from the protective action on the liver cells (2, 3). In this study, effects of TJN-101 on immunologically induced liver injuries were investigated.
in vivo and in vitro.

When a small dose of LPS was injected to mice previously treated with heat-killed \textit{P. acnes}, most of the mice died with massive necrosis in the liver. TJN-101, which was administered immediately after the injection of LPS, markedly inhibited the increase of the mortality of the mice, and histological and biological changes were prevented by the treatment of TJN-101. When TJN-101 was administered 2 or 4 hr after the injection of LPS, the mortalities of the mice were improved slightly. From these findings, it was supposed that liver injury was already in progress when TJN-101 was administered 2 or 4 hr after the injection of LPS. Histologically, focal necrosis was observed 2 to 4 hr after the injection of LPS.

It was reported that immunological liver cell injuries were produced by the ADCC reaction and activated macrophages (11–18). When the culture supernatant of the ADCC reaction mixture or activated macrophages reaction mixture was added to the isolated liver cell suspension, the protein synthesis that was determined by the uptake of (3H)-L-leucine into liver cells was reduced significantly. On the other hand, the protein synthesis of liver cells that had been incubated with TJN-101 was not reduced. These results suggest that TJN-101 can be protective against liver cell injuries with cytotoxic factors from the ADCC reaction and activated macrophages, but there was an optimal dose of TJN-101 for inducing these liver cell injuries.

From our results, it is supposed that TJN-101 shows an inhibitory effect on liver injuries which were induced by cytotoxic factors produced by immunological reactions.

References

1. Ikeya, Y., Taguchi, H., Yoshioka, I. and Kobayashi, H.: The constituents of \textit{Schizandra chinensis} Bail. I. Isolation and structure determination of five lignans, gomisin A, B, C, F and G, and the absolute structure of schizandrin. \textit{Chem. Pharm. Bull.} (Tokyo) 27, 1383–1394 (1979)

2. Maeda, S., Sudo, K., Miyamoto, Y., Takeda, S., Shinbo, M., Aburada, M., Ikeya, Y., Taguchi, H. and Harada, M.: Pharmacological studies on \textit{schizandra} fruits. II. Effects of constituents of \textit{schizandra} fruits on drug induced hepatic damage in rats. \textit{Yakugaku Zasshi} 102, 579–588 (1982) (Abs. in English)

3. Takeda, S., Maemura, S., Sudo, K., Kase, Y., Arai, I., Ohkura, Y., Funo, S., Fujii, Y., Aburada, M. and Hesooya, E.: Effects of gomisin A, a lignan component of \textit{schizandra} fruits, on experimental liver injuries and liver microsomal drug-metabolizing enzymes. \textit{Folia Pharmacol. Japon.} 87, 169–187 (1986) (Abs. in English)

4. Hikino, H., Kiso, Y., Taguchi, H. and Ikeya, Y.: Antitoxic actions of lignoids from \textit{Schizandra chinensis} fruits. \textit{Planta Med.} 50, 213–218 (1984)

5. Kiso, Y., Toshkin, M., Hikino, H., Ikeya, Y. and Taguchi, H.: Mechanism of antitoxic activity of Wuweizisu C and Gomisin A. \textit{Planta
Antihapatotoxic Action of TJN-101

Med. 51, 331–334 (1985)

6 Tsutsui, H., Mizoguchi, Y., Miyajima, K., Sakagami, Y., Higashimori, T., Seki, S., Yamamoto, S., Hara, H., Tatsumi, Y., Monna, T. and Morisawa, S.: Studies on the hepatocellular injury in an experimentally induced acute hepatic failure. I. Possible involvement of the activated adherent cells. Japan. J. Gastroenterol. 82, 603–609 (1985) (Abs. in English)

7 Tsutsui, H.: Studies on the experimentally induced acute hepatic failure in mice: The role of adherent cells in the induction of liver injury. Acta Hepatol. Japon. 26, 1438–1444 (1985) (Abs. in English)

8 Tsutsui, H., Mizoguchi, Y., Miyajima, K., Sakagami, Y., Seki, S., Yamamoto, S., Kinosita, H. and Morisawa, S.: Studies on experimentally induced acute hepatic failure in mice: Characteristic of the hepatocytotoxic factors released from the activated liver adherent cells. Japan. J. Gastroenterol. 83, 1161–1167 (1986) (Abs. in English)

9 Tsutsui, H., Mizoguchi, Y., Miyajima, K., Sakagami, Y., Seki, S., Yamamoto, S., Kinosita, H. and Morisawa, S.: Studies on experimentally induced acute hepatic failure in mice: Difference between hepatotoxic factor and tumor necrosis factor. Japan. J. Gastroenterol. 83, 1161–1167 (1986) (Abs. in English)

10 Mizoguchi, Y., Shiba, T., Monna, T., Yamamoto, S. and Morisawa, S.: The possible involvement of Kupffer cell-mediated hepatocytotoxicity in the pathogenesis of liver injuries. Gastroenterol. Japan. 16, 377–383 (1981)

11 Cochrane, A.M.G., Moussouros, A., Thomson, A.D., Eddleston, A.L.W.F. and Williams, R.: Antibody-dependent cell-mediated cytotoxicity against isolated hepatocytes in chronic active hepatitis. Lancet 28, 441–444 (1976)

12 Mizoguchi, Y., Shiba, T., Higashimori, T., Ohnishi, F., Monna, T., Yamamoto, S. and Morisawa, S.: Immunological studies on the chronic hepatitis. J. Japan. Soc. Intern. Med. 69, 1609–1617 (1980)

13 Mizoguchi, Y., Tsutsui, H., Sakagami, Y., Higashimori, T., Monna, T. and Yamamoto, S.: Studies on the immunological liver injury induced by antibody-dependent cell-mediated cytotoxicity and its augmentation by activation of macrophages. Japan. J. Gastroenterol. 79, 2085–2090 (1982) (Abs. in English)

14 Mizoguchi, Y., Monna, T., Yamamoto, S. and Morisawa, S.: Studies on the mechanism of liver injury by antibody-dependent cell-mediated cytotoxicity (ADCC)—Partial purification of cytotoxic factor detected in the culture supernatant of ADCC reaction. Gastroenterol. Japan. 17, 360–367 (1982)

15 Mizoguchi, Y., Tsutsui, H., Monna, T., Yamamoto, S. and Morisawa, S.: Lipid peroxide formation in liver cells, induced by antibody-dependent cell-mediated cytotoxic reaction. Japan. J. Allergol. 32, 979–984 (1983)

16 Mizoguchi, Y., Shiba, T., Ohnishi, F., Monna, T., Yamamoto, S., Otani, S. and Morisawa, S.: Immunological studies on drug-induced allergic hepatitis. Hepatocellular injury by macrophage-mediated cytotoxicity. Gastroenterol. Japan. 15, 14–19 (1980)

17 Mizoguchi, Y., Tsutsui, H., Monna, T., Yamamoto, S. and Morisawa, S.: Studies on the mechanism of liver injury by macrophage-mediated cytotoxicity. Partial purification of cytotoxic factor detected in the culture supernatant of activated macrophages. Gastroenterol. Japan. 18, 41–46 (1983)

18 Mizoguchi, Y., Tsutsui, H., Monna, T., Yamamoto, S. and Morisawa, S.: Modulation of the macrophage hepatocytotoxicity and plasminogen activator activity of activated macrophages from guinea pigs by serum components from normal human and patients with liver diseases. Japan. J. Med. 22, 112–116 (1983)

19 Reitmann, S. and Frankel, S.: A colorimetric method of serum oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol. 28, 56–63 (1977)

20 Meyer zum Büschenfelde, K.H. and Miescher, P.A.: Liver specific antigen. Purification and characterization. Clin. Exp. Immunol. 10, 89–102 (1972)

21 Bellemann, P., Gebhardt, R. and Mecke, D.: An improved method for the isolation of hepatocytes from liver slice. Selective removal of trypan blue-dyeable cells. Anal. Biochem. 81, 408–415 (1977)