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Protection against CCl_4-induced injury in liver by adenovirally introduced thioredoxin gene

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Abstract

Antioxidation therapy is a promising strategy for treating or preventing oxidative stress-related liver diseases. The human thioredoxin (TRX) gene was inserted into an adenovirus vector (Adv-TRX), which was administered to mice. The mice were treated with 1 ml/kg CCl_4 48 h after the infection. Blood samples were taken and the liver was excised 24 h after the CCl_4 treatment. Serum ammonia, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were determined, and liver sections were stained with hematoxylin and eosin. RT-PCR analysis showed that the introduced TRX gene was expressed only in the liver. Adv-TRX decreased the serum ammonia, AST, and ALT levels. Hematoxylin-eosin staining indicated that the CCl_4-induced injury was significantly prevented by the Adv-TRX infection. The gene delivery of TRX, which plays a central role in intracellular redox control, was shown to be effective in protecting the liver against oxidative stress-induced injury.

Keywords: Thioredoxin; Adenovirus vector; Gene therapy; Oxidative stress; Liver injury

The liver has versatile functions and plays important roles in metabolism, such as biosynthesis of plasma proteins, gluconeogenesis, and detoxification. Although the liver has the intensive regeneration ability, when cellular loss exceeds a certain threshold, the insufficient functions cause hepatic failure, leading to liver disease. Overdose of drug or ischemia/reperfusion induces necrotic and apoptotic cell death of hepatocytes and non-parenchymal liver cells. One promising approach to prevent liver injury or to treat the patient with liver disease is to confer the resistance against cell death on hepatocytes and non-parenchymal cells. In our previous study, gene delivery into hepatocytes of thioredoxin (TRX), which plays a central role in intracellular redox control, was shown to suppress oxidant-dependent apoptotic cell death in vitro [1]. The introduction of TRX gene also extended the life-span of hepatocytes in a conventional monolayer culture. TRX is a low-molecular-weight redox protein (12 kDa) found in both prokaryotic and eukaryotic cells [2]. Cystein residues in the conserved, Cys-Gly-Pro-Cys-Lys active site of TRX undergo reversible oxidation-reduction catalyzed by the NADPH-dependent flavoprotein, thioredoxin reductase. TRX was originally identified as a reducing cofactor for ribonucleotide reductase. More recently, it was shown to be involved in cellular protective mechanisms against various kinds of stresses, such as ischemia/reperfusion, X-ray irradiation, and inflammatory cytokines [3]. Therapeutic effects of TRX have been reported in various animal models. The pancreatic β cell-specific expression of TRX prevents autoimmune and streptozotocin-induced diabetes [4]. TRX overexpression in transgenic mice attenuates both

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focal ischemic damage [3] and adriamycin-induced cardiotoxicity [5]. Resistance to influenza virus-induced pneumonia and hepatotoxin-induced liver fibrosis is conferred in TRX transgenic mice [6,7]. In this study, we showed that TRX gene delivery into the liver was effective for treating carbon tetrachloride (CCl_4)-induced acute hepatitis, using an adenovirus vector that is well known to accumulate in the liver.

Materials and methods

Animals. BALB/c mice and Sprague–Dawley rats were purchased from SLC (Shizuoka, Japan). The animals were housed in an air-conditioned room at 22 ± 1 °C prior to the experiment. The experiments were conducted according to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University. Hepatic injury was elicited in 6-week-old male mice by the intraperitoneal administration of CCl_4 at 1 ml/kg body weight. Forty-eight hours before the CCl_4 treatment, 1×10^8 pfu of recombinant adenovirus was administered intraperitoneally.

Media. The basal medium consisted of 100 U/ml penicillin G, 100 μg/ml streptomycin, 50 ng/ml amphotericin B, and 100 ng/ml aprotinin (Nacalai Tesque, Kyoto, Japan) in William's medium E (WE, ICN Biochemicals, Costa Mesa, CA, USA). Medium A consisted of 10% fetal bovine serum (FBS, ICN Biochemicals) in basal medium. Medium B consisted of 1 nM insulin (Sigma Chemicals, St. Louis, MO, USA) and 1 nM dexamethasone (Nacalai Tesque, Kyoto, Japan) in Medium A.

Culture conditions. Hepatocytes were isolated from male Sprague–Dawley rats weighing 150–200 g by perfusing the liver with collagenase (from *Clostridium histolyticum* Type IV; Sigma Chemicals, St. Louis, MO, USA) according to the method of Seglen [8]. Cells were seeded at a density of 1×10^5 cells/cm^2 into 12-well polystyrene culture plates (Nippon Becton Dickinson, Tokyo, Japan). After 6 h in Medium B, the cells were cultured in Medium A. The medium was changed every 24 h.

Construction of recombinant adenoviral vectors. We constructed an E1- and E3-deleted recombinant adenovirus vector using the pALC3 cosmid, which was generated by removing the E3 region of the adenoviral genome from the pALC cosmid, which already lacks E1 [9]. The TRX gene expression cassette, shown in Fig. 1A, was flanked by *Swa*I sites and included the CAG promoter [10], the human TRX cDNA, an internal ribosome entry sequence, enhanced green fluorescent protein (EGFP) cDNA, and the rabbit β-globin polyA signal. The expression cassette was inserted into the unique *Swa*I site of pALC3, to make pALC3-TRX. The recombinant adenovirus vector expressing human TRX (Adv-TRX) was produced by infecting 293 cells with pALC3-TRX, and the titer of the virus stock was determined as described previously [9]. Briefly, virus suspensions were serially diluted with medium and added to a 96-well multiplate seeded with 293 cells. After 10 days, the virus titer was calculated by examining the wells for the presence or absence of a cytopathic effect. The control vector, Adv-lacZ, was constructed using the *Escherichia coli* lacZ gene instead of the TRX gene (Fig. 1B).

RT-PCR. The total RNAs were extracted from rat hepatocytes and mouse organs using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). The gene expression of TRX was analyzed using the following primers: forward 5′-TCTGACTGACCGCGTTACTC-3′ and reverse 5′-TCATCCA CATCTACTTCAAGGA-3′. β-Actin gene expression was analyzed using

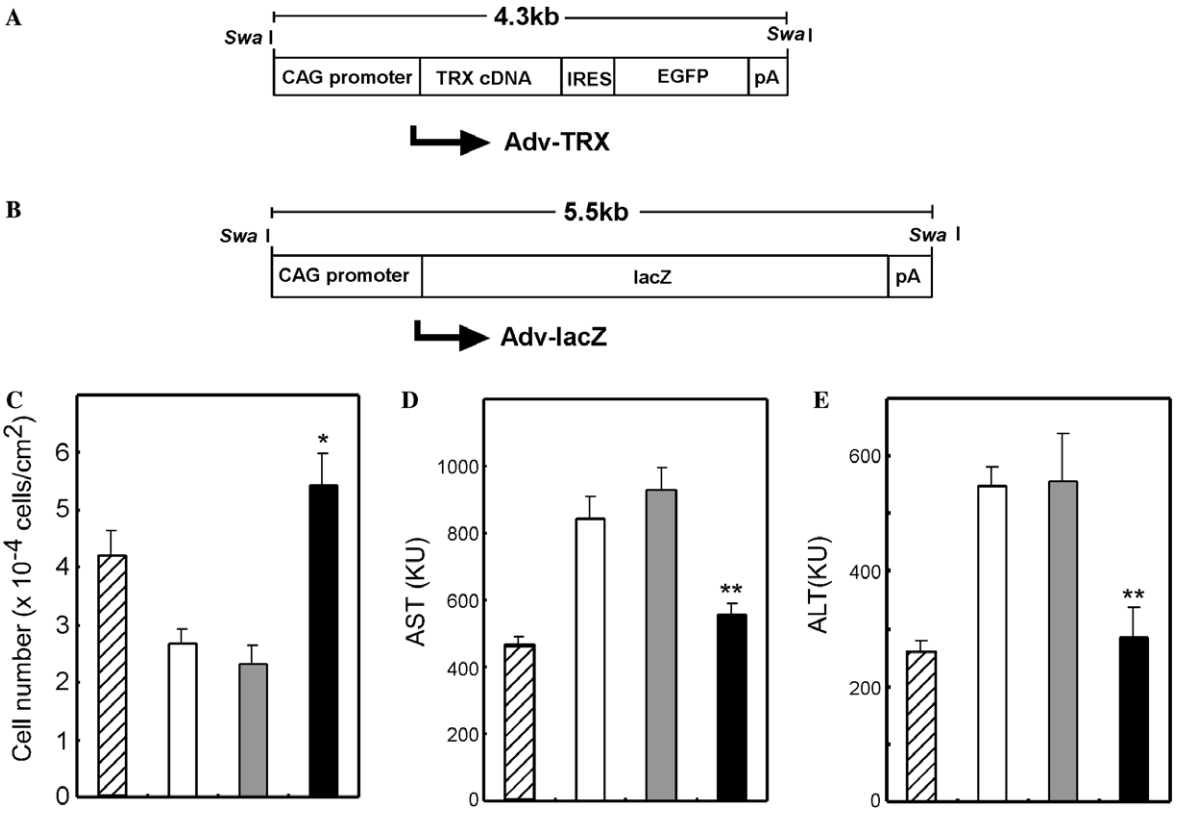

Fig. 1. Expression cassettes inserted into the *Swa*I site of the pALC3 cosmid and in vitro protective effect of Adv-TRX infection against CCl_4 treatment in rat hepatocytes. The cassette contained the human TRX (A) or lacZ (B) gene, resulting in Adv-TRX or Adv-lacZ, respectively. After 4 h of CCl_4 treatment, the number of viable cells was counted by the trypan blue dye exclusion test (C), and the activities of AST (D) and ALT (E) leaked into the medium were measured. Hatched bars indicate the values from a non-damaged control. Open, gray, and closed bars indicate the values from non-, Adv-lacZ-, and Adv-TRX-infected rat hepatocytes treated with CCl_4, respectively. Hepatocytes were infected with Adv-lacZ or Adv-TRX at an moi of 1. Values are means ± SD of three experiments. Asterisks indicate a value significantly different from that of the non-infected cells (**$p < 0.01$, *$p < 0.05$).

the following primers: forward 5′-CATCCCCCAAAGTTCTAC-3′ and reverse 5′-CCAAAGCCTTCATACATC-3′. RT was performed using a 1-μg total RNA sample with the BcaBEST RNA PCR kit (TaKaRa, Kyoto Japan). The PCR conditions were: (1) 94 °C for 1 min; (2) 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C; and (3) 72 °C for 5 min.

Assays. Adherent rat hepatocytes were treated with trypsin at 37 °C for 5 min, and viability was measured with the trypan blue dye exclusion test. The serum ammonia level was determined by the indophenol colorimetric method [11]. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using an assay kit (IATROZYME TA-Lq; Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). Human thioredoxin released into blood was determined using an assay kit (Redox Bio Science Inc., Kyoto Japan). Assay kits for IL-6 and TNF-α were purchased from BioSource International Inc. (CA, USA) and Pierce Biotechnology Inc., respectively.

Histological analysis. The excised liver was fixed in buffered formalin, embedded in paraffin, cut into 5-μm-thick sections, and examined with hematoxylin-eosin staining.

Statistics. The data were analyzed for statistical significance using Student's *t* test.

Results

Protective effect of Adv-TRX infection on hepatocytes

Isolated rat hepatocytes were infected with Adv-TRX, and the fluorescence derived from the co-translated EGFP was detected in almost all the cells attached to the polystyrene plate 24 h later (data not shown). We then examined whether the TRX gene had a protective effect by treating the gene-transfected hepatocytes with CCl_4. Fig. 1(C)–(E) shows the viable cell number and released transaminase level after 4 h of CCl_4 treatment. CCl_4 treatment caused considerable injury to the non-infected hepatocytes, which showed a decreased viable cell number and increased transaminase level compared with untreated cells. Likewise, the Adv-lacZ-infected hepatocytes did not show any resistance to the CCl_4 treatment. In contrast, the hepatocytes infected with Adv-TRX were significantly resistant to injury.

Protective effect on liver function in vivo

We next examined whether Adv-TRX had a protective effect on CCl_4-induced liver injury in vivo. Adv-TRX was first administered to normal mice, and 48 h later, the gene expression was analyzed in various organs by RT-PCR using primers for the human TRX gene. RNAs were extracted from the liver, small intestine, heart, kidney, and lung. As shown in Fig. 2A, TRX mRNA was detected only in the liver. Thus, the administered TRX gene appeared to be mainly delivered to and transcribed in the liver. Next we examined whether Adv-TRX administration had a protective effect on the detoxification function in the liver. Mice were infected with recombinant adenoviruses 48 h prior to the CCl_4 treatment. Blood samples were collected from mice 24 h after the CCl_4 treatment, and the serum human TRX level was determined. Human TRX was undetectable in serum from non- and Adv-lacZ-infected mice. In contrast, 7.25 ± 1.67 ng/ml human TRX was detected in the serum from Adv-TRX-infected mice. These

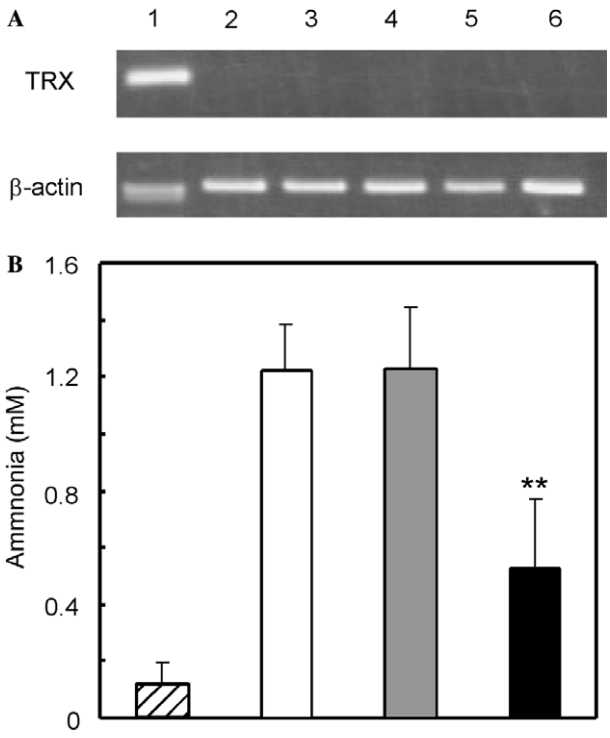

Fig. 2. (A) RT-PCR analysis of human TRX gene expression in various mouse organs. RNAs extracted from liver (lane 1), small intestine (lane 2), heart (lane 3), kidney (lane 4), and lung (lane 5) were analyzed using primers for human TRX and mouse β-actin. (B) Protective effect of Adv-TRX infection on ammonia removal function. Blood samples were collected 24 h after CCl_4 treatment from the experimental and normal mice. The hatched bar indicates the value of the serum ammonia concentration in normal mice. The open, gray, and closed bars indicate the values from non-, Adv-lacZ-, and Adv-TRX-infected mice treated with CCl_4, respectively. Values are means ± SD of four different animals. The asterisk indicates a value significantly different from that of the non-infected mice ($*p < 0.01$).

results clearly indicated that administered Adv-TRX was transcribed and translated in the liver cells to secrete human TRX protein into the blood. As shown in Fig. 2B, the serum ammonia level was markedly increased by the CCl_4 treatment. Although there was no significant difference in the serum ammonia level between the Adv-lacZ- and non-infected mice, Adv-TRX infection significantly decreased the ammonia level. Therefore, the Adv-TRX infection appeared to confer resistance to this toxic substance on hepatocytes in vivo.

Anti-inflammatory effect

The serum AST and ALT activities were measured 24 h after the CCl_4 injection (Fig. 3). The normal activities of AST and ALT in the sera from untreated mice were 65 ± 6 and 27 ± 4 (KU), respectively. The serum AST and ALT activities in injured mice were elevated to 8907 ± 1408 (KU) and $11{,}233 \pm 1153$ (KU), respectively. There was no significant difference in the serum AST and ALT activities between Adv-lacZ- and non-infected mice. In contrast, the serum AST and ALT activities were

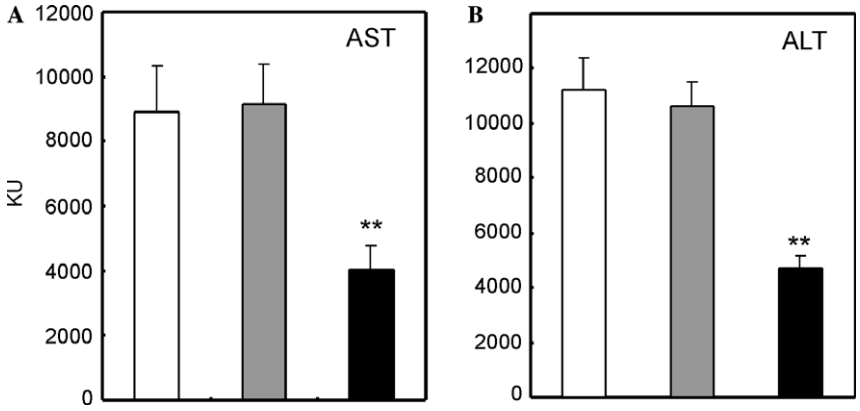

Fig. 3. Suppression of AST and ALT levels in CCl_4-injured mice caused by Adv-TRX infection. Blood samples were collected 24 h after CCl_4 treatment. Open, gray, and closed bars indicate the activities of AST (A) and ALT (B) in the serum obtained from non-, Adv-lacZ-, and Adv-TRX-infected mice treated with CCl_4, respectively. Values are means ± SD of four different animals. Asterisks indicate a value significantly different from that of the non-infected mice (**$p < 0.01$).

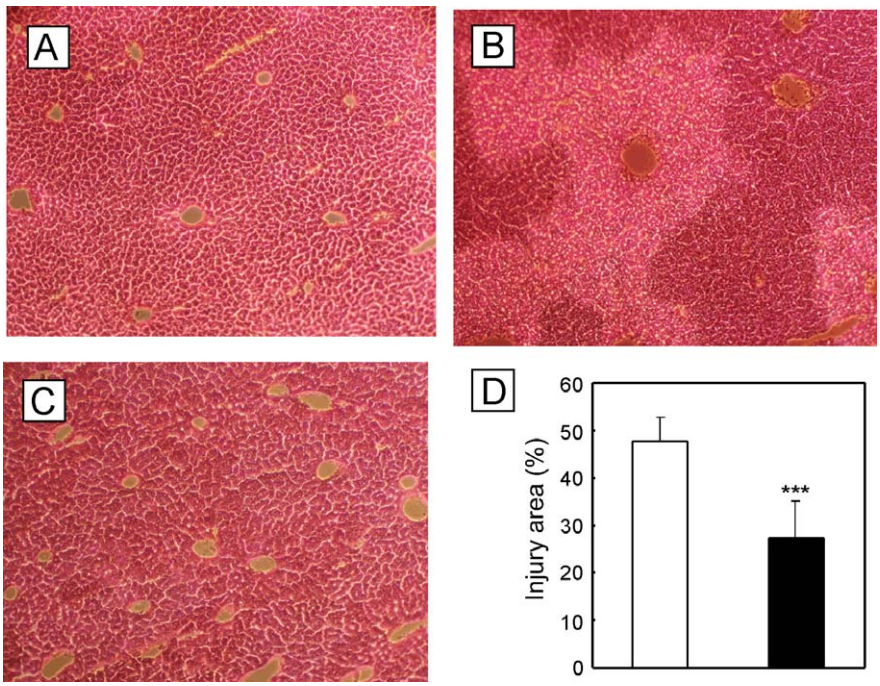

Fig. 4. Suppression of liver injury by Adv-TRX infection in CCl_4-treated mice. Hematoxylin-eosin staining was performed on paraffin-embedded sections of liver tissues. Sections from normal mouse liver were used as a control (A). The liver was excised from non- (B) and Adv-TRX- (C) infected mice 24 h after CCl_4 treatment. After the staining, the injured area, which appeared as a whitish region around the vessels, was digitalized (D). The open and closed bars represent liver sections obtained from non- and Adv-TRX-infected mice, respectively. Values are means ± SD of the injured area (%) using images from five different sections. Asterisks indicate a value significantly different from that of the non-infected mice (***$p < 0.005$).

significantly lower in the Adv-TRX-infected mice. The liver was excised 24 h after CCl_4 treatment and stained with hematoxylin and eosin to evaluate the extent of liver injury (Fig. 4). Extensive damage was apparent in the sections from non-infected mouse liver (Fig. 4B). The effect of Adv-TRX infection on the injury was evaluated by digitalization of the damaged area (Fig. 4D). The Adv-TRX infection significantly suppressed the liver injury. These results clearly indicated that the adenovirally introduced TRX gene could confer resistance against liver injury on the host. We measured the concentrations of inflammatory cytokines, IL-6 and TNF-α, in the serum. Both cytokines were undetectable in the serum from normal mice, and the CCl_4 treatment stimulated the secretion. The Adv-TRX infection significantly increased the IL-6 secretion and tended to suppress the TNF-α secretion (data not shown).

Discussion

The liver is acutely and/or chronically exposed to toxic substances including alcohol and therapeutic drugs, and excess reactive oxygen species (ROS) generated to metabolize xenobiotics by cytochrome P450 (CYP) enzymes cause

severe liver damage. CCl_4 is frequently used to make an experimental model for liver injury, since CCl_4 is activated by CYP2E1, CYP2B, or CYP3A to form various radicals, causing fatty degeneration, fibrosis, hepatocellular death, and carcinogenesis [12]. In this study, we showed that the human TRX gene was successfully delivered to the mouse liver using an adenovirus vector and that it protected the mouse from CCl_4-induced acute liver injury. Thus, overexpressed human TRX appears to scavenge radicals generated by the metabolism of CCl_4 to protect liver function and suppress tissue damage. In addition to the administration of antioxidants, this type of redox gene therapy is thought to be a promising strategy for preventing and/or treating ROS-mediated liver injury. Moreover, introduction of the TRX gene prior to liver surgery might be effective in preventing ischemia-reperfusion injury, which is caused by free radical generation.

As the vector, we used a mutant of adenovirus type 5 (Ad5) that has deletions in the E1 and E3 regions [9]. Studies show that Ad5 is immediately cleared from the bloodstream and accumulates in the liver, where it is mainly taken up by hepatocytes and Kupffer cells [13,14]. In agreement with these reports, the transferred TRX gene was expressed only in mouse liver 48 h after the administration of Adv-TRX. Although we did not confirm the cell type into which the TRX gene was introduced, Adv-TRX might infect non-parenchymal liver cells other than Kupffer cells. In our preliminary study, rat hepatic stellate cells (HSCs) were infected with Adv-TRX, and the effect on cell proliferation and activation was examined in vitro. Adv-TRX infection suppressed the cell proliferation and the gene expressions of the α-smooth muscle actin, transforming growth factor-β, and collagen-I, which are markers for activated HSCs. Thus, in vivo, Adv-TRX administration might prevent liver fibrosis by suppressing the activation of HSCs.

In this study, Adv-TRX infection was shown to decrease the serum levels of AST and ALT. This result indicates that hepatocytes were protected from CCl_4-induced necrosis by the overexpression of TRX. In our previous study we showed that Adv-TRX infection conferred a resistance against apoptosis caused by H_2O_2 treatment on rat primary hepatocytes [1]. Recently, extensive studies on the relationship between TRX and oxidative stress-induced apoptosis have been carried out [15,16]. In the absence of stress, apoptosis-signal-regulating kinase 1 (ASK1) exists as an inactive complex with the reduced form of TRX. Oxidative stress causes the oxidation of TRX, which disrupts the ASK-TRX complex, thereby activating ASK1 to induce apoptosis [17]. TRX overexpression appears to protect the TRX-ASK1 complex from such disruption. In this study serum IL-6 level was shown to increase by the Adv-TRX infection. When liver is damaged by partial hepatectomy and hepatotoxin administration, IL-6 plays an important role in regeneration and recovery of the liver [18]. On the contrary, Adv-TRX administration tended to decrease the concentration of TNF-α, which induces hepatocyte apoptosis. Therefore, the TRX gene, by virtue of its potency to protect against oxidative stress-mediated apoptotic and/or necrotic cell death, is considered to be a promising prodrug for liver protection therapy.

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