Tacrine, an Oral Acetylcholinesterase Inhibitor, Induced Hepatic Oxidative Damage, Which Was Blocked by Liquiritigenin through GSK3-beta Inhibition

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Although the cholinesterase inhibitor tacrine has been successfully used for the treatment of Alzheimer’s disease, it is known to have hepatotoxic effects. Liquiritigenin (LQ), an active flavonoid in Glycyrrhizae radix, exerts protective effects against liver damage. This study investigated the toxic effect of tacrine on hepatocytes and the beneficial effect of LQ on tacrine intoxication in vivo and vitro, and the underlying mechanism involved. In hepatocyte cell lines, tacrine induced cell death and oxidative stress, as indicated by decreases in cell viability and glutathione (GSH) contents, which were blocked by pretreatment with LQ. Fluorescent activated cell sorter (FACS) analysis revealed that LQ inhibited cellular H2O2 production and mitochondrial dysfunction induced by tacrine in HepG2 cells. Furthermore, LQ promoted inhibitory phosphorylation of glycogen synthase kinase-3β (GSK3β) and prevented decreases in GSK3β phosphorylation induced by tacrine. In rats treatment with tacrine at 30 mg/kg increased hepatic damage as assessed by blood biochemistry and histopathology. Administration of LQ (10 or 30 mg/kg/d, per os (p.o.)) or the hepatoprotective drug silymarin (100 mg/kg/d) for 3 d inhibited elevations in alanine aminotransferase, aspartate aminotransferase, and histological changes induced by tacrine. These results show that LQ efficaciously protects the rat liver against tacrine-induced liver damage, and suggest that LQ is a therapeutic candidate for ameliorating the hepatotoxic effects of tacrine.

Key words tacrine; liquiritigenin; glycogen synthase kinase-3β; liver; oxidative stress; mitochondria

Tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride) is recommended as the first-line treatment for Alzheimer’s disease by the US Food and Drug Administration. It is an active cholinesterase inhibitor that blocks the degradation of cholinergic nerves in the cerebral cortex and hippocampus to increase cholinergic transmission.1 However, although tacrine has positive therapeutic effects, it has been demonstrated to induce increased serum alanine aminotransferase (ALT) levels in about 30% of patients, and this seriously limits its clinical use.2 A number of authors have reported that tacrine-induced hepatotoxicity is related to cytochrome P450 1A2 in human liver microsomes and is associated with mitochondrial dysfunction.3–5 Studies also have showed that the hepatotoxic effect of tacrine is associated with oxidative stress, as demonstrated by increased reactive oxygen species (ROS) production and decreased intracellular glutathione (GSH).6–8 However, mechanism responsible for tacrine-induced hepatotoxicity has not yet been elucidated. Nevertheless many studies have shown that anthraquinones, chromone glycosides, phenolic amide, and an herbal medicine protect hepatocytes from the toxic effects of tacrine.9–12

Oxidative stress is state in which the balance between ROS production and antioxidants is shifted in favor of ROS, and is associated with severity of liver damage.13 ROS normally work as redox messengers in cellular signaling and the regulation of gene expression. However, excess ROS production influences cellular components and causes tissue injury via the oxidative modification of cellular macromolecules.14 Mitochondria are a major intracellular source of ROS, which are byproducts of oxidative respiration and continuously produced by mitochondria under normal conditions. Moreover, mitochondrial ROS can regulate inflammatory response and cell death.15,16 Furthermore, mitochondria can induce cell death due to the convergence of external and intracellular stress response pathways. Hence, ROS production and mitochondrial dysfunction can cause oxidative stress and cell death.17

Glycogen synthase kinase-3β (GSK3β) is a serine/threonine kinase and is constitutively activated in the normal state. Furthermore, GSK3β is a regulator of cell functions, such as, gene expression, cell cycle, and apoptosis. Oxidative stress stimulates GSK3β and causes GSK3β to translocate into mitochondria. Stimulated GSK3β phosphorylates the components of mitochondrial membrane pores, and thereby, provokes MMP transition. Thus, GSK3β is critically required for the regulation of MMP transition, and it is believed that the inhibitory phosphorylation of GSK3β is related to cell survival.18–20 Glycyrhiza radix (G. radix, Licorice) is one of the oldest, most frequently employed botanicals in herbal medicine in Asia, and currently occupies an important place among food products. In traditional medicine, it is used for its life-enhancing properties, detoxifying effects, and to treat injuries and swellings.21 G. radix contains flavonoids and pentacyclic triterpene saponins, such as, liquiritigenin (LQ), liquiritin, isoliquiritigenin, liquiritin apioside, glycyrrhizin, and glycycycromes.
Liquiritic acid, an aglycone of liquiritin, is a flavonoid found in *G. radix*. Our previous studies demonstrated the hepatoprotective effects of LQ against endotoxins and xenobiotics in *vivo* and *in vitro*. In the present study, we investigated the effects of LQ against tacrine-induced cell death and oxidative stress in rats and hepatocyte-derived cell lines, and the underlying mechanisms involved.

**MATERIALS AND METHODS**

**Materials** LQ was isolated from the chloroform-acetone fraction of *G. radix*, as previously described, and its chemical structure was verified by a variety of spectroscopic analyses. Antibody directed poly(ADP-ribose) polymerase (PARP), procaspase-3, procaspase-9, GSK3β, phospho-GSK3β and horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). Anti-Bad antibody was purchased from BD biosci (San Jose, CA, U.S.A.) and SB216763 was obtained from Calbiochem (San Diego, CA, U.S.A.). 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride (tacrine), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), rhodamine 123, 2',7'-dichlorofluorescein diacetate (DCFH-DA), anti-β-actin antibody, polyethylene glycol #400 (PEG), silymarin and other reagents were supplied from Sigma-Aldrich (St. Louis, MO, U.S.A.).

**Cell Culture** HepG2 (human), H4IIE (rat), AML12 (mouse) hepatocyte-derived cell lines were supplied from American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured in Dulbecco’s modified Eagle’s medium, which contains 100 U/mL penicillin, 10% fetal bovine serum and 100 µg/mL streptomycin at 37°C. For all experiments, the cells were grown to 80–90% confluency, incubated in medium without fetal bovine serum for 12 h, and then subsequently exposed to either tacrine or LQ+tacrine for the indicated time periods.

**MTT Assay** The cells were plated at a density of 5×10⁴ cells/well in 24-well plates. After cells were treated with the drugs, the cells were stained with MTT (0.5 mg/mL, 4 h) as described previously. The cell viability was defined as the % of untreated control cells [i.e., viability (% control)=100×(absorbance of treated sample)/(absorbance of control)].

**Sample Preparation and Immunoblot Analysis** Cells were lysed in radio immunoprecipitation assay (RIPA) buffer containing protease inhibitor and phosphatase inhibitor cocktail (Pierce, Woburn, MA, U.S.A.). Protein contents of samples were measured using bichoninic acid (BCA) assay kit (Pierce, Woburn, MA, U.S.A.). Protein was visualized by an enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, U.K.). Equal protein loading

![Fig. 1. Cytotoxicity of Tacrine in Hepatocyte Cell Lines](image)

(A) MTT assay. HepG2 cells were treated with 30–1000 µM tacrine for 12 h or 300 µM tacrine for 3–48 h. After incubation, cell viability was assessed by the MTT analysis. Data represent mean±S.D. of at least three separated experiment (significant as compared with control cells, **p<0.01, *p<0.05**).
among the samples in each gel was verified by β-actin. Density measurement was determined by using an image analyzing system (Ultra-Violet Products Ltd., Upland, CA, U.S.A.).

**Measurement of H$_2$O$_2$ Production** The level of H$_2$O$_2$ generation was monitored in HepG2 cells by adding the DCFH-DA. After treatment of 10–100 µM LQ for 12h prior to the addition of 300 µM tacrine. The cells were further incubated for 5h and then continuously stained with 50 µM DCFH-DA for 1h. H$_2$O$_2$ production was measured by the concomitant increase in DCF fluorescence. (B) GSH contents. The cells were treated with 10–100 µM LQ for 12h and then continuously exposed to 300 µM tacrine for 12h. After incubation, the GSH contents measured in cell homogenates. Data represent mean±S.D. of at least three separated experiment (significant as compared with control cells, **p<0.01; significant as compared with tacrine-treated cells, ##p<0.01, #p<0.05).

**Fluorescent Activated Cell Sorter (FACS) Flow Cytometry Analysis** Mitochondrial membrane potential (MMP) was measured with rhodamine 123. HepG2 cells were treated and stained with 0.05 µg/mL rhodamine 123 for 1h. The change in MMP was determined using a FACS flow cytometer (Partec, Münster, Germany). In each analysis, 10000 cells were recorded.

**Animal Treatment** Animal studies were conducted in accordance with the institutional guidelines of Daegu Haany University, and reviewed by institutional review board. Six-weeks-old Sprague-Dawley rats (140–160g) were provided by Hyochang Science (Daegu, Korea) and acclimatized for 1 week, and maintained in Daegu Haany University. Rats (N=25) were randomly divided into five groups and each group consisted of five animals. LQ (10, 30mg/kg) and silymarin (as reference standard of hepatoprotective activity, 100mg/kg) dissolved in 40% PEG was orally administrated to rats for 3 consecutive days. One hour after the last LQ administration, toxicant was induced by a single treatment of 30mg/kg tacrine (per os (p.o.), dissolved in 40% PEG) to the...
rats. All animals were fasted for 24 h. Blood and liver tissue samples were obtained 24 h after tacrine treatment.

**Blood Biochemistry** Serum ALT and aspartate amino transferase (AST) were assayed using analysis kits (Pointe Scientific Inc., Canton, MI, U.S.A.) as well as an analyzer for blood chemistry (Photometer 5010, Robert Riele GmbH & Co., KG, Berlin, Germany).  

**Histochemistry** The histochemistry has an index of criteria as previously described [i.e., the percentages of degenerative regions in hepatic parenchyma (%/mm²), the number of degenerative hepatocytes (cells/mm² of hepatic centrilobular regions), the number of inflammatory cells infiltrated (cells/mm² of hepatic portal area) and total hepatocyte counts (cells/mm² of hepatic centrilobular regions)]. 27–30 Samples from liver were separated and fixed, and then embedded and stained with hematoxylin and eosin. Each index was calculated using automated image analysis (DMI-300 Image Processing; DMI, Korea). 27,30 In addition, total hepatocyte numbers were also calculated according to previous established methods. 28,29

**Statistical Analysis** For each treatment showing a statistically significant effect, the Student’s t-test analysis was used for comparisons of group means.

**RESULTS**

**Tacrine-Induced Hepatic Damages in Vitro and Its Inhibition by LQ** Initially, we confirmed the effect of tacrine in vitro. We used a MTT assay to determine the cell viabilities of HepG2 (human), H4IIE (rat), and AML12 (mouse) hepatocyte-derived cell lines. HepG2 cells were treated with 30–1000 µM tacrine for 12h or 300 µM of tacrine for 3–48 h. Treatment with 300 µM tacrine for 12 h decreased cell viability by approximately 70% (Fig. 1A left and right). H4IIE and AML12 cells were also incubated with 30–1000 µM tacrine for 12h.
H4IIE and AML12 cells showed ca. 70% decreases in cell viability in 300 and 1000 µM tacrine, respectively (Fig. 1B left and right). Therefore, 300 µM tacrine for 12 h were selected for subsequent experiments.

We investigated the effect of LQ on the cytotoxicity of tacrine in HepG2 cells. We treated 3–100 µM LQ for 12 h prior to adding 300 µM tacrine. Treatment with tacrine significantly decreased cell viability. However, LQ pretreatment significantly and dose-dependently inhibited tacrine-induced decreases in cell viability. Strong inhibition of cell death was obtained by pretreating cells with 100 µM LQ (Fig. 2A). Next, the expressions of apoptosis-related proteins were monitored by immunoblot analysis. PARP cleavage and Bad protein expression were increased in tacrine-treated cells, but pretreatment with 100 µM LQ blocked tacrine-induced cell damage. HepG2 cells were treated with 100 µM SB216763 for 1h and then continuously exposed to 300 µM tacrine for 12h. After incubation, cell viability was assessed by the MTT analysis (left). For FACS analysis, HepG2 cells were pretreated with 10 µM SB216763 for 1h prior to the exposure of 300 µM tacrine (right). The cells were harvested after rhodamine 123 staining. The relative mitochondrial membrane permeability levels were assessed by the ratio with low rhodamine 123 fluorescence intensity. Data represent mean ± S.D. of at least three separated experiments (significant as compared with control cells, **p<0.01).

Tacrine-Induced Oxidative Stress and Mitochondrial Dysfunction and Its Inhibition by LQ

To determine the effect of LQ on tacrine-mediated oxidative stress, we measured intracellular levels of H₂O₂ with or without LQ pretreatment. H₂O₂ production was significantly induced in tacrine-treated cells, whereas pretreatment with 30 or 100 µM LQ blocked H₂O₂ production (Fig. 3A). In addition, we monitored intracellular levels of GSH, a major endogenous anti-oxidant, using a colorimetric method. Intracellular concentrations of GSH in HepG2 cells were decreased by tacrine, but these were inhibited dose-dependently by pretreatment with LQ (Fig. 3B).

It has been shown that mitochondrial dysfunction inhibits oxidative phosphorylation and increases ROS production. Therefore, we examined whether LQ protects mitochondria. The fluorescence intensities of HepG2 cells were measured by FACS after staining with rhodamine 123. Tacrine-treated cells increased the number of HepG2 cell populations with low rhodamine 123 fluorescence (RN1 fraction) as compared with non-treated controls (Fig. 4A). On the other hand, pretreat-
SB216763 significantly inhibited tacrine-inducible cell death in the liver damage caused by tacrine, and pretreatment with (a GSK3β inhibitor) was used to confirm the role of GSK3β in the liver damage caused by tacrine, and pretreatment with SB216763 significantly inhibited tacrine-inducible cell death and mitochondrial dysfunction (Fig. 5C).

**DISCUSSION**

Tacrine is the cholinesterase inhibitor which is used as treatment of Alzheimer’s disease by the US Food and Drug Administration. However, it has shown to the hepatotoxicity by increasing ALT levels and substantially was limited its clinical use. Although tacrine has detrimental effects on hepatic function, the mechanism responsible has not been explained. The results of this study show that increases hepatocyte death, ROS production, and mitochondrial damages in HepG2 (human), H4IIE (rat), AML12 (mouse) cells. In rats, tacrine increased markers of liver damages (ALT and AST), degenerative region areas, and numbers of infiltrating inflammatory cells. Accordingly, the study confirms that tacrine is hepatotoxic in vivo and in vitro.

In this study, the administration of tacrine to rats increased the serum levels of the enzymes ALT and AST, which are produced in damaged liver cells and released to blood. ALT is present in large quantities in the cytoplasm of hepatocytes and is considered as an excellent marker of liver damage, especially in animal species. AST is a mitochondria-bound enzyme and is found in several body tissues but its levels are particularly high in liver and striated muscle. Furthermore, serum AST activities are elevated in the presence of skeletal muscle necrosis or hepatocellular necrosis. The present study shows LQ has protective effects against tacrine-induced liver damage in rats, as assessed by the serum biomarkers ALT and AST.

Tacrine is known to induce hepatocyte necrosis and degeneration. In the present study, during the progress of tacrine...
Fig. 7. Histochemical Analysis

(A) Representative histological images of the rat liver in control, tacrine 30 mg/kg, tacrine+LQ 10 mg/kg, tacrine+LQ 30 mg/kg, and tacrine+silymarin 100 mg/kg treated groups. After 24h of tacrine treatment, liver were stained with hematoxylin and eosin (scale bars=160 µm). (B) Percentages of degenerative regions and numbers of degenerative hepatocytes. Data represent mean±S.D. of five animals (significant as compared with control group, **p<0.01; significant as compared with tacrine-treated group, ##p<0.01).

Table 1. Histomorphometrical Analysis in Tacrine-Treated Rat Livers

<table>
<thead>
<tr>
<th>Groups</th>
<th>Infiltrated inflammatory cells (cells/mm²)</th>
<th>Total centrolobular hepatocyte counts (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.50±4.04</td>
<td>583.50±112.35</td>
</tr>
<tr>
<td>Tacrine</td>
<td>76.17±10.65**</td>
<td>315.83±36.84**</td>
</tr>
<tr>
<td>Tacrine+LQ 10 mg/kg</td>
<td>52.67±12.08**</td>
<td>387.33±35.92**</td>
</tr>
<tr>
<td>Tacrine+LQ 30 mg/kg</td>
<td>32.83±9.02**</td>
<td>433.33±44.93**</td>
</tr>
<tr>
<td>Tacrine+Silymarin</td>
<td>34.00±4.10**</td>
<td>430.50±43.23**</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.D. of rat liver histological fields (significant as compared with control group, **p<0.01; significant as compared with tacrine-treated group, ##p<0.01, #p<0.05).
intoxication, degenerative changes in liver-centrolobular necrosis of hepatocytes were observed with portal inflammatory cell infiltrations and decreases in liver cells around central vein regions due to necrosis. Furthermore, tacrine-related centrolobular hepatic damage and portal hepatitis were reconfirmed histomorphometrically by changes in degenerative regions and numbers of degenerative hepatocytes and infiltrating inflammatory cells, which were significantly higher in the tacrine-treated group than in the control group. In our in vivo study, the oral administration of LQ (10 or 30 mg/kg/d, p.o. for 3 d) inhibited increases in degenerative regions and numbers of degenerative hepatocytes induced by tacrine. Moreover, LQ protected against inflammatory cell infiltration. Previously, we showed that LQ has anti-inflammatory effects through NF-κB inhibition in macrophage cell lines.35) Other studies performed by our group have confirmed that LQ has the ability to inhibit acute hepatitis induced by acetaminophen or galactosamine/LPS in rats.24,26) These findings show that LQ inhibits the degeneration of parenchymal cells and activation of inflammatory cells.

Mitochondria are a major source of ROS and oxidative stress.34) Furthermore, intracellular oxidative stress is associated with the severity of the liver damage caused by ROS generation. Although mitochondria act as a defense organelles against toxic injury, mitochondrial dysfunction has pathologic effects.35) The present study confirms that treatment with tacrine induces oxidative stress, mitochondrial dysfunction, and hepatocyte death. On the other hand, LQ pretreatment inhibited cell death and oxidative stress induced by tacrine in HepG2 cells, as indicated by increases in cell viability and GSH contents, and by decreases in apoptosis maker proteins and ROS production, which suggests that LQ inhibits ROS production, and thereby, inhibits mitochondria damage and cell death.

LQ is a flavonoid isolated from Glycyrrhiza radix and is used traditionally for detoxification and to treat injuries and swelling. Previously, we demonstrated the cytoprotective effects of LQ against cadmium-induced hepatotoxicity in hepatocyte-derived H4IIE cells and the hepatoprotective effect of LQ against acute injuries induced by acetaminophen with or without buthionine sulfoximine in rats.24,25) We also reported that LQ has anti-inflammatory effects in vivo and in vitro, and a choleretic effect involving the induction of transporters and phase-II enzymes in liver.25,26) Moreover, others have shown LQ is a plant-derived selective estrogen receptor-β agonist with neuroprotective effects on glutamate-induced apoptosis in hippocampal neuronal cells.36,37) In the present study, LQ protected hepatocytes from cytotoxicity induced by tacrine, as evidenced by the inhibition of cellular H₂O₂ production and mitochondrial dysfunction and by the restoration of the intracellular concentration of GSH. Pretreatment with LQ also inhibited tacrine-induced liver damage, as assessed by inhibitions of the up-regulations of ALT and AST and by hepatocyte degeneration and inflammation in liver tissue. Moreover, LQ has a possibility of having any effect on cholinesterase inhibition. In this study, the cells were treated with tacrine in the presence of LQ. So, LQ might affect the cell viability via directly interaction with tacrine. Therefore, it could be a next research project to determine the effect of LQ on cholinesterase in brain of rats in the future.

More importantly, tacrine decreased the phosphorylation of GSK3β, and this was blocked by LQ. Moreover, LQ markedly induced the phosphorylation of GSK3β and an inhibitor of GSK3β significantly blocked the ability of tacrine to induce mitochondrial dysfunction and cell death. These findings suggest GSK3β is a plausible target of the inhibition of tacrine-induced hepatotoxicity. Our findings may be informative not only with respect to the hepatotoxic effect of tacrine, but also in terms of the pharmacological effect and mechanism responsible for the inhibitor effect of LQ on liver damage induced by xenobiotics via GSK3β inhibition.

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Conflict of Interest The authors declare no conflict of interest.

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