Hepatoprotective Effects of 1-[(2-Thiazolin-2-yl)-amino]acetyl-4-(1,3-dithiol-2-ylidene)-2,3,4,5-tetrahydro-1H-1-benzazepin-3,5-dione Hydrochloride (KF-14363) in Various Experimental Liver Injuries

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ABSTRACT — Hepatoprotective effects of KF-14363 were investigated in the following experimental models. KF-14363 inhibited the increase in serum glutamate pyruvate transaminase (GPT) dose-dependently, and a significant inhibition was noted at a dose of 30 mg/kg or more. KF-14363 significantly inhibited the D-galactosamine (D-gal)-induced increase in serum transaminase by oral administration at 250 mg/kg × 1 and 250 mg/kg × 2 doses. The D-gal-induced decrease in total protein levels was inhibited at doses of 100 mg/kg × 2 and 250 mg/kg × 2. KF-14363 (100 mg/kg or more) significantly inhibited the increase in liver triglyceride levels induced by DL-ethionine (250 mg/kg × 3). KF-14363 (300 mg/kg) significantly inhibited the D-gal plus lipopolysaccharide-induced increase in GPT. At 100 mg/kg or less, however, an inhibiting tendency was noted, which was not significant as the values varied widely. KF-14363 (100 mg/kg) significantly inhibited Propionibacterium acnes plus lipopolysaccharide-induced mortality at 7 and 8 hr, and it showed an inhibitory tendency at 24 hr. These results demonstrate that KF-14363 is a compound that has a protective effect against the damage induced in various experimental liver injury models with different mechanisms.

It is known that liver injuries are induced by various chemicals such as carbon tetrachloride (CCl₄), D-galactosamine (D-gal) and DL-ethionine (Eth). These models are frequently used in prospective studies on liver protecting agents because of experimental ease and their mechanisms of action are well-documented (1–5). Recently, D-gal plus lipopolysaccharide (D-gal•LPS) (6, 7) -induced hepatitis and heat-treated Propionibacterium acnes (P. acnes) plus lipopolysaccharide-induced hepatitis and heat-treated Propionibacterium acnes (P. acnes) plus LPS (P. acnes•LPS) (8) -induced hepatitis have been used as immunological liver injury models. These models differ from others with respect to the absence of hepatotoxicity.

KF-14363 is a benzazepine derivative synthesized as a hepatoprotective agent at the Pharmaceutical Research Laboratories of Kyowa Hakko Kogyo Co., Ltd. (Fig. 1). In this study, the liver protecting effects of KF-14363 were investigated in the above experimental models.
MATERIALS AND METHODS

Animals
P. acnes·LPS-hepatitis was induced in male Balb/c mice at 8 weeks of age. Other experiments were conducted in male ddY mice weighing approximately 20 g and in male Wistar rats weighing 200–250 g. All animals were allowed free access to food (F-2, Funabashi Farm) and water.

Materials
KF-14363 was synthesized by Kyowa Hakko. Carbon tetrachloride (CCl₄, Kanto Chemical Co., Inc.), D-galactosamine (D-gal, Wako Pure Chemicals), DL-ethionine (Eth, Wako Pure Chemicals), and lipopolysaccharide (LPS, Difco) were used as liver injury inducing compounds.

Propionibacterium acnes (P. acnes), supplied by the Department of Bacteriology, Osaka City University Medical School, was cultured and attenuated by heat-treatment at Kyowa Hakko.

Methods
CCl₄-induced liver injury: Liver injury was induced by CCl₄ in 10 mice and 7 rats per group. They were intraperitoneally injected with 16 ml/kg of 0.3125% CCl₄ in olive oil (0.05 ml/kg b.w. as CCl₄) or 3 ml/kg of 50% CCl₄ in olive oil (1.5 ml/kg b.w. as CCl₄), respectively. The animals were decapitated 22 hr after injection, and sera were separated. Serum glutamate pyruvate transaminase (GPT) was measured with a Determiner GPT 755 (Kyowa Medex). KF-14363 was dissolved in olive oil and orally administered at the dose of 10, 30 or 100 mg/kg (10 ml/kg b.w.) 2 hr before CCl₄ injection. The control animals orally received the solvent (olive oil) in a similar manner.

D-gal-induced liver injury: Liver injury was induced by D-gal according to the method described by Hirooka et al. (9). Eight rats per group were intraperitoneally injected with 250 mg/kg of D-gal once every 4 hr (1000 mg/kg b.w., in total). They were decapitated 12 hr after the 4th injection of D-gal, and sera were separated. GPT and glutamate oxaloacetate transaminase (GOT) and total protein (TP) levels were determined with a biochemical autoanalyzer (AU510, Olympus). KF-14363, suspended in a 5% gum arabic solution, was orally administered at a dose of 100 or 250 mg/kg once at 6 hr before the 1st D-gal injection or twice at 6 hr before and at the same time as the 1st injection. The control group orally received the same volume of solvent (5% gum arabic) in the same manner as that of KF-14363.

Eth-induced liver injury: Liver injury was induced by Eth according to the method described by Nakayama et al. (10). Eth was intraperitoneally injected into 7 or 13 rats per group at a dose of 250 mg/kg per day for 3 days. KF-14363 was orally administered at a dose of 100 or 250 mg/kg together with the Eth injections, and the control group orally received the same volume of 5% gum arabic solution. The animals were sacrificed 22 hr after the final administration. Triglyceride (TG) level in isolated livers was determined in accordance with the method described by Forch et al. (11): a portion of the large median lobe of the liver was resected, weighed and homogenized in 10 ml CHCl₃:MeOH (2:1) solution. The homogenate was filtered and washed with 3 ml of the same solution. The filtrate volume was measured. To 2 ml of
solvent-free filtrate, 3 ml of 2-propranol was added and the mixture was agitated vigorously. TG level was determined with a biochemical autoanalyzer (AU510, Olympus). The values were converted into units of mg/g liver weight.

D-gal • LPS-induced liver injury: Liver injury was induced by D-gal • LPS according to the method described by Tiegs et al. (12). Twenty mice per group received an intraperitoneal injection of 700 mg/kg of D-gal and simultaneously an intravenous injection of 1 μg/kg of LPS. KF-14363 was orally administered 1 hr prior to the injection of D-gal. The control group was given the same volume of the solvent (purified water) in a similar manner. The animals were decapitated 8 hr after the D-gal injection and sera separated. Blood GPT levels were determined with a biochemical autoanalyzer (AU510, Olympus).

P. acnes • LPS-induced liver injury: Liver injury was induced by P. acnes • LPS in male Balb/c mice divided into groups of 20 to 30 mice each. They were intravenously injected through the tail vein with 1 mg of heat-inactivated P. acnes; and after 7 days, they were injected with 10 μg/mouse of LPS. The 24-hr survival rates after the LPS injection were calculated. KF-14363 was orally administered at a dose of 100 mg/kg (10 ml/kg b.w.) at the same time as the injection of LPS. The control group received the solvent (purified water) in a similar manner.

Histopathological examination
A portion of the median lobe of the liver was fixed in 10% neutralized formalin solution, embedded in paraffin, sectioned, and stained with hematoxilin-eosin (H.E.). Liver cell necrosis and fatty change were graded: (-) normal, (+) slight, (++) moderate, (+++) severe.

Statistical analysis
All the data are expressed as the mean ± S.E. The Dunnet and Scheffe multiple test and the χ² test were applied, and P < 0.05 was regarded as significant.

RESULTS

Effects of KF-14363 on CCl₄-induced liver injury in mice and rats
The GPT value of the control mice given solvent was 1538.1 ± 273.2 IU/l. KF-14363 significantly inhibited GPT leakage by 80.7% and 98.5% at doses of 30 and 100 mg/kg, respectively (Fig. 2).

In rats, the GPT of the control group was 433.2 ± 72.2 IU/l. There was a 61.1% and 70.7% inhibition of GPT leakage in the 30 mg/kg and 100 mg/kg groups, respectively (Fig. 3). The results of the histopathological grading in rat liver injury induced by CCl₄ are summarized in Table 1. KF-14363 pretreatment decreased the severity of the centrolobular necrosis and massive fatty degeneration induced by CCl₄ (Fig. 4).

Effects of KF-14363 on D-gal-induced liver injury
Figures 5 and 6 show the protecting effect of KF-14363 on the change of serum parameters caused by the administration of D-gal.

GPT: As compared with the control group, 250 mg/kg of KF-14363 significantly (P <
0.01) inhibited GPT leakage by about 89%. In the 100 mg/kg group, an approximate 38% decrease was observed, which was not significant. The 250 mg/kg × 2 group showed an approximate 84% inhibition which was significant (P < 0.01), but the 100 mg/kg × 2 group indicated a decreasing tendency of 46%.

**GOT:** A similar tendency towards GPT was noted. KF-14363 significantly (P < 0.05 and P < 0.01) inhibited GOT leakage by about 81% and 80% at doses of 250 mg/kg × 1 and 250 mg/kg × 2, respectively. On the other hand, the 100 mg/kg × 1 and the 100 mg/kg × 2 groups showed a decreasing tendency of approximately 34% and 47%, respectively, which was not significant.

**TP:** In the 100 mg/kg × 1 and the 250 mg/kg × 1 groups, an 8.6% increase in TP was observed. The 100 mg/kg × 2 and the 250 mg/kg × 2 groups showed a significant (P < 0.05 and P < 0.01) increase of about 11.1% and 19.2%, respectively.

**Effect of KF-14363 on Eth-induced liver injury**

Liver TG levels were approximately 21 mg/g liver weight after the 3-day treatment with 250 mg/kg Eth (Table 2). KF-14363, administered at 100 and 250 mg/kg at the same time as the Eth treatment, significantly inhibited (P < 0.05 and P < 0.01) lipid accumulation by about 32% and 62%, respectively.

**Effect of KF-14363 on D-gal•LPS liver injury**

The GPT level was elevated to about 380 IU/l 8 hr after treatment with D-gal plus LPS in mice (Fig. 7). The GPT increase in the groups orally given 30 and 100 mg/kg of KF-14363 1 hr prior to the D-gal treatment was 18.6% and 47.0% inhibited, respectively, but there was no significant difference as the values varied widely. KF-14363 inhibited the GPT increase by 73.5% at a dose of 300 mg/kg (P < 0.01).

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**Fig. 3.** Effect of KF-14363 on GPT in the serum of rats treated with CCl₄. KF-14363 (10–100 mg/kg, p.o.) or vehicle was administered 2 hr before CCl₄ (1.5 ml/kg, p.o) injection and the animals were sacrificed 22 hr after CCl₄ injection. Each value shows the mean ± S.E. of 7 rats. Significantly different from the CCl₄-control value, *P < 0.05 and **P < 0.01.

**Table 1.** Effect of KF-14363 on histopathological changes in the liver of rats with liver injury induced by CCl₄

<table>
<thead>
<tr>
<th>Necrosis</th>
<th>Fatty degeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
</tr>
<tr>
<td>KF-14363 (10 mg/kg)</td>
<td>1</td>
</tr>
<tr>
<td>KF-14363 (30 mg/kg)</td>
<td>2</td>
</tr>
<tr>
<td>KF-14363 (100 mg/kg)</td>
<td>3</td>
</tr>
</tbody>
</table>

Grade designation of the histopathological findings: (−) normal, (+) slight, (+++) moderate, (++++) severe. Each value is the number of animals with grading changes.
Fig. 4. Effect of KF-14363 on histopathological changes in rat liver induced by CCl₄. H.E. stain; ×25, (a) Liver of untreated rat. (b) Liver of a vehicle-pretreated rat 22 hr after CCl₄ administration. Centrilobular necrosis and inflammatory infiltration are noted. (c) Liver of a KF-14363 (100 mg/kg)-pretreated rat at 22 hr after CCl₄ administration. The centrilobular necrosis is less extensive, and the periportal zones are well-preserved.
Fig. 5. Effects of KF-14363 on serum parameters of rats treated with D-galactosamine. KF-14363 (100 or 250 mg/kg, p.o.) or vehicle was administered 6 hr before the 1st D-gal (250 mg/kg × 4, once every 4 hr, i.p.) injection, and the animals were sacrificed 12 hr after the 4th injection of D-gal. Each value shows the mean ± S.E. of 8 rats. TP: Total protein. Significantly different from the control value, *P < 0.05 and **P < 0.01.

Fig. 6. Effects of KF-14363 on serum parameters of rats treated with D-galactosamine. KF-14363 (100 or 250 mg/kg × 2, p.o.) or vehicle was administered 6 hr before and at the same time as the 1st D-gal (250 mg/kg × 4, once every 4 hr, i.p.) injection, and the animals were sacrificed 12 hr after the 4th injection of D-gal. Each value shows the mean ± S.E. of 8 rats. KF-14363 was administered orally twice at a dose of 100 or 250 mg/kg. Significantly different from the control value, *P < 0.05 and **P < 0.01.
Table 2. Effect of KF-14363 on liver triglyceride content of rats treated with DL-ethionine

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Dose (mg/kg × 3)</th>
<th>n</th>
<th>Liver TG (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>13</td>
<td>21.29 ± 2.68</td>
</tr>
<tr>
<td>KF-14363</td>
<td>100</td>
<td>13</td>
<td>14.49 ± 1.46*</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>7</td>
<td>20.60 ± 4.45</td>
</tr>
<tr>
<td>KF-14363</td>
<td>250</td>
<td>7</td>
<td>7.93 ± 1.62**</td>
</tr>
</tbody>
</table>

KF-14363 (100 or 250 mg/kg × 3, p.o.) or vehicle was administered together with the ethionine (250 mg/kg × 3, every day, i.p.) injection, and the animals were sacrificed 22 hr after the final administration. Each value shows the mean ± S.E. Significantly different from the control value, *P < 0.05 and **P < 0.01.

**Effect of KF-14363 on P. acnes·LPS-induced liver injury**

The control animals received heat-inactivated P. acnes (1 mg/mouse); and after 7 days, they were given LPS (10 μg/mouse) by intravenous injection. The animals started to die within 5 hr after the LPS injection. The 24-hr survival rate was 16.7%. In the group given 100 mg/kg of KF-14363 at the same time as the LPS injection, a significant inhibition of mortality was noted at 7 hr and 8 hr as compared to the control group. However, at 24 hr, an inhibiting tendency of only 20% was noted, which was not significant (Fig. 8).
DISCUSSION

KF-14363 is a new compound proposed for the treatment of liver disease. Abnormalities in the subacute oral toxicity test of KF-14363 (400 mg/kg/day, for 28 days) were not seen and also the LD$_{50}$ of KF-14363 is a high dose (>2000 mg) in mice (K. Nakamoto, an unpublished study), so KF-14363 is considered to be a relatively safe compound. We investigated the liver protecting effects of KF-14363 in various experimental liver injury models.

It has been considered that in liver injury induced by CCl$_4$ a ·CCl$_3$ radical is formed by a metabolic enzyme found in intrahepatocellular microsomes. This radical production then induces the peroxidization of the unsaturated fatty acids that constitutes the cell membrane, which leads to membrane injury (13, 14). From the observation that KF-14363 significantly inhibited the increase in serum transaminase activity, it is speculated that this agent inhibits ·CCl$_3$ formation or the radical peroxidization of membrane lipids. Also, the effects of KF-14363 on serum parameters coincided with those observed by histopathological examination.

An explanation of the mechanism of D-gal-induced liver injury is that D-gal causes a disruption of liver glucose metabolism which leads to a decrease in RNA and protein syntheses, resulting in necrotized hepatocytes (15–18). Based on the fact that malotilate, regarded as effective against this model, promotes liver RNA (19) and protein (20) syntheses, Hirooka et al. have reported the possibility that this agent inhibits ·CCl$_3$ formation or the radical peroxidization of membrane lipids. Also, the effects of KF-14363 on serum parameters coincided with those observed by histopathological examination.

It is known that Eth, which acts as a metabolic antagonist of methionine in vivo, inhibits the metabolism of methionine by ethylating the regions of proteins, lipids, nucleic acids, etc. that normally undergo methylation involving methionine (21–23). Eth has been reported to produce S-adenosyl ethionine (SAE) (24) and the accumulated SAE in the liver inhibits oxidative phosphorylation to cause a decrease in ATP (25–28), which results in the inhibition of RNA synthesis (29) and a fatty liver (30). In this study, KF-14363 inhibited the development of fatty liver induced by Eth. Its mechanism of action remains to be determined, but it can be speculated to be as follows: As described above, the mechanism by which Eth induces liver injury is through a decrease in ATP. It is known that KF-14363 promotes ATP production in the liver (I. Yoshitake, unpublished study). Therefore, it is thought that this effect antagonizes the ATP decrease and as a result inhibits liver injury. In addition Raick (31) have reported that a sufficient level of glucose induces an excessive secretion of insulin which promotes protein synthesis in the liver, thus reducing the effect of Eth. KF-14363 tended to improve the decrease in blood insulin that occurs after partial hepatectomy (I. Yoshitake, unpublished study). It is likely that a general effect of these above actions leads to the inhibition of Eth-induced fatty liver.

Tiegs and Wendel (32) have reported the involvement of leukotriene D$_4$ (LTD$_4$) in liver injury induced by simultaneous administration of D-gal and LPS. In addition, Tiegs et al. (12) and Wang and Wendel (33) have indicated that tumor necrosis factor (TNF) also plays an important role in this injury. Based
on the observation that KF-14363 was effective against this model although at high doses, it is possible to speculate that this agent exerts the liver protecting effect through the inhibition of LTD₄ and TNF syntheses.

Most of mice or rats given heat-treated P. acnes and after 7 days, a very small amount of LPS by intravenous injection into the tail vein develop extensive hepatic necrosis and die (34–36). It has been considered that the mechanism of this acute hepatic failure is that adherent cells which undergo priming and accumulate in liver by intravenous P. acnes are further activated by LPS to produce a cell injury factor. This model resembles fulminant hepatitis with respect to the sudden onset of hepatocellular injury, although differing in the pathological state. Therefore, it differs from other experimental hepatotoxicity models such as liver injury induced by CC14 and D-gal. KF-14363 also decreased mortality in this P. acnes·LPS model. This suggests that KF-14363 will be useful in the treatment of liver injuries in which the immune system is involved and that this compound will be promising as a broad spectrum hepatoprotective agent.

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REFERENCES