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Role of enzymatic N-hydroxylation and reduction in flutamide metabolite-induced liver toxicity

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Running title: Role of reduction of FLU-1 N-OH in flutamide liver injury

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Non-Standard Abbreviations:

ALT, alanine aminotransferase; FLU-1, 5-amino-2-nitrobenzotrifluoride; FLU-1 N-OH,
N-hydroxy-5-amino-2-nitrobenzotrifluoride; FLU-3,
5-amino-2-nitro-4-hydroxybenzotrifluoride; GSH, glutathione; GSTs, glutathione
S-transferases; CDNB, 1-chloro-2,4-dinitrobenzene; TCPOBOP,
1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; HPLC, high performance liquid
chromatography; β -NF, β -naphthoflavone; PCN, pregnenolone-16 α -carbonitrile; BSA,
bovine serum albumin; KLH, keyhole limpet hemocyanin.

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Abstract

Flutamide is used for prostate cancer therapy, but occasionally induces severe liver injury. Flutamide is hydrolyzed in the body into 5-amino-2-nitrobenzotrifluoride (FLU-1) and then further oxidized. In our previous study, N-hydroxy FLU-1 (FLU-1 N-OH) was detected in the urine of patients, and exhibited cytotoxicity in rat primary hepatocytes. In the present study, we have assessed the roles of FLU-1 N-oxidation and hepatic glutathione (GSH) depletion in liver injury. FLU-1 (200 mg/kg, p.o.) was administered to C57BL/6 mice for five days together with TCPOBOP (3 mg/kg, i.p.) for the first three days. Mice were fasted for the last two days to deplete hepatic GSH. Administration of FLU-1 alone didn't affect serum alanine aminotransferase activities (ALT), while coadministration of FLU-1 and TCPOBOP significantly increased ALT in fasted mice, but not in non-fasted mice. Microsomal FLU-1 N-hydroxylation was enhanced about five-times by TCPOBOP treatment. Flutamide metabolite-protein adducts were detected in liver microsomes incubated with FLU-1 N-OH, but not with FLU-1 and flutamide, by immunoblotting using anti-flutamide antiserum. In the presence of mouse liver cytosol, FLU-1 N-OH was reduced back into FLU-1. This enzymatic reduction required NAD(P)H as a cofactor. The reduction was enhanced by the coexistence of NAD(P)H and GSH while it was markedly inhibited by allopurinol (20 μ M). By using purified bovine xanthine oxidase, the reduction was observed in the presence of NAD(P)H. These results suggest that FLU-1 N-OH is involved in flutamide-induced hepatotoxicity and that cytosolic reduction of FLU-1 N-OH plays a major role in protection against flutamide-induced hepatotoxicity.

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Introduction

Flutamide is a non-steroidal antiandrogen that has been used for prostate cancer therapy. Flutamide is reported to induce severe liver dysfunction in patients, although hepatotoxicity was not recognized in preclinical studies using experimental animals. About 3 per 10,000 users of flutamide were estimated to develop severe liver injury (Wysowski and Fourcroy, 1996). In most cases, flutamide caused liver dysfunction after a latency period (approximately 16 weeks). This flutamide-induced liver injury is not acute but delayed (Thole et al., 2004; Manso et al., 2006). Although reactive metabolites of flutamide have been considered to be involved in hepatotoxicity induction (Berson et al., 1993; Fau et al., 1994; Matsuzaki et al., 2006; Tevell et al., 2006; Kang et al., 2007), the mechanism of flutamide-induced hepatotoxicity in humans remains obscure.

Flutamide is rapidly absorbed from the gastrointestinal tract after oral administration and metabolized by hepatic CYP1A2 into 2-hydroxyflutamide (OH-flutamide) (Schulz et al., 1988; Shet et al., 1997; Aizawa et al., 2003; Takashima et al., 2003) (Fig.1). Flutamide and OH-flutamide are partially hydrolyzed into an arylamine metabolite, 5-amino-2-nitrobenzotrifluoride (FLU-1) (Schulz et al., 1988; Goda et al., 2006). Plasma concentration of FLU-1 was considerably higher in patients who had exhibited liver dysfunction and lower CYP1A2 activity in patients was found to be associated with onset of liver injury (Ozono et al., 2002; Aizawa et al., 2003). Additionally, *Cyp1a2*-null mice that mainly produced FLU-1 displayed hepatotoxicity by continuous administration of flutamide in contrast to the metabolites formed by wild type mice (Matsuzaki et al., 2006). These findings suggest that FLU-1 may possibly mediate flutamide-induced liver injury. Although 5-amino-2-nitro-4-hydroxybenzotrifluoride (FLU-3) is the main metabolite in the urine of patients (Goda et al., 2006), FLU-3 did

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not exhibit obvious cytotoxicity. The other metabolite, FLU-1 N-OH, is considered to be involved in flutamide hepatotoxicity.

Arylamines are metabolically activated by CYP-mediated N-hydroxylation. Electrophilic N-hydroxylamine is a generally unstable and highly reactive intermediate that reacts with intracellular molecules such as proteins, lipids and DNA to induce various types of toxicity including hepatotoxicity (Kato and Yamazoe, 1994; Ju and Uetrecht, 2002; Turesky, 2002; Uetrecht, 2002). In our previous study, the N-hydroxy metabolite of FLU-1 (FLU-1 N-OH) was detected in urine of patients taking flutamide as FLU-1 N-OH glucuronide (Goda et al., 2006). Metabolism of FLU-1 into FLU-1 N-OH was catalyzed mainly by CYP3A4 in humans (Goda et al., 2006). FLU-1 N-OH showed a potent cytotoxicity in rat primary hepatocyte (D. Nagai, R. Goda, E. Ichimura, A. Akiyama, C. Nishimura, K. Nishikawa, M. Miyata, and Y. Yamazoe, manuscript submitted for publication). However, FLU-1 N-OH was quite stable. Amount of FLU-1 N-OH (free plus conjugates) in urine did not differ between patients with and without hepatic dysfunction (Goda et al., 2006). The role of FLU-1 N-OH in liver injury has not been fully identified.

Glutathione (GSH) is a ubiquitous tripeptide and biosynthesized from cysteine, glycine and glutamate in mammalian tissues (Meister and Anderson, 1983). GSH is particularly present at a high concentration in liver and participates in cellular protection by scavenging radicals and reacting with electrophiles (Wu et al., 2004). Depletion of GSH is known to increase sensitivity to hepatotoxic compounds (Yang et al., 2002; Watanabe et al., 2003; Akai et al., 2007; McConnachie et al., 2007). Nucleophilic attack of GSH to electrophiles is catalyzed by glutathione S-transferases (GSTs) (Hayes et al., 2005). The role of GSH in flutamide-induced liver injury has not been sufficiently identified.

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In the present study, we have demonstrated the involvement of N-hydroxy metabolite and protective role of cytosolic reduction of the metabolite in flutamide-induced liver injury using a mouse model.

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Materials and Methods

Materials.

FLU-1 N-OH (>95%) and anti-flutamide antiserum were provided by Nippon Kayaku Co., Ltd (Tokyo, Japan). 5-Amino-2-nitrobenzotrifluoride (FLU-1), glutathione (reduced form) (GSH), glutathione (oxidized form) (GSSG) and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Flutamide, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), β -naphthoflavone (β -NF), pregnenolone-16 α -carbonitrile (PCN), 2,4-dinitrofluorobenzene, allopurinol, pyrazole, dicumarol (3,3'-methylene-bis(4-hydroxycoumarin)), human glutathione S-transferase (G8642), equine glutathione S-transferase (G6511), rabbit glutathione S-transferase (G8261), xanthine oxidase from bovine milk (X4500) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Japan K. K. (Tokyo Japan). Umbelliferone was purchased from Nacalai Tesque (Kyoto, Japan). Cellulose membrane was purchased from Viskase Com., Inc. (Darien, IL).

Animals and treatments.

C57BL/6 Cr Slc male mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan) and housed under standard 12 hr light/12 hr dark cycle with access to a standard rodent diet CE-2 (Clea, Tokyo, Japan) and water *ad libitum*. Age-matched groups of 9- to 10-week-old mice were used in all experiments.

Flutamide (200 mg/kg) or FLU-1 (200 mg/kg) was suspended in 0.5% carboxymethylcellulose solution and administered orally to mice for five days. These are toxicological doses. TCPOBOP (3 mg/kg) was suspended in corn oil and administered intraperitoneally for the first three days. Mice were fasted for the last two

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days to deplete hepatic GSH and were euthanized 4 hr after the last administration. The experimental protocol was shown as Fig. 2. Animal experiments were performed under the instruction of Tohoku University Animal Care and Use Committee.

ALT activities in serum.

Blood was obtained from inferior vena cava of mice. Serum was prepared from blood by centrifugation at $20,400 \times g$ for 20 min. Serum alanine aminotransferase (ALT) activities were measured using a commercial kit, Transaminase CII-B-test Wako (Wako Pure Chemicals, Osaka, Japan).

Preparation of mouse liver microsomes and cytosols.

Mouse livers were homogenized in 75 mM potassium-phosphate buffer (pH 7.4) containing 75 mM KCl and centrifuged at $9,000 \times g$ for 20 min at 4°C . The supernatant was centrifuged at $105,000 \times g$ for 60 min at 4°C . For microsome preparation, the final pellet was washed with 75 mM potassium-phosphate buffer (pH 7.4) containing 75 mM KCl twice and suspended in 75 mM potassium-phosphate buffer (pH 7.4) containing 20% glycerol. Cytosolic fraction was obtained from $105,000 \times g$ centrifugation during microsome preparation. Protein concentration was determined by the method of Lowry *et al.* with bovine serum albumin (BSA) as the standard (Lowry *et al.*, 1951).

Metabolism of FLU-1 in mouse liver microsomes.

The reaction mixture consisted of 20 μg microsomal protein, 200 μM FLU-1, 0.1 M potassium-phosphate buffer (pH 7.4), 4.8 mM MgCl_2 , 0.32 mM NADP^+ , 2.4 mM glucose 6-phosphate and 0.26 units/ml glucose-6-phosphate dehydrogenase in a final volume of 0.2 ml. The mixture was incubated for 10 min at 37°C . The reaction was

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terminated by addition of 0.4 ml ice-cold methanol containing 100 μ M ascorbic acid and 2 nmol of umbelliferone was added to the mixture as an internal standard. The mixture was centrifuged at $3,000 \times g$ for 5 min and the supernatant was analyzed by high performance liquid chromatography (HPLC) as described below.

HPLC conditions.

HPLC analysis was performed with Jasco intelligent model PU-980 pump (Jasco, Tokyo, Japan) and UV-970 detector (Jasco, Tokyo, Japan). Metabolites were separated by ODS-80 T_M column (4.6×150 mm, 5 μ m, TOSOH, Tokyo, Japan) and monitored by UV detection at 366 nm at room temperature. Flow rate was 0.75 ml/min with mobile phase of ammonium-acetate buffer (25 mM, pH 6.5) and methanol (68:32).

Detection of Cyp3a or Cyp1a in mouse liver microsomes by immunoblot analysis.

Microsomal proteins boiled for two min were separated by SDS-PAGE with 8% acrylamide gel and transferred to nitrocellulose membrane. The membrane was incubated for two hr in blocking solution consisting of PBS, Tween 20 [0.05% (v/v)] and fetal calf serum [20% (v/v)]. The membrane was then treated with anti-rat CYP3A2 antibody at 1:1000 dilution or anti-rat CYP1A1 antibody at 1:10000 dilution in PBS-Tween for two hr at room temperature. Subsequently, the membrane was treated with alkaline phosphatase conjugated goat anti-rabbit IgG at 1:3000 dilution in PBS-Tween for two hr at room temperature. Thereafter, the membrane was washed five times in PBS-Tween and antibody reactivity was visualized using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) staining. The stained membranes were scanned with an Epson GT-8700 scanner, and the band intensities were measured using NIH image (version 1.59) software (Bethesda, MD).

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Interaction between flutamide and its metabolites with BSA.

The reaction mixture consisted of 10 μ M FLU-1 or FLU-1 N-OH, 0-1 mg/ml BSA and 0.1 M potassium-phosphate buffer (pH 7.4) in a final volume of 0.1 ml. Incubation was carried out for the indicated period at 37°C. To remove the metabolite binding to BSA, 200 μ l of ice-cold methanol was added and the reaction mixture was centrifuged at $3,000 \times g$ for 5 min. Unbound form of metabolite in supernatant was measured by HPLC.

Detection of flutamide-protein adducts by immunoblot analysis.

Detection of flutamide-protein adducts was carried out as described previously with some modification (D. Nagai et al., manuscript submitted for publication). Briefly, FLU-1 N-OH, FLU-1, fltuamide or OH-flutamide (500 μ M) was incubated with microsomal protein (2 mg/ml) for two hr at 37°C. Microsomal proteins (50 μ g) boiled for two min were separated by SDS-PAGE with 10% acrylamide gel and transferred to nitrocellulose membrane. The membrane was incubated for two hr in blocking solution as described above. The membrane was treated with anti-flutamide antiserum at 1:3000 dilution in 0.1% BSA/PBS-Tween for 90 min at room temperature. Subsequently, the membrane was treated with alkaline phosphatase conjugated goat anti-rabbit IgG for 60 min. Thereafter, the membrane was washed five times in PBS-Tween and antibody reactivity was visualized using BCIP/NBT staining.

Metabolism of FLU-1 N-OH.

The reaction mixture consisted of 0.1 M potassium-phosphate buffer (pH 7.4), 4.8 mM MgCl₂, 50 μ M FLU-1 N-OH and 20 μ g cytosolic protein or 1.1 μ g (1.5 mU)

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purified xanthine oxidase protein with and without 10 mM NAD(P)H or 10 mM GSH in a final volume of 0.2 ml. The mixture was incubated for the indicated period at 37°C. For inhibition study, CDNB (50 μ M and 1.5 mM), allopurinol (20 μ M), pyrazole (5 mM) and dicumarol (15 μ M) were added three min prior to the start of incubation. The reaction was terminated by addition of 0.4 ml ice-cold methanol containing 100 μ M ascorbic acid and 2 nmol of umbelliferone was added to the mixture as an internal standard. The mixture was then centrifuged at 3,000 \times g for 5 min and the supernatant was analyzed by HPLC. For the preparation of dialyzed cytosolic protein, cytosolic fraction was dialyzed three times using cellulose membrane (UC20-32) with 0.1 M potassium-phosphate buffer (pH 7.4) containing 4.8 mM MgCl₂ for 2 hours at 4°C.

Measurement of hepatic GSH level and GSH/GSSG level in reaction mixture.

Liver homogenates were diluted with 10% trichloroacetic acid (1:4) and centrifuged at 9,000 \times g for 5 min to obtain non-protein supernatants. GSH levels were determined by the method of Tietze *et al* (Tietze, 1969). GSH/GSSG levels in reaction mixture were measured by HPLC after the amino groups of GSH/GSSG were derivatized with 2,4-dinitrofluorobenzene (Reed *et al.*, 1980).

Statistical analysis.

Data are shown as the mean \pm S.D. Statistical differences were determined by either an unpaired Student's t test or one-way ANOVA followed by Tukey's multiple comparison with PRISM 4.0 software (GraphPad, san Diego). $P < 0.05$ was considered to be statistically significant.

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Results

Animal experiment with flutamide and FLU-1.

Fasting decreased hepatic GSH levels about 60% in C57BL/6 male mice, but did not affect ALT (data not shown). TCPOBOP, a drug metabolizing enzyme inducer, was reported to aggravate liver injury induced by acetaminophen or cocaine through CYPs-mediated bioactivation (Wei et al., 2000; Zhang et al., 2002). Administration of TCPOBOP or FLU-1 alone did not increase ALT, but coadministration of FLU-1 and TCPOBOP significantly increased ALT compared to vehicle control (Fig. 3). Coadministration of flutamide and TCPOBOP was also attempted, but did not increase ALT compared to vehicle control (data not shown).

Influence of TCPOBOP on microsomal FLU-1 N-hydroxylation.

Formation of FLU-1 N-OH was tested in mouse liver microsomes by HPLC. FLU-1 N-OH and FLU-3 were detected as microsomal metabolites of FLU-1 (Fig. 4A). Administration of TCPOBOP (3 mg/kg for three days) to mice increased about five-fold microsomal FLU-1 N-hydroxylation compared to vehicle treatment (Fig. 4B). FLU-1 administration (200 mg/kg for five days) also increased FLU-1 N-hydroxylation, but less than the TCPOBOP administration. Administration of TCPOBOP significantly increased hepatic expression of Cyp1a and Cyp3a in liver microsomes (Fig. 4C). FLU-1 also increased Cyp1a expression. Treatment with rodent Cyp1a and Cyp3a inducer, β -naphthoflavone (β -NF) and pregnenolone-16 α -carbonitrile (PCN) respectively, also increased hepatic FLU-1 N-hydroxylation (data not shown).

Covalent binding of FLU-1 N-OH to mouse liver microsomes.

To investigate the ability of FLU-1 N-OH to interact with protein, FLU-1 N-OH was

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incubated with BSA. Amount of FLU-1 N-OH in supernatant was decreased with incubation time and BSA concentration (Fig. 5A). Although FLU-1 N-OH in supernatant was not altered within 24-hr incubation in the absence of BSA, only about 10% of FLU-1 N-OH was recovered after 24-hr incubation with 1 mg/ml BSA. Similar results were obtained from the experiment in which heat-treated microsomes were used instead of BSA (data not shown). In contrast, FLU-1 was completely recovered regardless of incubation time and BSA concentration (Fig. 5B). Flutamide and OH-flutamide also did not show any interaction with BSA as well as FLU-1 (data not shown).

Furthermore, flutamide metabolite-protein adducts were detected by immunoblot analyses using a rabbit polyclonal antisera raised against a synthetic flutamide-KLH (keyhole limpet hemocyanin) conjugate. Hapten ({N-[4-nitro-3-(trifluoromethyl)phenyl]succinic acid}) was conjugated with keyhole limpet hemocyanin. The antiserum was developed by immunization of the hapten keyhole limpet hemocyanin-conjugate. Several protein adducts were detected by the antisera between 37-75 kDa after incubation of microsomal protein with FLU-1 N-OH, but not with FLU-1, flutamide and OH-flutamide (Fig. 6). The adduct formation was prevented by addition of GSH in the reaction mixture (Nagai et al, manuscript submitted for publication).

Protective role of hepatic GSH in liver injury.

To determine the protective role of hepatic GSH against onset of liver injury, FLU-1 and TCPOBOP were coadministered to fasted or non-fasted mice. Coadministration of FLU-1 and TCPOBOP significantly increased ALT in fasted mice compared to non-fasted mice (Fig. 7). Furthermore, hepatic GSH levels were not changed by

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administration of TCPOBOP or FLU-1 alone in fasted mice, but hepatic GSH levels were significantly decreased about 50% in mice treated with both FLU-1 and TCPOBOP compared to FLU-1-treated mice (Fig. 8A) and an inverse relationship ($r = 0.71$; $P < 0.001$) was observed between hepatic GSH levels and serum ALT activities in those groups (Fig. 8B). These results suggest the protective role of hepatic GSH in flutamide-induced liver injury.

Enzymatic reduction of FLU-1 N-OH with GSH and NAD(P)H

When FLU-1 N-OH was incubated with hepatic cytosol, FLU-1 was detected as a metabolite in a time-dependent manner (Fig. 9A). Reduction of FLU-1 N-OH to FLU-1 accounted for approximately 40-60% loss of FLU-1 N-OH. The relative rates of FLU-1 formation to the disappearance of FLU-1 N-OH were decreased in a time-dependent manner. To study the influence of the presence of cofactors in the formation of FLU-1, FLU-1 N-OH was incubated with NADH or GSH in the presence of mouse liver cytosol. The formation of FLU-1 was not observed in a system of FLU-1 N-OH incubation with GSH or NADH alone (Fig. 9B, C) or with GSH or NADH and heat-treated cytosols (data not shown). It was, however, enhanced by addition of GSH or NADH and cytosols. Amount of FLU-1 N-OH did not change in the incubation with GSH or NADH alone.

To further verify the electron donor for the formation of FLU-1, dialyzed cytosols were used to measure FLU-1 formation. This FLU-1 formation using dialyzed cytosols was reduced to 7% of native cytosols. FLU-1 formation was increased up to 6.9-fold and 8.3-fold by addition of NADPH (10 mM) or NADH (10 mM), respectively, to the reaction mixture containing the dialyzed cytosols (Fig. 10). Although addition of GSH (10 mM) alone did not increase such formation in the dialyzed cytosols, the addition of both GSH and NADH increased the formation, compared to that of NADH alone.

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To establish which enzyme was involved in the formation of FLU-1, representative inhibitors of NAD(P)H-dependent cytosolic enzymes were added to the reaction mixture. The formation was strongly inhibited by addition of allopurinol (25 μ M), a potent inhibitor of xanthine or aldehyde oxidase while it was only weakly (less than 11%) inhibited by addition of pyrazole (5 mM), a potent inhibitor of alcohol dehydrogenase (Table 1). CDNB (1-chloro-2,4-dinitrobenzene), a glutathione S transferases (GSTs) substrate, inhibited FLU-1 formation. The formation was inhibited about 30% by addition of CDNB (50 μ M).

To test for the involvement of xanthine oxidase or glutathione S-transferase (GST) in FLU-1 N-OH reduction, purified xanthine oxidase from bovine, GST proteins from human placenta (GSTM1 and GSTP1), equine liver and rabbit liver were used. By using purified bovine xanthine oxidase, the FLU-1 formation was observed in the presence of NADH (37.7 nmol/mg protein/min). This specific activity was 61-fold higher than that of cytosolic protein from mouse liver. The NADPH-dependent FLU-1 formation was 4-fold lower than that of NADH. The FLU-1 formation was not observed in the absence of NAD(P)H. By using any of GST proteins, FLU-1 formation from FLU-1 N-OH was not found. Furthermore, GSH consumption and GSSG formation during FLU-1 formation were not observed in the reaction mixture containing cytosol and FLU-1 N-OH.

The rate of FLU-1 N-hydroxylation per g liver was enhanced by TCPOBOP or FLU-1 administration (Table 2). On the other hand, the rate of FLU-1 N-OH reduction per g liver showed similar values (about 40 μ mol/g liver/min) among all groups.

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Discussion

The present study using a mouse model has suggested that N-hydroxy flutamide metabolite, FLU-1 N-OH, is involved in flutamide-induced liver injury. Furthermore, we demonstrate that FLU-1 N-OH is reduced to FLU-1 in the presence of cytosol and NAD(P)H. Coexistence of GSH increased the rate of the reaction.

Coadministration of FLU-1 and TCPOBOP elevated ALT in fasted mice (Fig. 3). On the other hand, FLU-1 administration alone or coadministration of flutamide and TCPOBOP did not increase ALT in fasted mice. These results suggest involvement of reactive intermediates from FLU-1, whose production is enhanced by treatment with TCPOBOP, in flutamide hepatotoxicity.

Formation of FLU-1 N-OH was detected in mouse liver microsomes (Fig. 4A) as well as previously reported in humans (Goda et al., 2006). Formation of FLU-1 N-OH by liver microsomes was enhanced approximately five-fold after TCPOBOP treatment (Fig. 4B). Flutamide metabolite-protein adducts were detected by immunoblot analyses using anti-flutamide antibody after incubation of microsomal protein with FLU-1 N-OH (Fig. 6). In contrast FLU-1 itself had little bonding to microsomal proteins and did not interact with BSA in a manner similar to that observed with flutamide and OH-flutamide. It is proposed that FLU-1 N-hydroxylation causes intracellular protein modification through the covalent binding to cause the liver dysfunction. Addition to FLU-1 N-OH, nitroso and quinone imine derivatives of FLU-1 N-OH are possible to react with proteins. Further study is required to understand the reaction between FLU-1 N-OH and macromolecules.

Treatment with rodent Cyp3a or Cyp1a inducer, PCN or β -NF respectively, also enhanced hepatic FLU-1 N-hydroxylation as well as TCPOBOP treatment (data not shown). Berson and Fau et al reported that flutamide-mediated cytotoxicity in rat

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hepatocytes and covalent binding to microsomal proteins were increased remarkably by preadministration of dexamethasone (an inducer of Cyp3a) and moderately by β -NF (Berson et al., 1993; Fau et al., 1994). These reports are consistent with our hypothesis that FLU-1 N-OH participates in liver injury through protein binding to intracellular components.

Coadministration of FLU-1 and TCPOBOP elevated ALT in fasted mice, but not non-fasted mice (Fig. 7), suggesting that partial depletion of hepatic GSH during fasting enhances liver dysfunction associated with FLU-1 administration. Moreover, hepatic GSH levels were significantly decreased in mice treated with both FLU-1 and TCPOBOP compared to FLU-1-treated mice (Fig. 8A). An inverse correlation was observed between hepatic GSH levels and serum ALT in those mice (Fig. 8B). In vitro experiments, adduct formation was prevented by addition of GSH in the reaction mixture (Nagai et al, manuscript submitted for publication). Coexistence of GSH enhanced NADH-dependent FLU-1 formation from FLU-1 N-OH (Fig.10). It has been reported that two GSH adducts were detected in human liver microsomal incubation of FLU-1 in the presence of NADPH and GSH (Kang et al., 2008). These results suggest the protective role of hepatic GSH in flutamide-induced liver injury. Age-related decline of intracellular GSH is reported (Hazelton and Lang, 1980; Hernanz et al., 2000), although the mechanism is not fully identified. Loss of GSH biosynthesis may be associated with decrease of GSH levels in elders (Nakata et al., 1996; Suh et al., 2004). It is speculated that these elder patients tend to exhibit flutamide-induced hepatotoxicity due to lower hepatic GSH levels.

Unlike other N-hydroxy arylamines, FLU-1 N-OH did not spontaneously decompose in water as detected by HPLC analysis. This chemical contains nitro and amino groups in the molecule, but is not mutagenic in the Ames test using *Salmonella typhimurium*

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TA98 (unpublished data). Bulky trifluoromethyl group ortho to nitro group causes perpendicular arrangement of nitro group to benzene ring. In addition to electron-withdrawing effect of nitro and trifluoromethyl group, these substitutions are likely to contribute to the stability of this N-hydroxylamine.

FLU-1 N-OH was enzymatically reduced into FLU-1 in the presence of NAD(P)H (Fig. 10). The cytosolic reduction was markedly inhibited by allopurinol. Furthermore, bovine xanthine oxidase catalyzed the reduction in an NAD(P)H-dependent manner. Thus, xanthine oxidase likely plays an important role in the cytosolic reduction of FLU-1 N-OH. Xanthine oxidase, in the presence of an adequate electron donor, can mediate the reduction of aromatic nitro compounds (Kitamura et al., 2006). In human, several studies on the possibility of variation in levels of xanthine oxidase have been reported (Grant et al., 1983; Kalow and Tang, 1991; Guerciolini et al., 1991). In Japanese population, eleven percent of subjects were determined to be putative poor metabolizers (Saruwatari et al., 2002). Further studies are necessary to understand the relation between xanthine oxidase polymorphism and susceptibility to flutamide-induced hepatotoxicity. Furthermore, it remains unclear how GSH enhances the cytosolic NAD(P)H-dependent reduction. The rate of FLU-1 N-OH reduction per g liver showed relatively high values among all groups (Table 2). FLU-1 N-OH is expected to accumulate in the body and impair hepatocyte unless removed by enzymatic detoxification pathway. FLU-3 formation from FLU-1 is a major metabolic pathway in humans and mice, compared to FLU-1 N-OH formation from FLU-1. Because FLU-3 is efficiently conjugated and removed from the body, FLU-1 formation from FLU-1 N-OH likely plays a crucial role in elimination of FLU-1 N-OH.

Fasted mice coadministered with FLU-1 and TCPOBOP showed slight increase of ALT (about 100 IU/L). The mouse model developed in the present study is considered

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to reproduce mild hepatotoxicity which is often observed in patients taking flutamide. Idiosyncratic hepatotoxicity induced by flutamide may require somewhat “second-hit” such as cytokine- and immune-mediated mechanism, followed by binding of reactive intermediate of flutamide to hepatocellular macromolecule.

In conclusion, we have investigated the hepatotoxicity of FLU-1 N-OH and the metabolism of FLU-1 and FLU-1N-OH. Proposed mechanism of flutamide-induced liver toxicity is shown as Fig. 11. Our *in vivo* study suggests that FLU-1 N-OH is involved in the onset of flutamide-induced liver injury. Binding of FLU-1 N-OH to intracellular proteins is proposed as one of the mechanisms by which flutamide induces hepatotoxicity. The present study demonstrates that FLU-1 N-OH is reduced into FLU-1 by cytosolic enzyme, xanthine oxidase in the presence of NAD(P)H. This reduction of FLU-1 N-OH is proposed as one of the important detoxification pathways of FLU-1 N-OH in flutamide-induced liver injury. The present study suggests that the balance between hepatic FLU-1 N-OH production and reduction is a critical factor for flutamide-induced liver injury.

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Footnotes

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Figure legends

Figure 1: Metabolic pathways of flutamide.

Figure 2: Experimental protocol to develop mouse model for flutamide-induced liver injury.

C57BL/6 male mice (10-week-old) were used. Flutamide or FLU-1 (200 mg/kg) was given p.o. for five days and TCPOBOP (3 mg/kg weight) was given i.p. for the first three days. Mice were fasted for the last two days to deplete hepatic GSH and were euthanized 4 hr after the last treatment and blood and livers were collected.

Figure 3: Influence of TCPOBOP administration on FLU-1-induced liver injury in fasted mice.

FLU-1 and TCPOBOP were coadministered to mice as shown in Fig. 2. Mice were fasted for the last two days. Blood was collected 4 hr after last administration. Data are shown as mean \pm S.D. (n = 5-6) and analyzed by Tukey's test. ***, $P < 0.001$. (Significant difference between the indicated groups).

Figure 4: Influence of TCPOBOP administration on FLU-1 N-hydroxylation and CYPs expression in mouse liver microsomes.

(A) HPLC chromatogram of FLU-1 metabolites in mice. FLU-1 (200 μ M) was incubated for 10 min at 37°C with microsomes (0.1 mg/ml) prepared from TCPOBOP-administered mouse livers. Reaction mixture was analyzed by HPLC ($\lambda = 366$ nm). I.S., Internal standard (Umbelliferone). (B) Rate of microsomal FLU-1 N-hydroxylation was determined by HPLC ($\lambda = 366$ nm). FLU-1 (200 μ M) was incubated with mouse liver microsomes (0.1 mg/ml) prepared from mice for 10 min at

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37°C. Data are shown as mean \pm S.D. (n = 5-6) and analyzed by Tukey's test. ***, $P < 0.001$. (Significant difference between the indicated groups). (C) Hepatic Cyp3a and Cyp1a expression levels were determined by immunoblot analysis. Microsomal proteins (5 μ g for Cyp3a and 20 μ g for Cyp1a) were separated by SDS-PAGE with 8% acrylamide gel. Anti-rat CYP3A2 antibody and anti-rat CYP1A1 antibody were used for determination of mouse Cyp3a and Cyp1a respectively. Data are shown as two representative samples. Relative expression levels are indicated.

Figure 5: Interaction between flutamide metabolites and BSA.

(A) FLU-1 N-OH or (B) FLU-1 (10 μ M) was incubated with BSA at 37°C for indicated period. To remove BSA, reaction mixture was centrifuged after methanol was added. FLU-1 N-OH or FLU 1 in supernatant was determined by HPLC. Data are shown as the mean \pm S.D. (n = 3).

Figure 6: Detection of flutamide metabolite-protein adducts using anti-flutamide antiserum.

FLU-1-N-OH, FLU-1, flutamide or OH-flutamide (500 μ M) was incubated with liver microsomal proteins (2 mg/ml) at 37°C for 2 hours. Microsomal proteins were separated by SDS-PAGE with 10% acrylamide gel. Drug-protein complexes were detected by immunoblot analysis with anti-flutamide antibody. Data are shown as two representative samples.

Figure 7: Influence of fasting on liver injury induced by FLU-1- and TCPOBOP-coadministration.

Coadministration of FLU-1 and TCPOBOP was carried out as shown in Fig. 1. FLU-1-

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and TCPOBOP-coadministered mice were fasted for two days (fasted group) or continued to be fed *ad libitum* (non-fasted group). Blood samples were collected 4 hr after euthanasia and serum ALT activities were determined. Data are shown as the mean \pm S.D. (n = 4-5). Data are analyzed by Student's *t* test . *, $P < 0.05$. (Significant difference between the groups).

Figure 8: Influence of FLU-1- and TCPOBOP-coadministration on hepatic GSH levels in fasted mice.

(A) FLU-1 and TCPOBOP were coadministered to mice as shown in Fig. 1. Mice were fasted for the last two days. Hepatic GSH levels were determined as reported by Tietze *et al* (Tietze, 1969). Data are shown as the mean \pm S.D. (n = 4-5) and analyzed by Tukey's test. **, $P < 0.01$; ***, $P < 0.001$. (Significant difference between the indicated groups). (B) Relationship between hepatic GSH levels and serum ALT activities in FLU-1-administered mice and FLU-1- and TCPOBOP-coadministered mice. Data obtained from two separate experiments are shown. □, FLU-1; ■, FLU-1 and TCPOBOP.

Figure 9: Reduction of FLU-1 N-OH to FLU-1.

(A-B) FLU-1-N-OH (50 μ M) was incubated with GSH (10 mM) and/or hepatic cytosolic protein (0.1 mg/ml) prepared from mouse livers of control group. Reaction mixture was analyzed by HPLC ($\lambda = 366$ nm). Formation of FLU-1 and residual FLU-1 N-OH were determined for indicated incubation period. (C) FLU-1-N-OH (50 μ M) was incubated with NADH (10 mM) and hepatic cytosolic protein (0.1 mg/ml) prepared from mouse livers of control group. Formation of FLU-1 was determined for indicated incubation period.

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Figure 10: Reconstitution of reductase activity

Cytosolic fraction was dialyzed three times with 0.1 M potassium-phosphate buffer (pH 7.4) containing 4.8 mM MgCl₂. FLU-1-N-OH (50 μM) and dialyzed cytosol ((0.1 mg/ml) were incubated with NAD(P)H (10 mM) and/or GSH (10 mM) for 10 min. Cont indicates cytosol alone.

Figure 11: Proposed mechanism of flutamide-induced liver injury.

FLU-1 hydrolyzed from flutamide or OH-flutamide is oxidized mainly by CYP3A and partially CYP1A into FLU-1 N-OH which possesses a capacity to bind to intracellular proteins. FLU-1 N-OH is detoxified by NAD(P)H-dependent reduction, but in the case that FLU-1 N-OH formation is increased and/or NAD(P)H-dependent FLU-1 N-OH reduction is decreased, FLU-1 N-OH escapes these detoxification pathways and binds to intracellular proteins to induce hepatotoxicity. GSH depletion likely suppresses FLU-1 N-OH reduction and also facilitates protein binding.

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Table1 Effect of inhibitors on cytosolic FLU-1 N-OH reduction.

FLU-1 N-OH reduction was determined by HPLC ($\lambda = 366$ nm). FLU-1 N-OH (50 μ M) was incubated in cytosol (0.1 mg/ml) at 37°C for 10 min. Inhibitors were added three min prior to start of incubation. Each value represents the mean of two independent experiments.

Addition	Concentration (μ M)	Inhibition (%)
CDNB	50	29.7
	1500	83.9
Allopurinol	20	80.1
Dicumarol	15	38.6
Pyrazole	5000	10.5

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Table 2. Comparison of FLU-1 N-hydroxylation with FLU-1 N-OH reduction in mouse livers. FLU-1 N-hydroxylation and FLU-1 N-OH reduction were determined by HPLC ($\lambda = 366$ nm). FLU-1 (200 μ M) was incubated in microsomes (0.1 mg/ml) at 37°C for 10 min. FLU-1 N-OH (50 μ M) was incubated in cytosol (0.1 mg/ml) at 37°C for 20 min. Data are shown as the mean of the activity per mg liver \pm S.D. (n = 4-5).

	Control	TCPOBOP	FLU-1	FLU-1 + TCPOBOP
	(nmol/g liver/min)			
FLU-1 N-hydroxylation	4.4 \pm 0.7	36.3 \pm 1.5	13.2 \pm 1.6	40.5 \pm 4.7
FLU-1 N-OH reduction	35.8 \pm 1.8	41.5 \pm 11.7	48.3 \pm 3.6	33.2 \pm 7.6

Figure 1

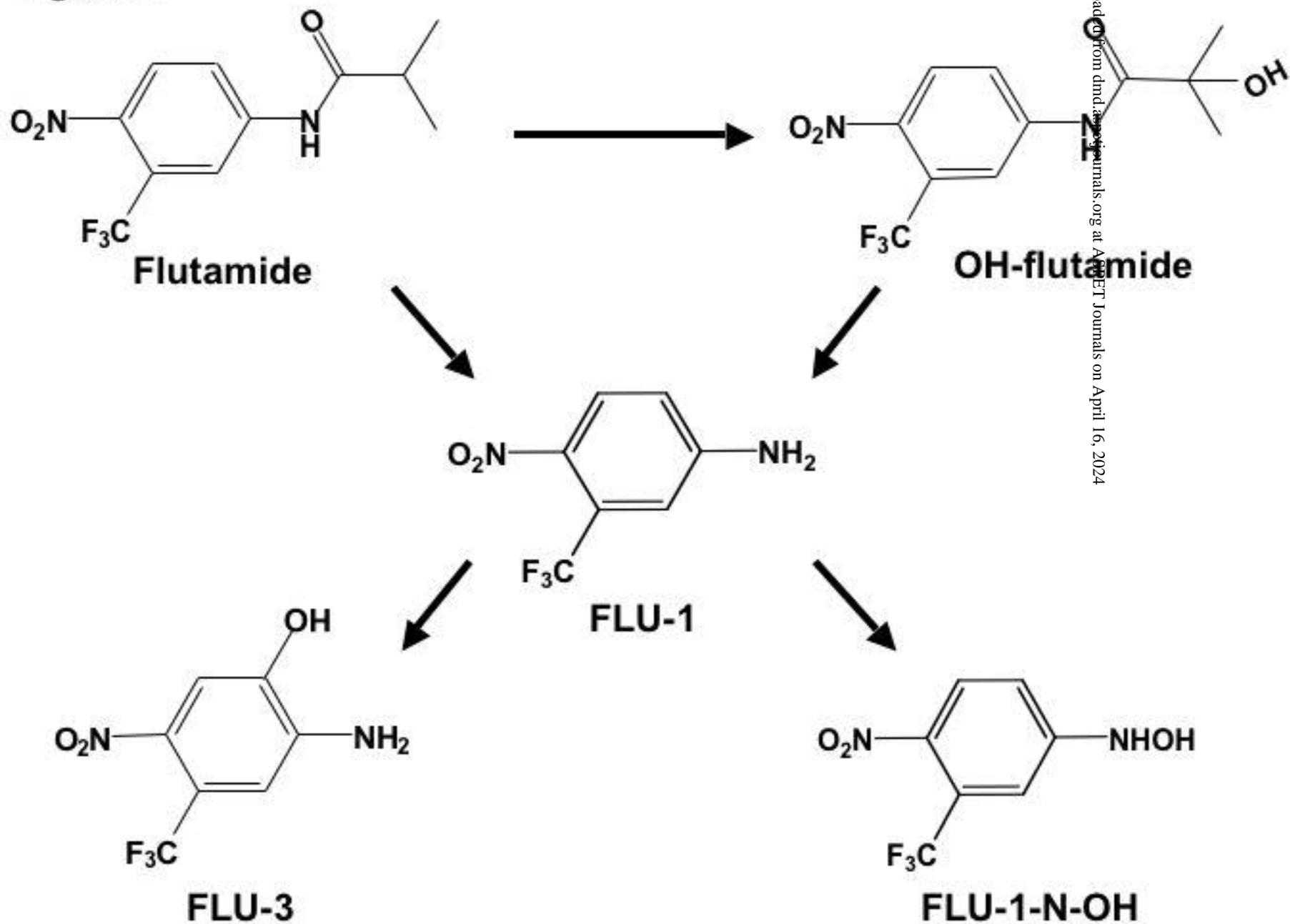


Figure 2

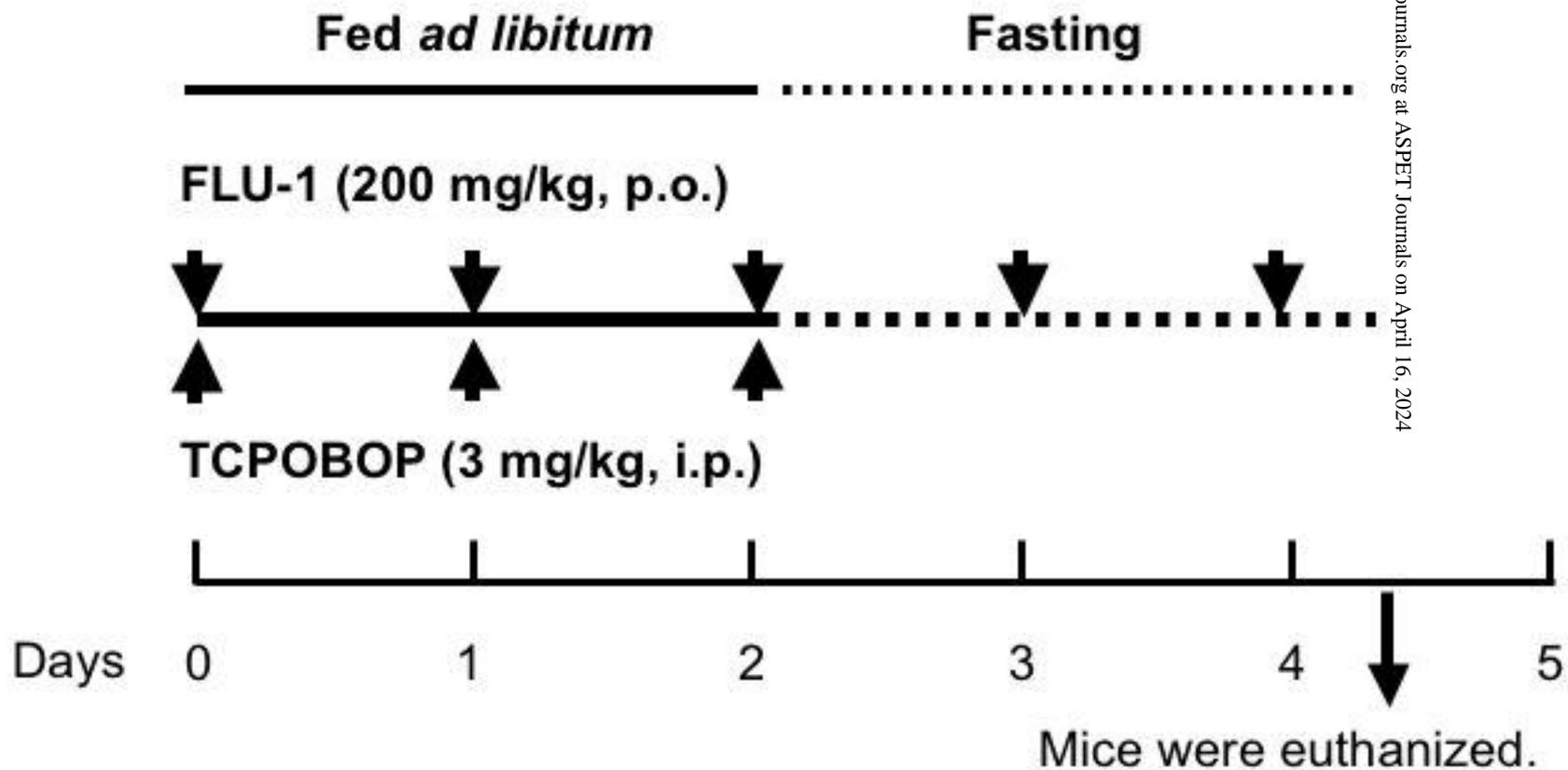


Figure 3

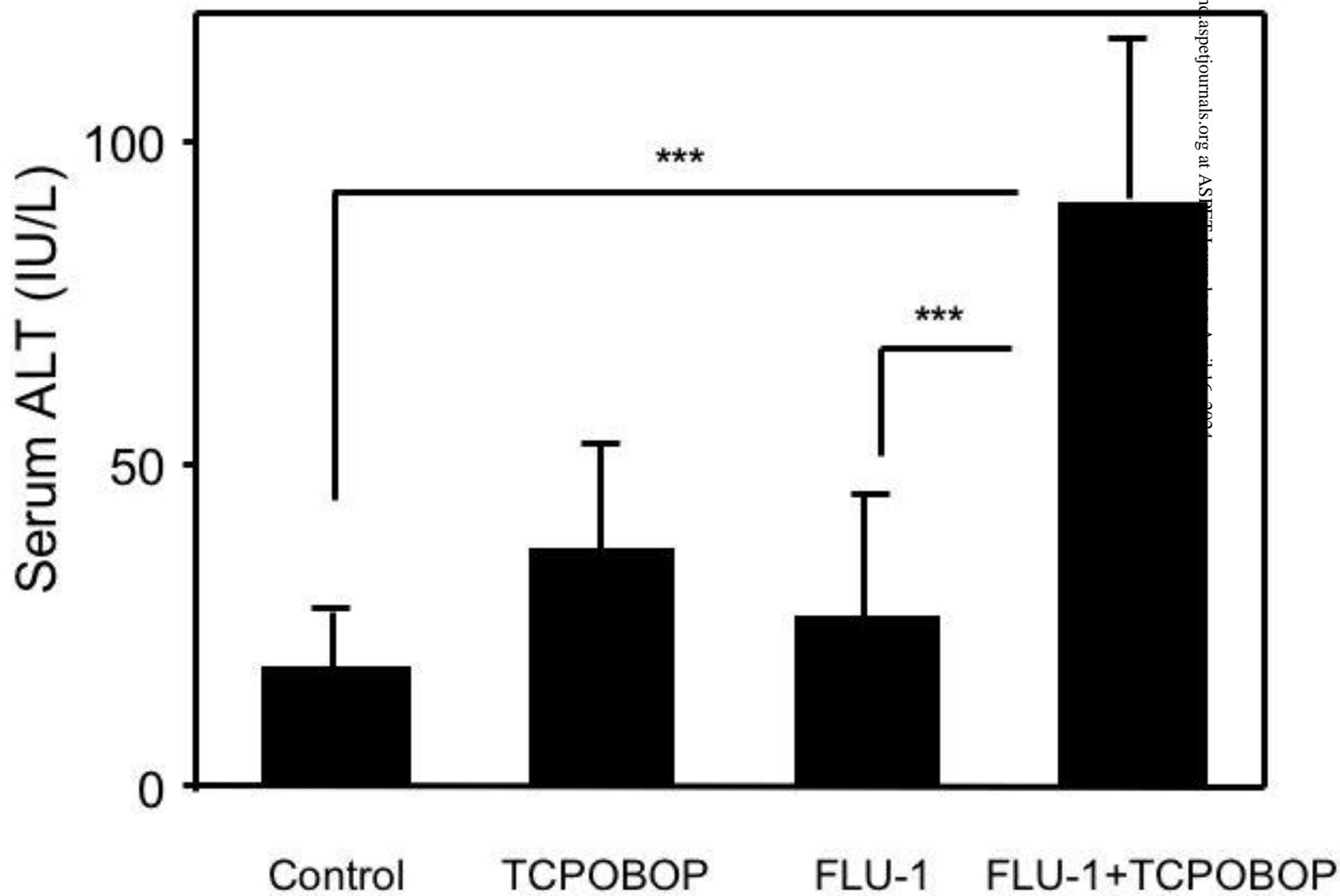


Figure 4 (A)

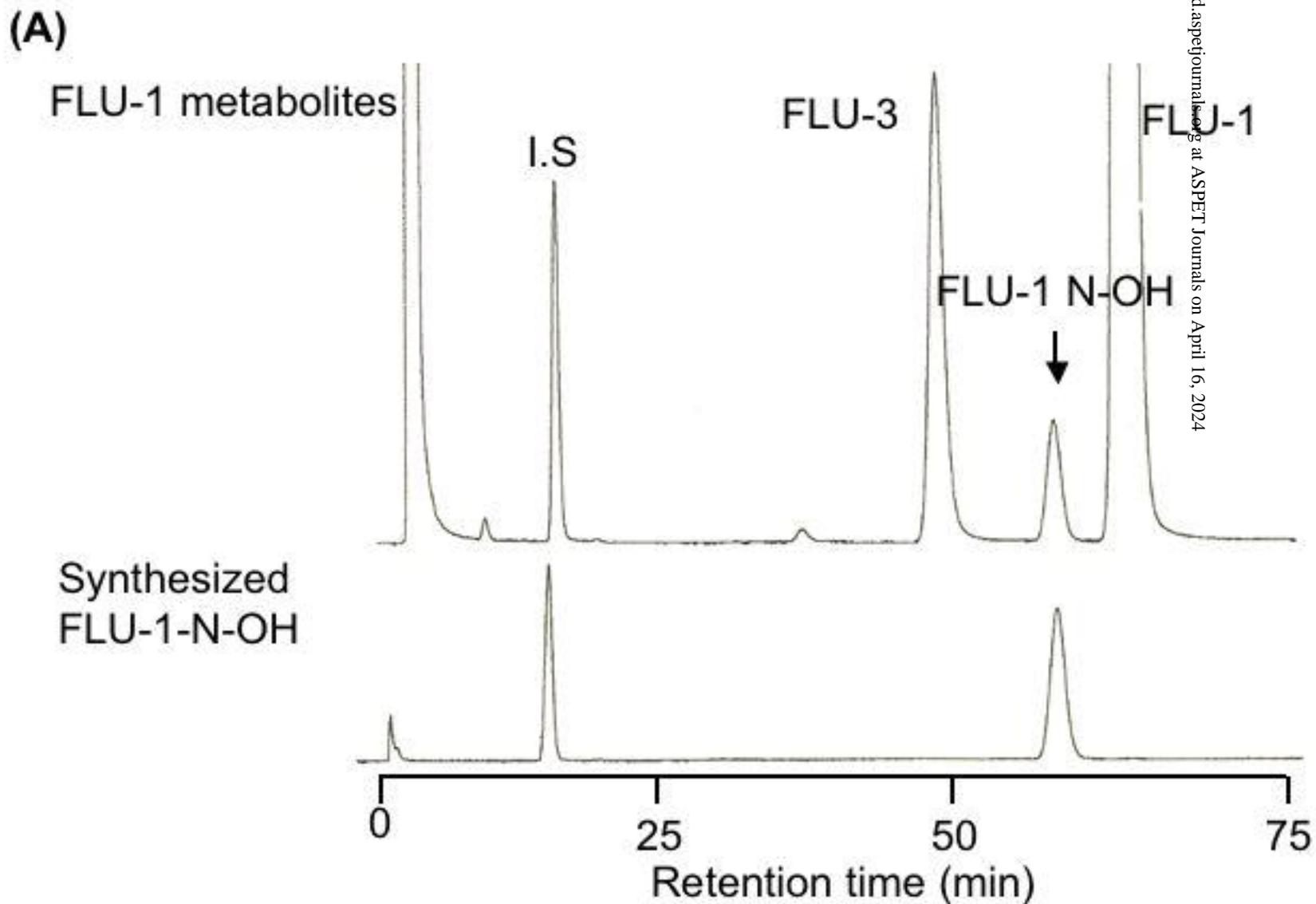


Figure 4 (B)

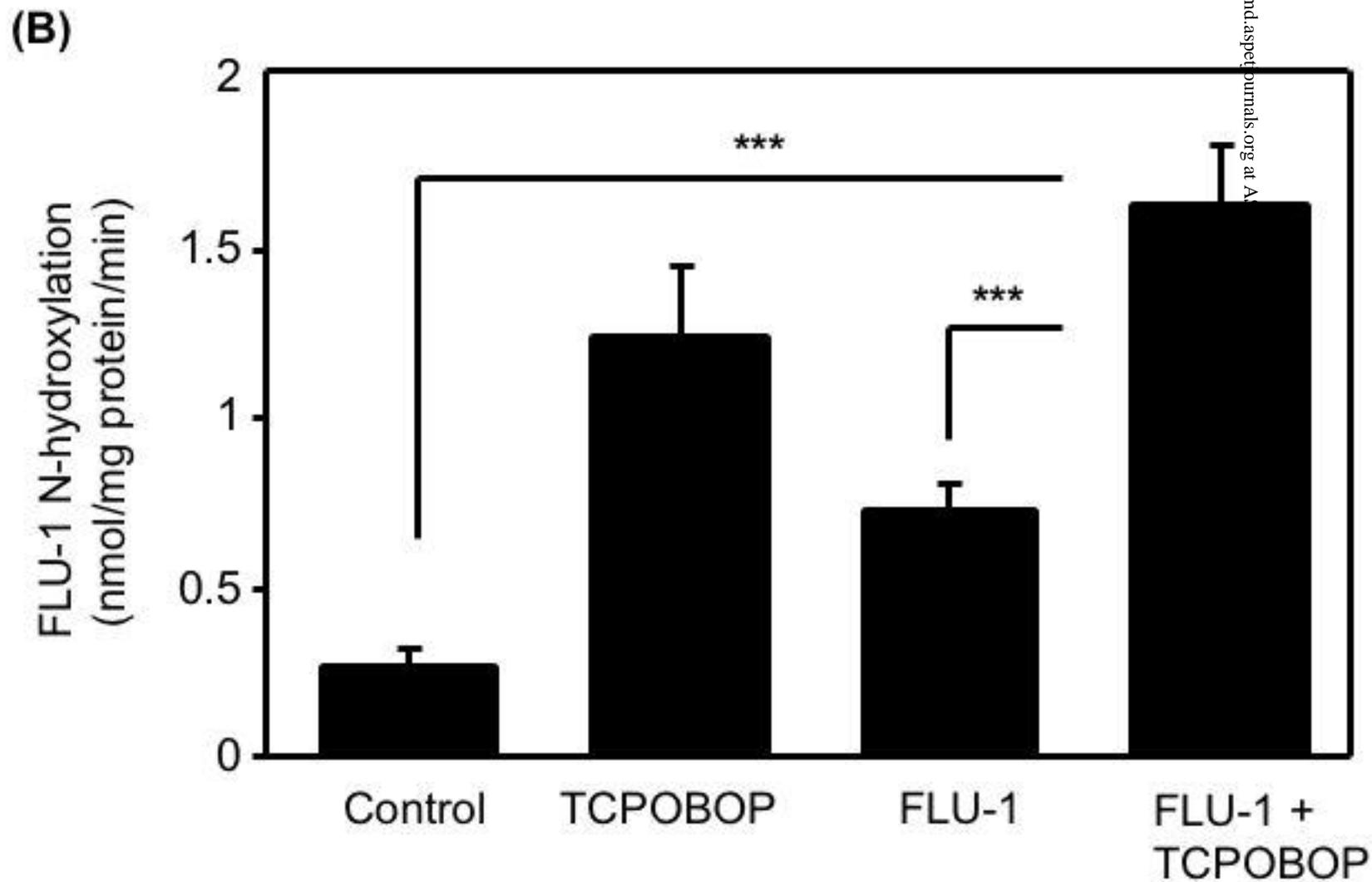


Figure 4 (C)

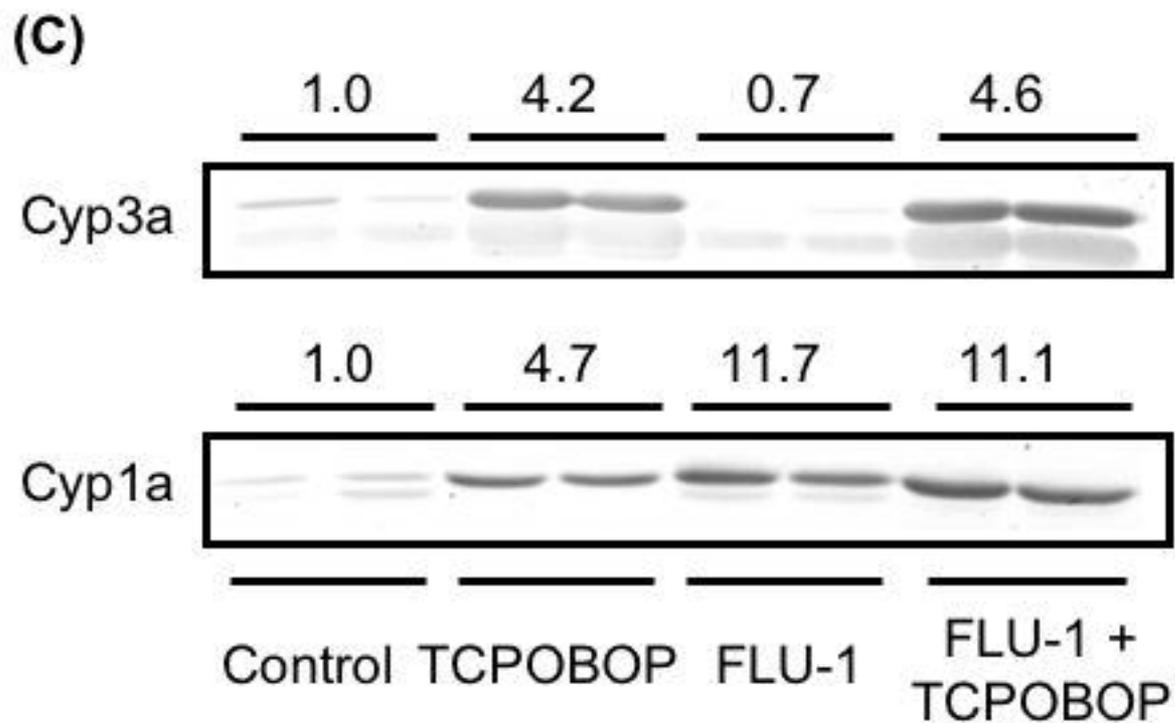


Figure 5

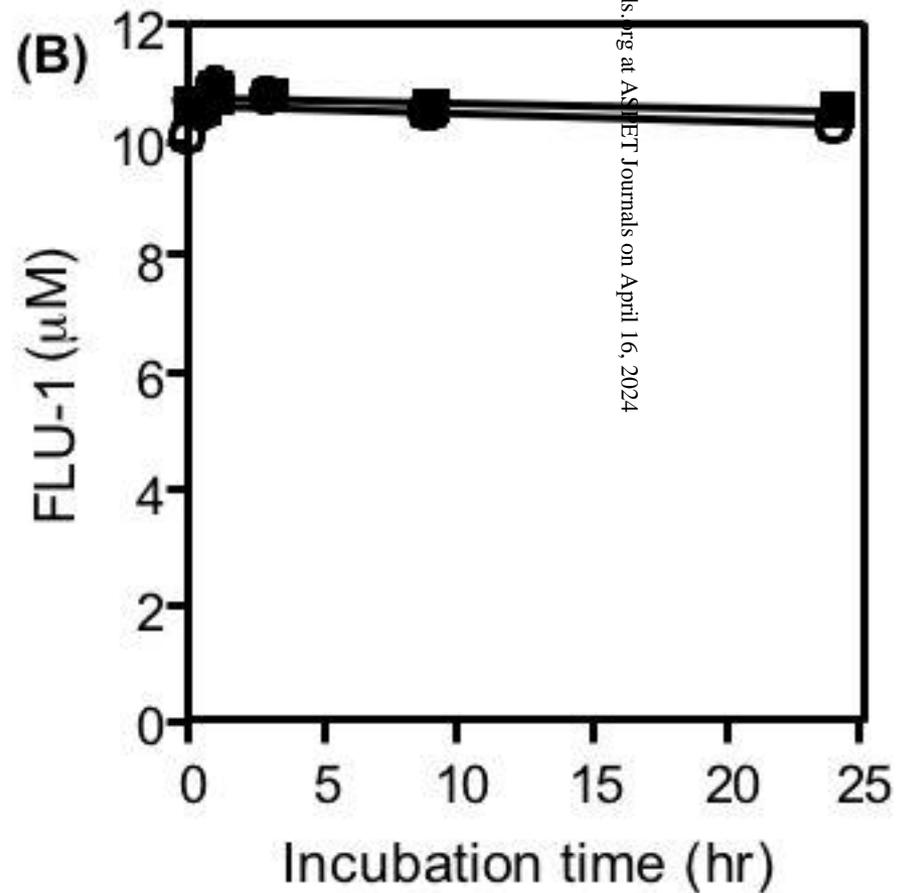
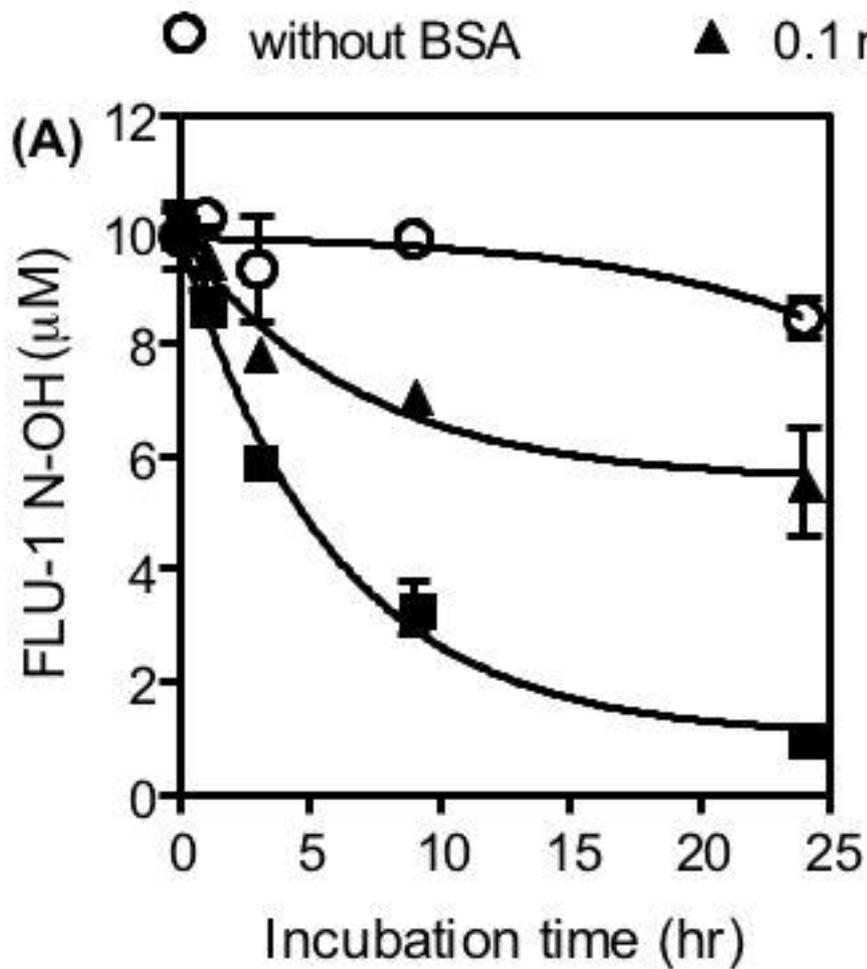


Figure 6

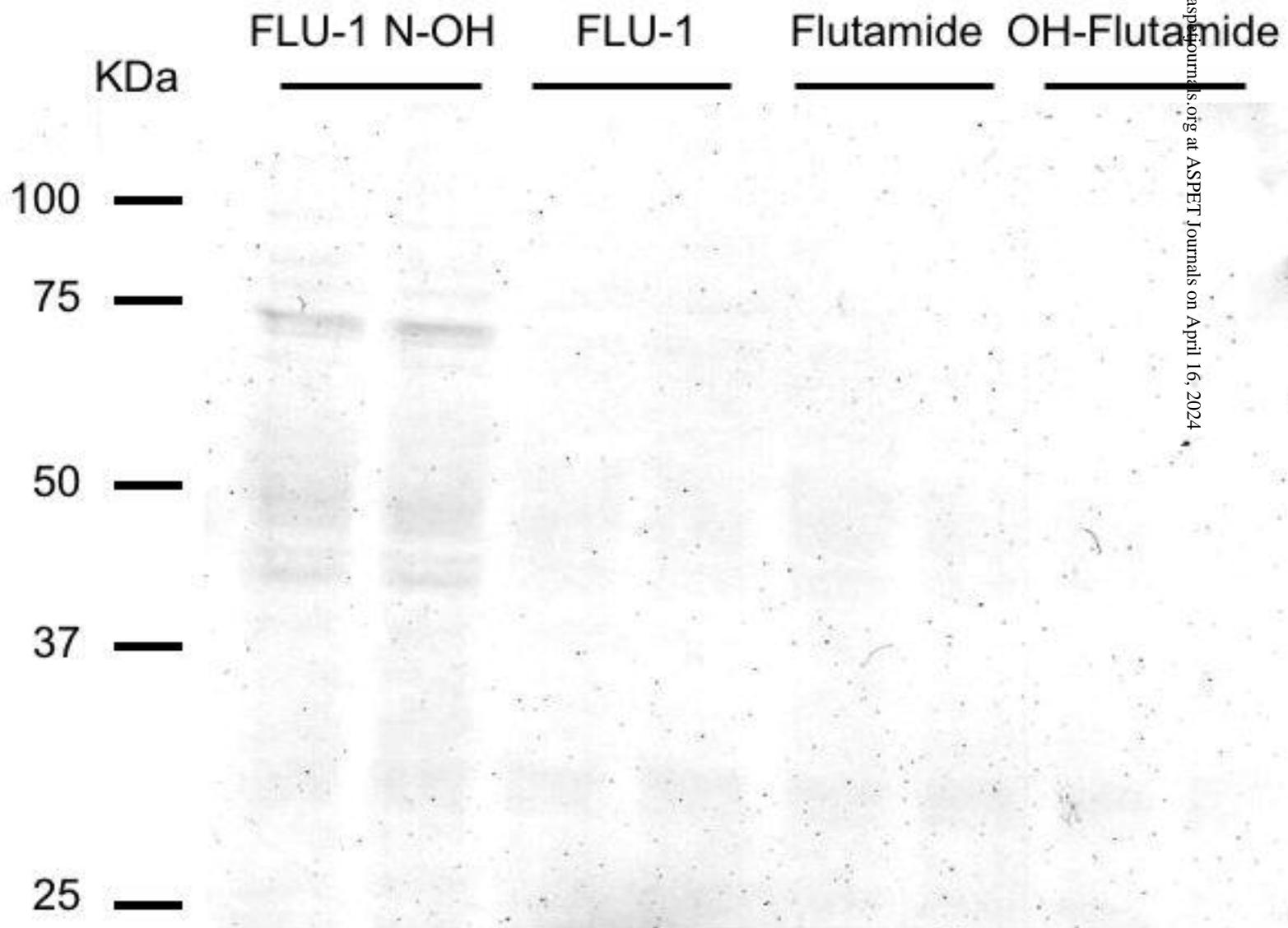


Figure 7

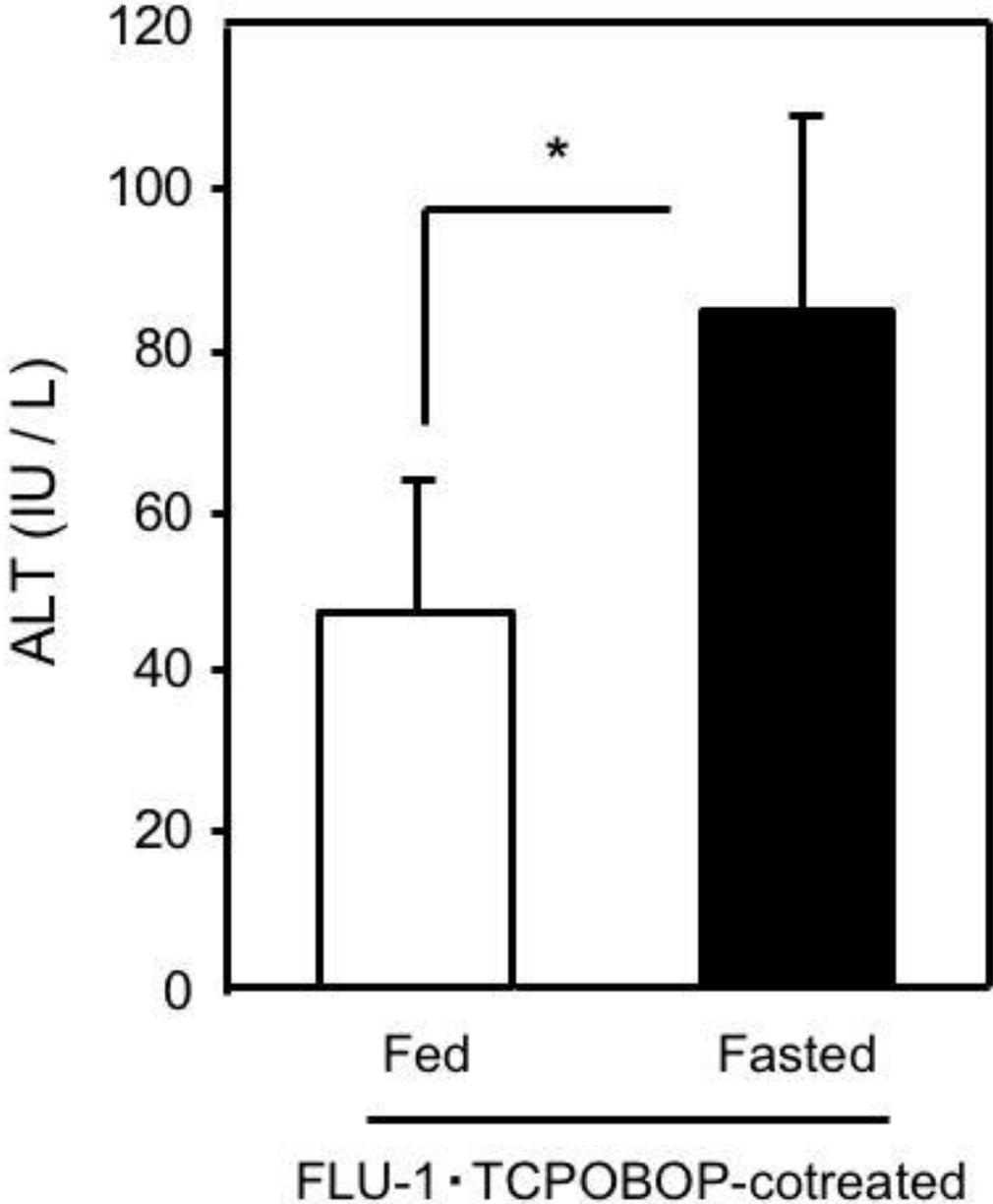


Figure 8(A)

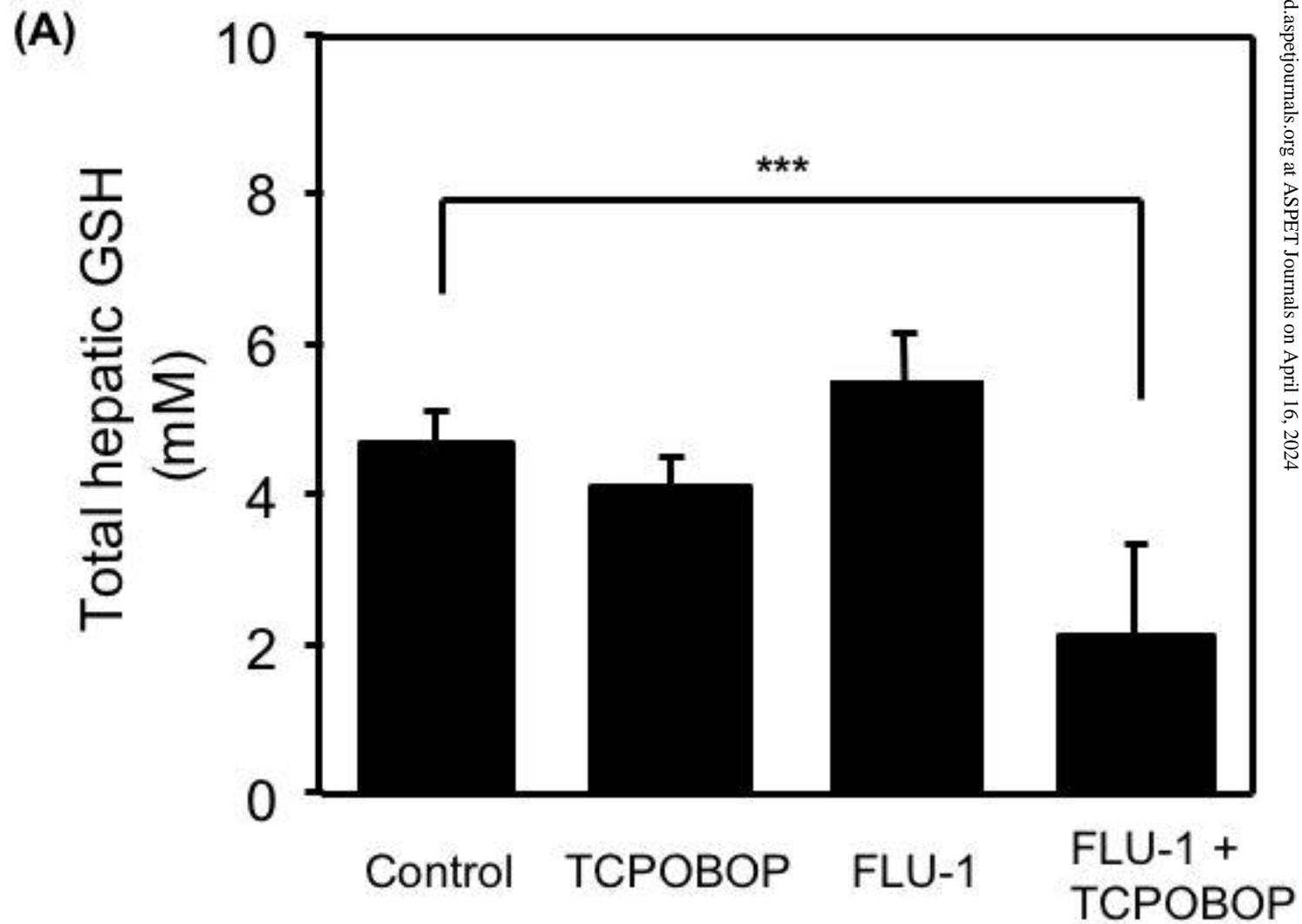


Figure 8(B)

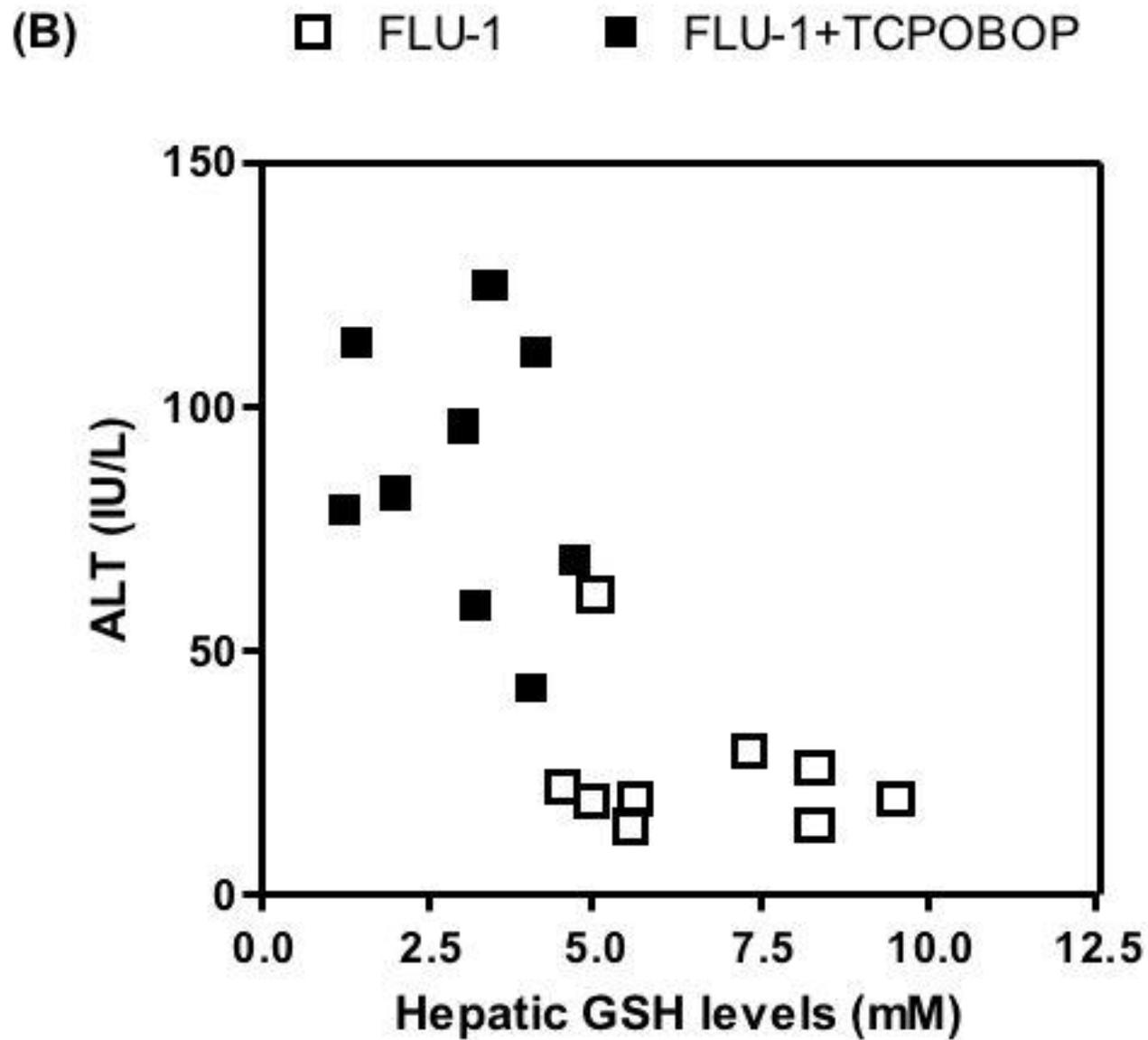


Figure 9

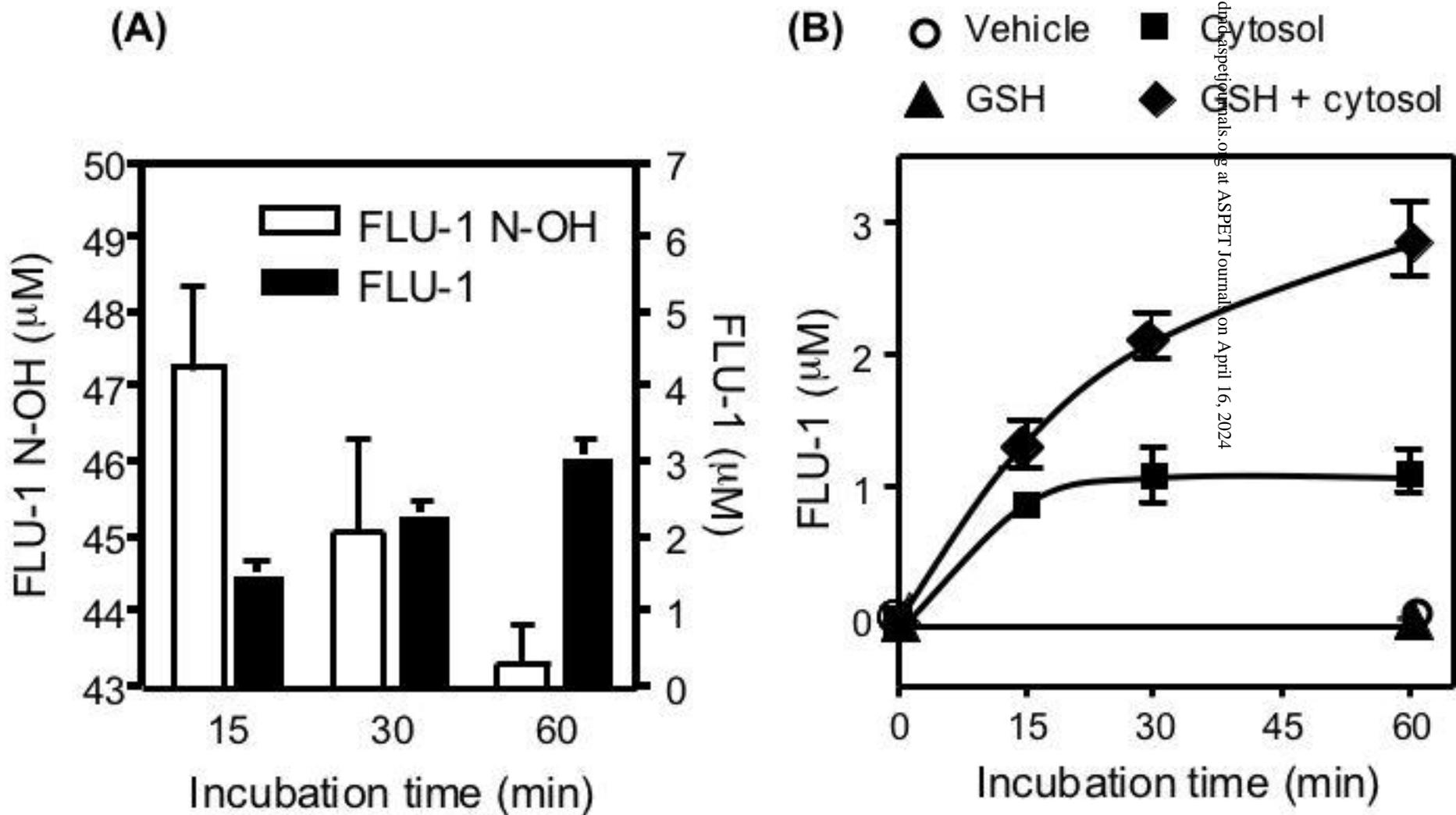


Figure 9

(C) ○ Vehicle ■ Cytosol
 ▲ NADH ◆ NADH + cytosol

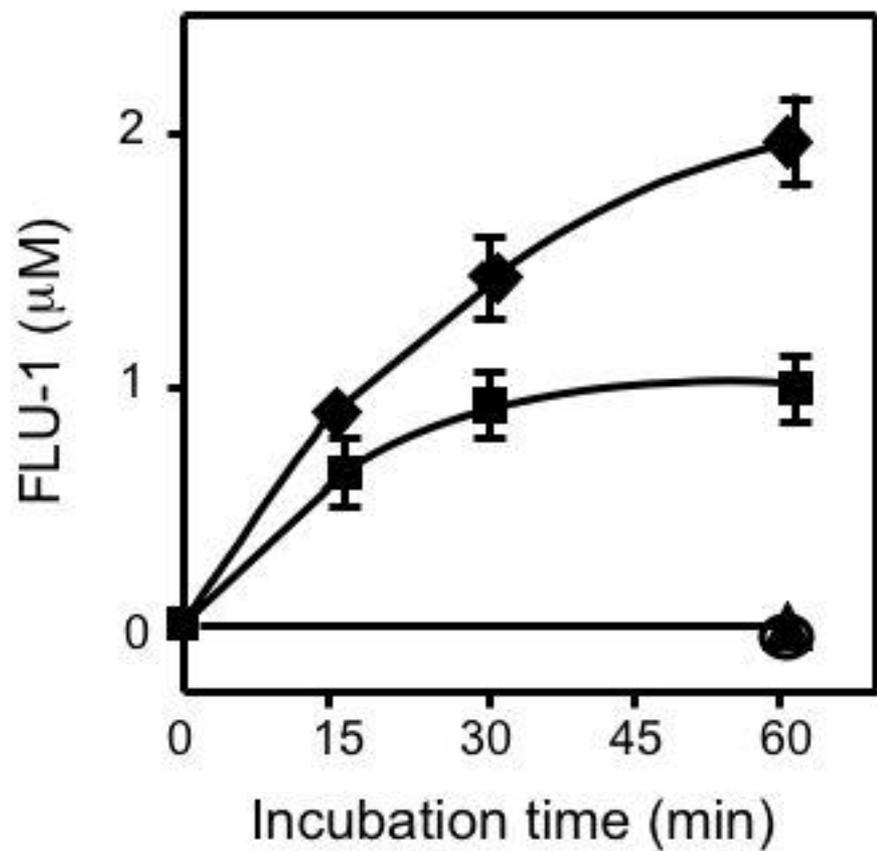


Figure 10

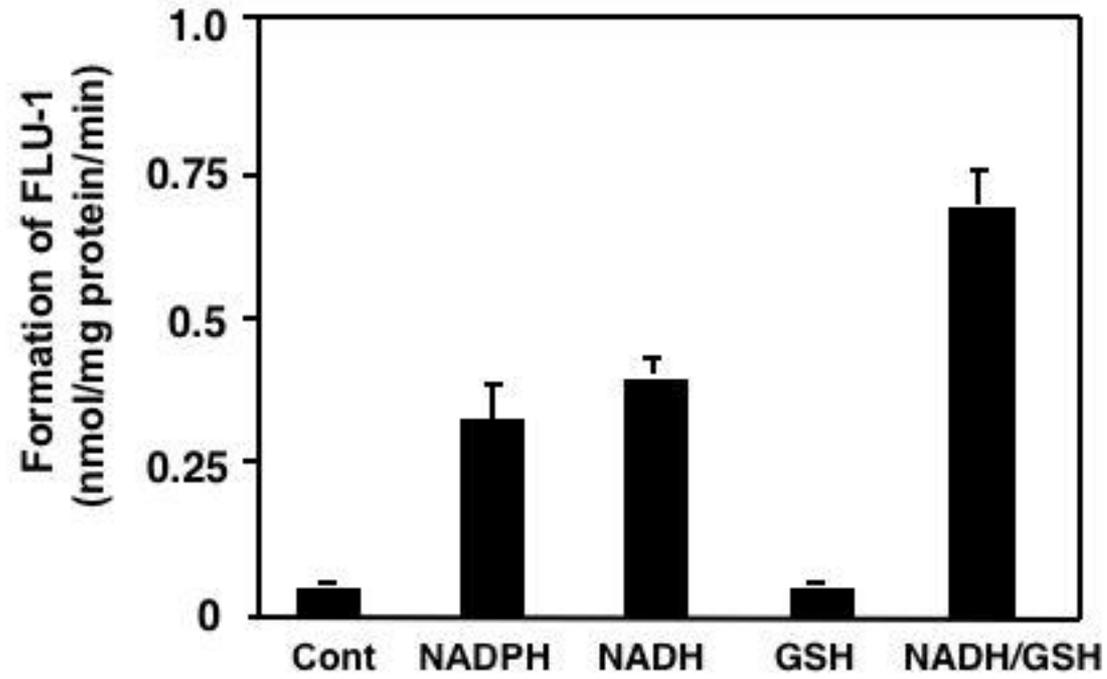


Figure 11

