Protective Effects of Exogenous Glutathione and Related Thiol Compounds against Drug-Induced Liver Injury

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An overdose of acetaminophen (APAP) causes liver injury both in experimental animals and humans. *N*-Acetylcysteine (NAC) is clinically used as an antidote for APAP intoxication, and it is thought to act by providing cysteine as a precursor of glutathione, which traps a reactive metabolite of APAP. Other hepatoprotective mechanisms of NAC have also been suggested. Here, we examined the effects of thiol compounds with different abilities to restore hepatic glutathione, on hepatotoxicity of APAP and furosemide in mice. Overnight-fasted male CD-1 mice were given APAP or furosemide intraperitoneally. NAC, cysteine, glutathione, or glutathione-monoethyl ester was administered concomitantly with APAP or furosemide. All thiol compounds used in this study effectively protected mice against APAP-induced liver injury. Only glutathione-monoethyl ester completely prevented APAP-induced early hepatic glutathione depletion. Cysteine also significantly restored hepatic glutathione levels. NAC partially restored glutathione levels. Exogenous glutathione had no effect on hepatic glutathione loss. NAC and glutathione highly stimulated the hepatic expression of cytokines, particularly interleukin-6, which might be involved in the alleviation of APAP hepatotoxicity. Furosemide-induced liver injury, which does not accompany hepatic glutathione depletion, was also attenuated by NAC and exogenous glutathione, supporting their protective mechanisms other than replenishment of glutathione. In conclusion, exogenous thiols could alleviate drug-induced liver injury. NAC and glutathione might exert their effects, at least partially, *via* mechanisms that are independent of increasing hepatic glutathione, but probably act through cytokine-mediated and anti-inflammatory mechanisms.

Key words hepatotoxicity; glutathione; *N*-acetylcysteine; acetaminophen; furosemide; interleukin-6

Acetaminophen (APAP), a commonly used analgesic, is usually safe when administered at therapeutic doses. However, APAP overdose causes liver injury both in experimental animals and humans. APAP has been used extensively to develop an animal model of drug-induced liver injury. The toxicity is initiated by cytochrome P450 (CYP) metabolism into *N*-acetyl-*p*-benzoquinone imine (NAPQI), and the high reactivity of NAPQI with sulfhydryl groups results in depletion of reduced glutathione (GSH) in hepatocytes, followed by covalent binding to intracellular proteins.[1—3] Therefore, hepatic GSH levels are important in protecting against APAP hepatotoxicity, and enhancement of hepatic GSH is a reasonable strategy for the treatment of APAP intoxication.

N-Acetylcysteine (NAC) is clinically used as an antidote for APAP intoxication.[4] It is thought that NAC provides cysteine (CYS) as a precursor of GSH which traps NAPQI, leading to a decrease in toxicity (Fig. 1).[5] However, NAC was shown to improve patient outcome after late administration[6]; this could not be explained by trapping NAPQI. In fact, treatment of mice with NAC after APAP administration protected against hepatic necrosis without reducing covalent binding, suggesting that mechanisms other than GSH replenishment are involved in the hepatoprotective effects of NAC.[7—9]

In contrast to NAC, GSH itself is not used as an antidote for APAP intoxication. It is believed that intraperitoneal administration of GSH is less effective in restoring hepatic GSH, probably because exogenous GSH can not be taken up by hepatocytes, whereas ethyl esters of GSH are proposed as GSH precursors that are taken up and hydrolyzed by hepatocytes into GSH (Fig. 1).[10] On the other hand, intravenously administered GSH was found to protect against APAP hepatotoxicity.[11] It is considered that intravenous GSH rapidly degrades in the kidney, is absorbed as amino acids, and is used to synthesize GSH in the liver, which could led to hepatoprotection. These studies suggest the potential importance of restoring hepatic GSH levels in the protection against APAP hepatotoxicity by exogenous thiol compounds.

The objective of this study was to clarify the role of GSH supplementation in the hepatoprotective action of NAC and other thiol compounds, which could lead to the development of a new hepatoprotective agent. We found that a relatively low dose of thiol compounds, GSH, NAC, CYS, and glutathione-monoethyl ester (GSH-EE), protected mice against APAP hepatotoxicity together with different abilities to re-

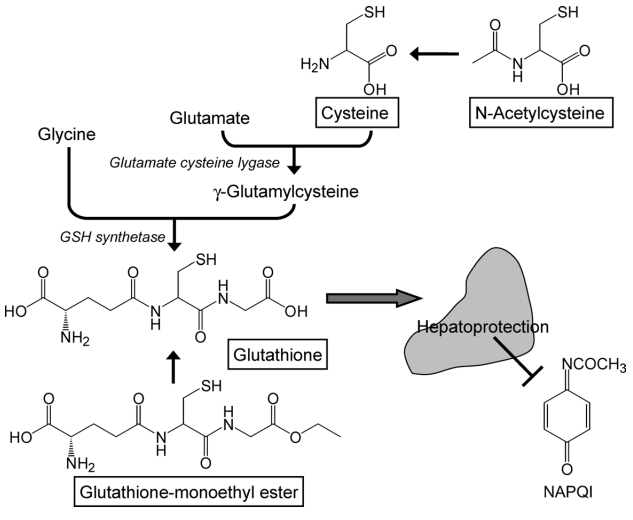

Fig. 1. Pathways for Biosynthesis of GSH and Chemical Structures of Thiol Compounds Used in This Study

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store hepatic GSH. We also examined the effects of these thiol compounds on hepatic cytokine expression, which is involved in pathogenesis and prevention of APAP hepatotoxicity. Furthermore, the effects of thiol compounds were examined using furosemide (FS) as a hepatotoxic agent which does not deplete GSH prior to the development of hepatotoxicity.

MATERIALS AND METHODS

Chemicals APAP was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). GSH, NAC, CYS, FS, diethyldithiocarbamic acid, and ketoconazole were purchased from Wako Pure Chemical Ind. (Osaka, Japan). GSH-EE was purchased from Bachem AG (Bubendorf, Switzerland). All chemicals and solvents used in this study were of analytical grade.

Animals and *in Vivo* Treatment Male and female CD-1 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were acclimatized for at least 1 week in a climate-controlled room on a 12-h light–dark cycle and were fed *ad libitum*. The mice were used in our experiment at 9—10 weeks of age. All procedures and care techniques were performed according to the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals.' The mice were fasted for 16 h before experimentation to sensitize them to APAP hepatotoxicity by decreasing basal levels of liver GSH. The mice received either 300 mg/kg APAP or 400 mg/kg FS in 10 ml/kg saline intraperitoneally (i.p.). Control mice received only 10 ml/kg saline vehicle. NAC (250 mg/kg, i.p.) or an equimolar (1.5 mmol/kg) dose of another thiol compound (CYS, GSH, or GSH-EE) was coadministered with APAP or FS. In some experiments, diethyldithiocarbamic acid (200 mg/kg, i.p.), or ketoconazole (50 mg/kg, i.p.) was coadministered with APAP or FS. The mice were then sacrificed to obtain their blood and livers at 3 or 24 h after the administration of APAP or FS. Blood samples were allowed to coagulate and then were centrifuged to obtain serum.

Assessment of Hepatotoxicity Serum alanine aminotransferase (ALT) activity was assayed as a marker of drug-induced hepatotoxicity. Assays were run on a test kit (Sigma Diagnostics, St. Louis, MO, U.S.A.).

Assay of Hepatic GSH Levels Liver tissues (100 mg) were homogenized in 1.0 ml 0.5 % sulfosalicylic acid. GSH concentrations in liver homogenates were determined by the dithionitrobenzoic acid-glutathione disulfide reductase recycling assay.[12] Assays were run on a GSH test kit (Dojindo Laboratories, Kumamoto, Japan).

RNA Isolation from Liver Liver tissues (approximately 60 mg) were homogenized in 1.2 ml RLT buffer (Qiagen Inc., Valencia, CA, U.S.A.) including 1% mercaptoethanol, and total cellular RNA was extracted according to the manufacturer's instructions. The isolated RNA was quantitated by spectrophotometric analysis at 260 nm.

mRNA Quantification of Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Real-time RT-PCR was performed to quantify the mRNA expression of TNF-α and IL-6 genes with modifications of described methods.[13] Total RNA (0.8 μg) from each sample was transcribed into complementary DNA (cDNA) using high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, U.S.A.). Real-time RT-PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) for mouse TNF-α (Mm00443258_m1), mouse IL-6 (Mm99999064_m1), and mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH, Mm99999915_g1). The reaction mixture contained one of these probes, TaqMan Universal PCR Master Mix (Applied Biosystems), and cDNA obtained as described above. Amplification and further analysis were performed by the ABI 7500 Real-Time PCR System (Applied Biosystems), which were initiated at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The intensity for mRNA expression of TNF-α and IL-6 thus obtained were normalized relative to G3PDH expression for each sample.

Statistical Analysis Statistical comparisons between two groups were performed using Student's *t*-test. Analyses involving more than two groups were compared using analysis of variance, followed by Dunnett's multiple comparison test to determine significant differences between group means.

RESULTS

Protective Effects of GSH and Its Related Thiols against APAP Hepatotoxicity and APAP-Induced Hepatic GSH Depletion Overnight-fasted male CD-1 mice were administered 300 mg/kg APAP. NAC, CYS, GSH, or GSH-EE were administered concomitantly with APAP, and hepatotoxicity was assessed at 3 and 24 h after APAP administration (Fig. 2). Treatment of mice with APAP caused liver injury as assessed by increases in serum ALT levels, and all thiols used in this study protected mice against APAP-induced liver injury almost completely. Hepatic GSH rapidly declined but recovered thereafter, as assessed at 3 and 24 h after APAP administration (Fig. 3). Only GSH-EE completely prevented APAP-induced early hepatic GSH depletion. Cysteine also significantly but not completely restored hepatic glutathione levels. NAC produced partial glutathione restoration. However, GSH administration was ineffective in protecting

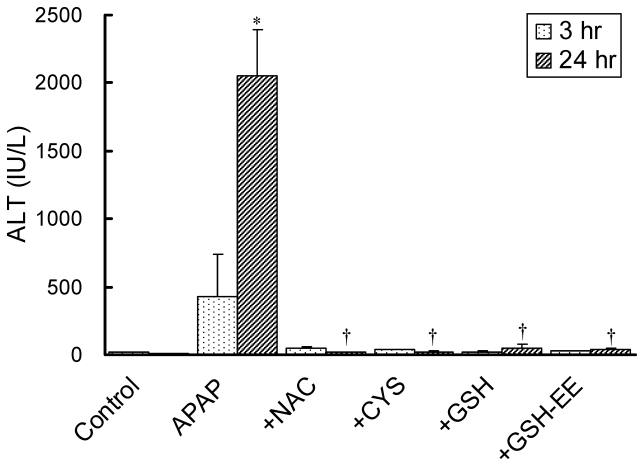

Fig. 2. Protection against APAP-Induced Liver Injury by Thiol Derivatives

Mice were administered APAP 300 mg/kg alone or with various thiol derivatives, and were sacrificed 3 or 24 h after administration for assay of serum ALT. Results are means±S.E. Numbers of mice used are 6 (APAP) and 3 (other groups). $*p<0.05$ *vs.* control; $\dagger p<0.05$ *vs.* APAP alone.

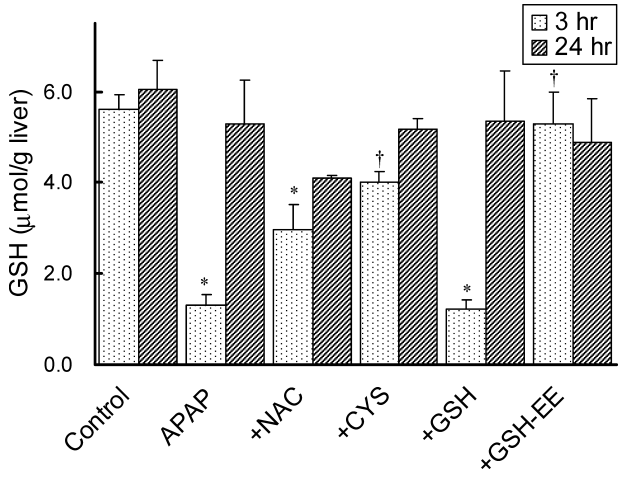

Fig. 3. Protection against APAP-Induced Hepatic GSH Depletion by Thiol Derivatives

Mice were administered APAP 300 mg/kg alone or with various thiol derivatives, and were sacrificed 3 and 24 h after administration for assay of liver GSH. Results are means±S.E. Numbers of mice used are 4 (APAP) and 3 (other groups). $*p<0.05$ *vs.* control; $\dagger p<0.05$ *vs.* APAP alone.

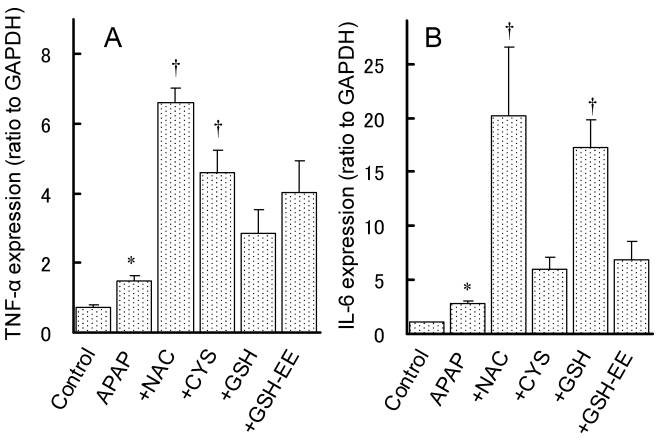

Fig. 4. Hepatic TNF-α and IL-6 Expression in APAP-Treated Mice and Additional Effects of Thiol Compounds

Mice were administered APAP 300 mg/kg alone or with various thiol compounds, and were sacrificed 3 h after administration. Hepatic mRNA expression of TNF-α (A) and IL-6 (B) are presented as ratios to GAPDH. Results are means±S.E. Numbers of mice used are 4 (APAP) and 3 (other groups). $*p<0.05$ *vs.* control; $\dagger p<0.05$ *vs.* APAP alone.

against hepatic GSH loss by APAP treatment, which is consistent with previous observations that exogenous GSH is less effective in restoring hepatic GSH than GSH-EE.[10]

Altered Ratio of Hepatic Cytokine Expression Following Administration of GSH Liver TNF-α and IL-6 mRNA expression in mice administered APAP with or without a thiol compound was measured by real-time RT-PCR. TNF-α is a typical proinflammatory cytokine, and some studies suggest its involvement in the progression of APAP hepatotoxicity.[14,15] On the other hand, IL-6 is a pleiotropic cytokine, which may behave as an anti-inflammatory cytokine and protect mice against APAP hepatotoxicity.[16] Hepatic mRNA expression of these two cytokines was measured 3 h after APAP administration (Fig. 4). Both TNF-α and IL-6 were modestly but significantly induced by administration of APAP alone. Coadministration of every thiol with APAP potentiated the induction of hepatic TNF-α and IL-6. Notably,

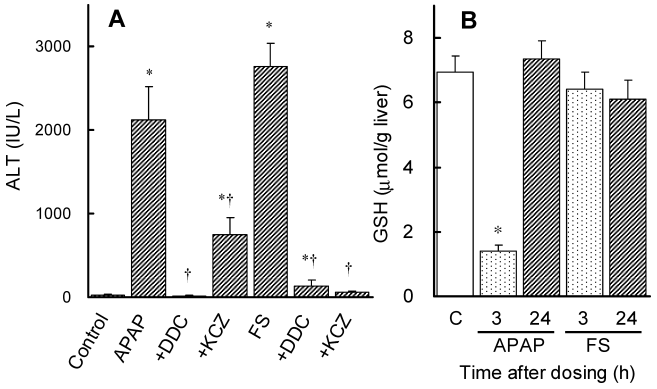

Fig. 5. Roles of Reactive Metabolites in APAP- and FS-Induced Liver Injury

Mice were administered APAP 300 mg/kg alone or FS 400 mg/kg alone, or with diethyldithiocarbamate (+DDC) or ketoconazole (+KCZ), and were sacrificed 3 or 24 h after administration. (A) Serum ALT 24 h after administration. Results are means±S.E. Numbers of mice used are 5 (APAP and FS) and 3 (other groups). $*p<0.05$ *vs.* control; $\dagger p<0.05$ *vs.* no inhibitor. (B) Liver GSH levels 3 or 24 h after administration. Results are means±S.E. of 3 mice. $*p<0.05$ *vs.* control (C).

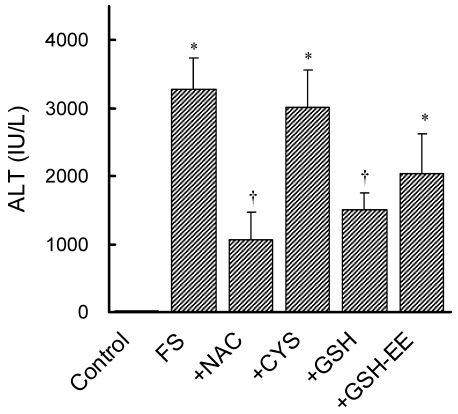

Fig. 6. Protection against FS-Induced Liver Injury by Thiol Compounds

Mice were administered FS alone or with various thiol derivatives, and were sacrificed 24 h after administration for assay of serum ALT. Results are means±S.E. Numbers of mice used are 6 (FS) and 3 (other groups). $*p<0.05$ *vs.* control; $\dagger p<0.05$ *vs.* FS alone.

coadministration of NAC and GSH markedly potentiated the induction of hepatic IL-6. Accordingly, a ratio of TNF-α to IL-6, a postulated proinflammatory/anti-inflammatory index, was decreased in those mice administered APAP with GSH (0.166) or NAC (0.327), compared with APAP administration alone (0.512). None of the thiol compounds affected the hepatic expressions of TNF-α or IL-6 in the mice not given APAP (data not shown).

Protective Effects of NAC and GSH against FS Hepatotoxicity FS, a loop diuretic, is converted into a reactive metabolite and causes hepatic necrosis in mice as well as APAP.[17] In the present study, FS at a dose of 400 mg/kg was subjected to induce hepatotoxicity. Diethyldithiocarbamate or ketoconazole, inhibitors of mouse CYP2E1[18] and CYP3A11,[19] respectively, protected mice against FS- as well as APAP-induced hepatotoxicity (Fig. 5A). Despite a potential role of the P450-mediated reactive metabolite in FS hepatotoxicity, hepatic GSH was unchanged in those mice treated with FS (Fig. 5B), which agrees with previous observations.[20,21] We examined the protective effects of thiol compounds on FS hepatotoxicity, which does not require hepatic

GSH depletion prior to the development of hepatotoxicity. Among the four thiols, NAC and GSH alleviated FS hepatotoxicity, whereas no significant effect of GSH-EE or CYS was observed (Fig. 6). This also supports the notion that hepatoprotection by NAC and GSH could be mediated by a mechanism other than GSH supplementation.

DISCUSSION

In the present study, coadministration of a relatively low dose (1.5 mmol/kg) of NAC, CYS, GSH, and GSH-EE effectively protected mice against APAP-induced liver injury. By contrast, their effects on an APAP-induced early decrease in hepatic GSH were distinctly different from each other. Only GSH-EE completely prevented APAP-induced early hepatic GSH depletion. CYS was also effective in preventing an APAP-induced decrease in hepatic GSH, while NAC showed a partial GSH restoration. Unlike the other thiols, exogenous GSH had no effect on hepatic GSH loss in APAP-treated mice. Therefore, the protective action of GSH-EE and CYS can be mainly explained by GSH replenishment. NAC exerts its effects not only *via* GSH replenishment but also other mechanisms. Exogenous GSH could protect mice against APAP hepatotoxicity without replenishing hepatic GSH levels.

The major mechanism underlying protection against APAP hepatotoxicity by NAC is thought to provide CYS as a precursor of GSH, which traps NAPQI.[5] However, treatment of mice with NAC 1—2 h after APAP protected against hepatic necrosis without preventing covalent binding, suggesting that mechanisms other than GSH replenishment are involved in the hepatoprotective effects of NAC.[7—9] These include inhibiting the production of reactive nitrogen species and/or proinflammatory cytokines,[7] which are pathogenic events in APAP hepatotoxicity.[14,15] In the present study, coadministration of NAC with APAP highly potentiated the early induction of hepatic TNF-α and IL-6; the latter was more pronounced, leading to a decreased ratio of TNF-α/IL-6, an index of the Th1/Th2 cytokine balance. Disruption of this balance is responsible for the pathogenesis of APAP hepatotoxicity,[13] and IL-6 is one of the protective factors against it,[16] Therefore, NAC could exert its hepatoprotective effects by increasing IL-6 expression and improving the Th1/Th2 response. The induction of cytokines, including IL-6, in APAP-treated mice and their suppression by NAC at relatively later time points might be due to the response to hepatotoxicity and its attenuation by NAC.[7]

Intravenous injections of GSH have been reported to protect mice against APAP hepatotoxicity by restoring hepatic GSH levels.[11] The mechanisms underlying exogenous GSH are explained as follows: GSH is rapidly degraded after intravenous administration, and is taken up by the liver as amino acids where it is regenerated in hepatocytes. On the other hand, the present study shows the lack of restoration of hepatic GSH after intraperitoneal administration of GSH. This may be due to differences in the route of administration, because the absence of any effect following the intraperitoneal injection of GSH on hepatic GSH levels is consistent with a previous report, which suggested that GSH could not be taken up by hepatocytes.[10] It is thus suggested that GSH acts from outside of the hepatocyte membrane, and/or GSH-induced hepatoprotection is mediated by other endogenous components. GSH administration induced IL-6, and this selectivity (ratio to TNF-α) is more pronounced than NAC administration. Therefore, GSH could exert its effects mainly *via* an increased production of the anti-inflammatory cytokine. The present results provide novel insight into the hepatoprotection mechanisms of thiol compounds, particularly GSH, which are independent of supplementation of hepatic GSH.

To verify further these findings, we next evaluated effects of the thiol compounds on a non-APAP model of hepatotoxicity, which is not accompanied by depletion of hepatic GSH. FS is a loop diuretic frequently used in the treatment of cardiovascular and renal diseases. It was reported to be converted into a reactive metabolite and shown to cause hepatic necrosis as well as APAP in mice.[17] In the present study, we confirmed that hepatotoxicity requires metabolic activation, possibly by CYP2E1 and CYP3A11, because of its protection by diethyldithiocarbamate and ketoconazole. Unlike APAP, FS hepatotoxicity did not accompany changes in hepatic GSH levels as previously observed.[20,21] Interestingly, only NAC and GSH, both of which could protect mice against APAP hepatotoxicity *via* GSH-independent mechanisms as described above, attenuated FS hepatotoxicity. Thus, the FS model supports the idea that a protective mechanism of NAC and GSH involves factors other than GSH replenishment, such as cytokine modulation.

In summary, exogenous thiols alleviate APAP-induced hepatotoxicity, some of which mainly mediate GSH replenishment, and others of which mediate without increasing hepatic GSH, probably through cytokine-mediated and anti-inflammatory mechanisms. A simple and reasonable theory classifying APAP-induced liver injury has been proposed to involve two stages[22]: stage I includes metabolic activation, covalent binding, and mitochondrial dysfunction, while stage II involves subsequent processes of adaptation or failure of the response in modifying essential cellular processes, which may include cytokine modulation. The latter mechanism could provide the rationale for late administration of NAC as an antidote of APA hepatotoxicity. Furthermore, because NAC is associated with various adverse effects,[23] GSH could also be considered as an antidote for APAP intoxication.

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REFERENCES

1) Mitchell J. R., Jollow D. J., Potter W. Z., Davis D. C., Gillette J. R., Brodie B. B., *J. Pharmacol. Exp. Ther.*, **187**, 185—194 (1973).
2) Jollow D. J., Mitchell J. R., Potter W. Z., Davis D. C., Gillette J. R., Brodie B. B., *J. Pharmacol. Exp. Ther.*, **187**, 195—202 (1973).
3) Dahlin D. C., Miwa G. T., Lu A. Y., Nelson S. D., *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 1327—1331 (1984).
4) Prescott L. F., Park J., Ballantyne A., Adriaenssens P., Proudfoot A. T., *Lancet*, **2**, 432—434 (1977).
5) Corcoran G. B., Wong B. K., *J. Pharmacol. Exp. Ther.*, **238**, 54—61 (1986).
6) Harrison P. M., Keays R., Bray G. P., Alexander G. J., Williams R., *Lancet*, **335**, 1572—1573 (1990).
7) James L. P., McCullough S. S., Lamps L. W., Hinson J. A., *Toxicol. Sci.*, **75**, 458—467 (2003).

8) Salminen W. F. Jr., Voellmy R., Roberts S. M., *J. Pharmacol. Exp. Ther.*, **286**, 519—524 (1998).
9) Dambach D. M., Durham S. K., Laskin J. D., Laskin D. L., *Toxicol. Appl. Pharmacol.*, **211**, 157—165 (2006).
10) Puri R. N., Meister A., *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 5258—5260 (1983).
11) Knight T. R., Ho Y. S., Farhood A., Jaeschke H., *J. Pharmacol. Exp. Ther.*, **303**, 468—475 (2002).
12) Anderson M. E., *Methods Enzymol.*, **113**, 548—555 (1985).
13) Masubuchi Y., Sugiyama S., Horie T., *Chem. Biol. Interact.*, **179**, 273—279 (2009).
14) Blazka M. E., Wilmer J. L., Holladay S. D., Wilson R. E., Luster M. I., *Toxicol. Appl. Pharmacol.*, **133**, 43—52 (1995).
15) Ishida Y., Kondo T., Tsuneyama K., Lu P., Takayasu T., Mukaida N., *J. Leukoc. Biol.*, **75**, 59—67 (2004).
16) Masubuchi Y., Bourdi M., Reilly T. P., Graf M. L., George J. W., Pohl L. R., *Biochem. Biophys. Res. Commun.*, **304**, 207—212 (2003).
17) Mitchell J. R., Nelson W. L., Potter W. Z., Sasame H. A., Jollow D. J., *J. Pharmacol. Exp. Ther.*, **199**, 41—52 (1976).
18) Carlson G., *J. Toxicol. Environ. Health A*, **67**, 905—909 (2004).
19) Martignoni M., Groothuis G., de Kanter R., *Drug Metab. Dispos.*, **34**, 1047—1054 (2006).
20) Wong S. G., Card J. W., Racz W. J., *Toxicol. Lett.*, **116**, 171—181 (2000).
21) Randle L. E., Goldring C. E., Benson C. A., Metcalfe P. N., Kitteringham N. R., Park B. K., Williams D. P., *Toxicology*, **243**, 249—260 (2008).
22) Bessems J. G., Vermeulen N. P., *Crit. Rev. Toxicol.*, **31**, 55—138 (2001).
23) Kao L. W., Kirk M. A., Furbee R. B., Mehta N. H., Skinner J. R., Brizendine E. J., *Ann. Emerg. Med.*, **42**, 741—750 (2003).