SATURATION TOXICOKINETICS OF THIOACETAMIDE: ROLE IN INITIATION OF LIVER INJURY

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ABSTRACT:

Thioacetamide (TA), a potent centrilobular hepatotoxicant, undergoes a two-step bioactivation mediated by microsomal CYP2E1 to TA sulfoxide (TASO), and further to TA-S,S-dioxide (TASO2), a reactive metabolite that initiates cellular necrosis. Our earlier studies showed that bioactivation-mediated liver injury of TA is not dose-proportional. The objective of this study was to examine whether increasing doses of TA lead to enzyme saturation, thereby resulting in lack of dose-response for injury: bioactivation of TA → TASO → TASO2 may follow zero-order kinetics. A 12-fold dose range of TA (50, 300, and 600 mg/kg i.p.) was injected into male Sprague-Dawley rats. TA and TASO were quantified in plasma, liver, and urine by high-performance liquid chromatography. With increasing doses, the apparent elimination half-lives of TA and TASO increased linearly, indicating that TA bioactivation exhibits saturation kinetics. Increasing TA dose resulted in greater-than-proportional increases in plasma TA and TASO levels. The TASO/TA ratio was inversely proportional to the dose of TA. Covalent binding of 14C-derivative radiolabel to liver macromolecules showed a less-than-dose-proportionate increase with a 12-fold higher dose. Less than dose-proportional covalent binding was confirmed in liver microsomal incubations with 14C-MA. Three-fold higher excretion of TASO was seen in urine at the highest dose (600 mg/kg) compared with the lowest dose (50 mg TA/kg). Incubation of TA with rat liver microsomes and purified baculovirus-expressed rat and human CYP2E1 Supersomes, over a concentration range of 0.01 to 10 mM, revealed saturation of TA conversion to TASO at and above 0.05 mM TA concentration, comparable to in vivo plasma and liver levels achieved upon administration of higher doses. Calculated Km values for TA (0.1 mM) and TASO (0.6 mM) suggest that the second step of TA bioactivation is 6-fold less efficient. Collectively, the findings indicate saturation of CYP2E1 at the first (TA to TASO) and second (TASO to TASO2) steps of TA bioactivation.

The toxicokinetic profile of a compound becomes very important especially when its toxicity depends on its bioactivation to a reactive intermediate. When increasing doses of toxicants are administered, the rate of bioactivation may be limited as a result of enzyme saturation, and thus, production of reactive metabolites does not increase proportionately with dose (saturation, zero-order kinetics). Thioacetamide (TA) is a centrilobular hepatotoxicant, widely used as a model compound to induce acute and chronic liver disease (Mangipudy et al., 1998; Wasser and Tan, 1999; Al-Bader et al., 2000; Chu et al., 2000; Ramaiah et al., 2000). Numerous investigators use TA to study mechanisms of hepatic necrosis because of its relatively short half-life (Porter and Neal, 1978; Porter et al., 1979), well known ability to cause acute toxicity (Mehendale et al., 1997), and a large window of time between its necrogenic effects and liver failure (Mehendale, 2005). Liver injury begins with bioactivation of TA to thioacetamide sulfoxide (TASO) (step I) and further to thioacetamide-S,S-dioxide (TASO2) (step II) (Fig. 1). Thioacetamide-S,S-dioxide, an unstable, reactive metabolite, is thought to initiate necrosis by covalently binding to liver macromolecules (Hunter et al., 1977; Porter and Neal, 1978; Porter et al., 1979). The advantages of using TA as a model hepatotoxicant include high specificity for liver as a target organ, except at excessively high doses, when it can also cause marginal but transient renal injury (Barker and Smuckler, 1974), and regiospecificity for the perivenous region. When a lethal dose is administered to rats, death occurs 3.5 to 7 days after administration due to fulminant hepatic failure (Chanda and Mehendale, 1994, 1995; Mangipudy et al., 1995). This allows the luxury of a long window of time for studying the incline and decline slopes of injury and recovery by conducting time course studies after administration of low to moderate doses (Mangipudy et al., 1995, 1998; Mehendale, 2005).

TA is bioactivated by hepatic microsomal CYP2E1 to sulfoxide and further to TASO2 (Wang et al., 2000; Ramaiah et al., 2001). CYP2E1 metabolizes a large number of low-molecular-weight compounds, many of which are industrial solvents, chemical additives, halogenated anesthetics, and drugs (Tanaka et al., 2000). Most importantly, CYP2E1 activates many xenobiotics to hepatotoxic or carcinogenic products (Lieber, 1997). Also, a wide variety of CYP2E1 substrates are known to exhibit saturation kinetics (Ramsey and Young, 1978; Kirschman et al., 1986; Kreiling et al., 1986; Lof and Johanson, 1993; Ramaiah et al., 2000; and Ramaiah et al., 2000). CYP2E1 is known to be saturable toward TA and thioacetamide sulfoxide (TASO) (Mangipudy et al., 1995; 1998; Meherdale, 2005). These studies were supported by National Institute of Environmental Health Sciences Grant ES-09870, and H.M.M. was partly supported by the Louisiana Sciences Grant ES-09870, and H.M.M. was partly supported by the Louisiana Sciences Grant ES-09870. A series of studies (Chilakapati et al., 2000; Porte and Neal, 1978; and Porte et al., 1979) has shown that TA induces necrotic cell death in rodent liver.

The toxicokinetic profile of a compound becomes very important especially when its toxicity depends on its bioactivation to a reactive intermediate.

REFERENCES:

Mangipudy et al., 1995; 1998; Meherdale, 2005; Ramsey and Young, 1978; Kirschman et al., 1986; Kreiling et al., 1986; Lof and Johanson, 1993.

ABBREVIATIONS: TA, thioacetamide; TASO, thioacetamide sulfoxide; TASO2, thioacetamide-S,S-dioxide; SD, Sprague-Dawley; HPLC, high-performance liquid chromatography; 14C-MA, thio[1-14C]acetamide; TCA, trichloroacetic acid; AUC, area under the curve.
Dekant et al., 1995; Whysner et al., 1996; Lee et al., 2000; Vittozzi et al., 2000; Rappaport et al., 2002).

Although TA hepatotoxicity is well characterized and its metabolism well studied, information on dose-response for injury and toxicokinetics is very sparse. Porter et al. (1979) reported the half-lives of TA and TASO to be 1 to 1.5 h and 1.5 to 2 h, respectively, after administration of a single dose (200 mg/kg) of TA. The extent to which these half-lives remain consistent upon administration of lower or higher doses of TA has not been reported.

A 12-fold dose range of TA (50–600 mg/kg) failed to elicit a typical dose-response relationship with respect to initial liver injury as measured by plasma alanine aminotransferase, sorbitol dehydrogenase, and histopathology (Mangipudy et al., 1995). The lethal dose (600 mg/kg) caused lower injury at 12 h than did the other three doses. Injury continued to progress well after 36 h, when the compound was totally eliminated from the body (t1/2 ~3 h). Perhaps other mechanisms that are independent of the offending toxicant, such as leakage of destructive hydrolytic proteases like calpains released from dying cells or secondary inflammatory mechanisms, lead to progression of injury (Limaye et al., 2003).

Liver injury of a 6-fold dose range of TA (50, 150, and 300 mg/kg) did not differ between doses during the incline or decline phases of injury. Compensatory tissue repair data revealed a dose-proportional increase over a 6-fold dose range (50–300 mg/kg), but marked delay and inhibition after the highest dose (600 mg/kg). These observations run in opposition to the commonly accepted paradigm of higher doses yielding higher metabolic activation and, therefore, lower as expected bioactivation-based liver injury.

Materials and Methods

Animals. Male Sprague-Dawley (SD) rats (250–275 g) were obtained from Harlan (Indianapolis, IN) and were maintained on sawdust bedding, free of any chemical contaminants on a 12-h photoperiod in our central animal facility at 21 ± 1°C and 50 to 80% relative humidity. Rats were acclimated for a week before use in experiments. The animals had free access to water and commercial rat chow (no. 7001; Harlan Teklad, Madison, WI) before and during the treatment. Animal maintenance and treatments were in accordance with the National Institutes of Health Guide for Animal Welfare, as approved by the Institutional Animal Care and Use Committee.

Chemicals. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Thioacetamide was 99.7% (HPLC grade) pure. TASO was synthesized using the method described by Porter and Neal (1978). Thio [1-14C]acetamide (14C-TA, 5 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO).

Toxicokinetic Parameters of TA. Blood samples were collected (100 μl via retro-orbital sinus) in heparinized tubes over a time course from male SD rats treated with 50, 300, and 600 mg/kg TA intraperitoneally. Three rats were used to complete the entire time course once (n = 1). One rat was used to collect blood samples for four time points, overlapping one time point between rats. Three such replicate time courses were performed to achieve n = 3. Plasma was separated from heparinized blood by centrifugation for 10 min at 10,000g. Plasma half-lives (t1/2l) for TA and TASO were calculated using the terminal elimination constant (β), where t1/2l = 0.693/β. The value of β was estimated using a semilogarithmic plot of time versus concentration using JMP-IN statistical software (SAS Institute, Cary, NC).

In a separate set of experiments, liver samples were collected from rats after diethyther euthanasia at various time points after intraperitoneal TA (50, 300, and 600 mg/kg) administration. The liver samples were homogenized in saline (1 ml saline/g liver). The liver homogenate was centrifuged and the supernatant analyzed for TA and TASO concentrations.

For urinary analysis of TA and TASO, urine was collected by housing animals in separate metabolic cages (Nalgene; Nalg Nunc, Rochester, NY). Preliminary experiments confirmed that collecting urine at room temperature did not affect the stability of TA and TASO for 24 h. This was done by adding known amounts of TA and TASO to normal rat urine and storing the urine samples either at room temperature or at −20°C. TA and TASO were analyzed by HPLC at 0 and 24 h later and did not detect any significant differences between the two time points regardless of room temperature or cold storage.

We therefore did not find it necessary to collect the urine over dry ice. Urine was collected 12 and 24 h after treatment with TA. Standards were prepared by including known amounts of TA and TASO in plasma, liver homogenate, and urine from untreated rats. Levels of TA and TASO were estimated using a reverse-phase HPLC assay as described below.

Plasma Protein Binding of TA. The protein unbound fraction (fu) of TA in rat plasma was measured by the ultrafiltration method with a Centrifree (Millipore Corporation, Billerica, MA). TA was mixed with the rat plasma at 0.5, 5, and 10 nM concentrations and incubated at 37°C for 30 min. A 100-μl aliquot of the incubate was placed in the sample reservoir of the Centrifree tube and was analyzed for TA and TASO concentrations. To quantitate TA and TASO in plasma, liver, and urine, a reverse-phase HPLC assay was developed using 7% acetonitrile, 50 mM sodium sulfate, and 50 mM potassium phosphate buffer as the mobile phase (Shankar et al., 2003). An SPS-ODS column (5 μm; Regis Technologies, Morton Grove, IL) was used to separate the components at 1 ml/min. TA was detected by UV absorption at 212 nm, and TASO was detected by a photodiode array detector (PDA 100; Dionex Corporation, Sunnyvale, CA). Retention times for TA and TASO were approximately 4.1 and 3 min, respectively.

HPLC-Based Assay for Quantitation of TA and TASO. To quantitate TA and TASO in plasma, liver, and urine, a reverse-phase HPLC assay was developed using 7% acetonitrile, 50 mM sodium sulfate, and 50 mM potassium phosphate buffer as the mobile phase (Shankar et al., 2003). An SPS-ODS column (5 μm; Regis Technologies, Morton Grove, IL) was used to separate the components at 1 ml/min. TA was detected by UV absorption at 212 nm, and TASO was detected by a photodiode array detector (PDA 100; Dionex Corporation, Sunnyvale, CA). Retention times for TA and TASO were approximately 4.1 and 3 min, respectively.

Covalent Binding of 14C-TA to Liver Tissue. To examine covalently bound TA-reactive intermediate, livers from rats treated with 14C-TA (50 and 600 mg/kg i.p., 5 mCi/mmol, 25 μCi/rat) were flash frozen with liquid nitrogen over a time course (3–96 h) after TA treatment. 14C-TA-derived radioactivity...
covalently bound to liver macromolecules was estimated using the procedure described earlier (Shankar et al., 2003). Briefly, liver tissue (200 mg) was homogenized in 1 ml of 0.9% saline, and total protein was precipitated using 2 ml of 0.9 M trichloroacetic acid (TCA). Samples were centrifuged at 1000g for 15 min at room temperature. The supernatant was discarded and the protein precipitate was resuspended in 3 ml of 0.6 M TCA, mixed on an agitating mixer (Thermolyne, Dubuque, IA) for 1 min, and centrifuged at 1000g for 3 min. After two more washings with 0.6 M TCA (3 ml per wash), the pellet was washed six times with 80% methanol (5 ml per wash). It was found that after six methanol washings no radioactivity was detected in the supernatant. The remaining pellet was dissolved in 2 ml of 1 M sodium hydroxide (NaOH), and the 14C content in an aliquot was estimated using a liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Total protein concentrations were estimated using Bradford’s method (Bradford, 1976) using Bio-Rad reagent. Results are expressed as the amount of TA covalently bound to protein. The linear trapezoidal rule was applied to calculate the area under the curve.

Preparation of Microsomes. Untreated rats (250–275 g) were euthanized under diethyl ether anesthesia. Livers were perfused in situ with ice-cold 0.9% saline, rapidly excised, blotted dry, weighed, minced, and homogenized (1:5 w/v) in ice-cold Tris-acetate buffer (pH 7.4) containing 1.15% KCl. The homogenate was centrifuged at 10,000g for 30 min at 4°C. The supernatant was then centrifuged at 100,000g for 60 min at 4°C. Microsomes were resuspended by homogenization in a Potter-Elvehjem glass homogenizer and again centrifuged at 100,000g. Microsomal pellets were recovered, quick frozen with liquid nitrogen, and stored at −80°C for later use.

Microsomal Metabolism of TA. To investigate saturation of TA metabolism to TASO in vivo, TA was incubated with hepatic microsomes. TA (0.01–10 mM) (0.1 M potassium phosphate buffer, pH 7.4) was incubated with liver microsomes (2 mg of protein) and an NADPH-regenerating system (1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM MgCl₂). Each reaction mixture had a total volume of 3 ml and reactions were stopped at 10 min following the protocol used in the previous section and TASO levels were estimated at the end of 10 min.

In vivo Measurements of Plasma TA and TASO Levels. Mean plasma concentrations of TA and its major metabolite TASO are shown in Fig. 2, A and B, respectively. Plasma TA levels peaked by 5 min after each of the three doses. TA had a half-life of 16 min at the lowest dose (50 mg/kg). After administration of the higher doses, i.e., 300 and 600 mg/kg, TA levels did not decrease at a statistically significant rate from one time point to the other between 5 and 60 min and 15 and 120 min, respectively, suggesting saturation of its metabolism. Thereafter, the levels declined slowly, presumably due to conversion to TASO or excretion. Plasma TA AUC was more than dose-proportional with increase in dose. The plasma clearance of TA and TASO decreased with increasing doses, implying saturation of metabolism (Table 2).

TAOS was readily detected in plasma as early as 5 min, indicating its rapid formation. The plasma levels peaked around 60 to 90 min for the 50 mg/kg dose, whereas maximum concentrations were reached at 180 min for the 300 and 600 mg/kg doses. There was no difference in TASO concentration between the 30 and 300 mg/kg doses until 15 min after the administration and between the 300 and 600 mg/kg doses until 60 min after the administration. After reaching peak levels, TASO remained constant for 3.75 h (300 mg/kg) and 9 h (600 mg/kg), respectively, suggesting saturation of the TASO metabolism to TASO₂⁻, declining rapidly thereafter. Less than dose-proportional increase in peak plasma TASO levels was found with increase in dose. The metabolite to parent compound ratio was highest with the lowest dose (50 mg/kg) at all time points, indicating that the rate of metabolism (TASO) production was highest at this dose (Table 1). We could thus infer that there is saturation at the first step of TA metabolism. Plasma TASO AUC was more than dose-proportional with increase in dose, suggesting saturation of further metabolism of TASO. Toxicokinetic parameters of TA and TASO are given in Table 2. Dose-normalized AUC for TA and TASO showed greater values with higher doses (Table 2). With increasing doses, apparent half-lives of TA (16 ± 1 to 164 ± 7 min) and TASO (36 ± 1.6 to 247 ± 41.7 min) progressively increased (Table 2), a hallmark of zero-order kinetics. Saturable metabolism of TA as well as TASO was readily evident as the dose increased.

Plasma Protein Binding of TA. The plasma protein binding (fb percentage) of TA was low (9.93 ± 1.16%) and concentration-independent for TA. The plasma protein binding values (fb percentage) for TA over a 20-fold concentration range, i.e., 0.5, 5, and 10 mM
Liver Levels of TA and TASO. Liver TA (Fig. 2C) and TASO (Fig. 2D) levels were undetectable 8 h after treatment with 50 mg/kg. Hepatic TA levels remained constant until 6 h after the administration of 50 and 300 mg TA/kg doses (Fig. 2C), and until 3 h after the highest dose. Increases in AUC for hepatic TA and TASO were disproportionately higher with increase in dose (data not shown). TASO levels increased for the high dose only after TA levels began to decline in the plasma (Fig. 2A). Hepatic TASO levels peaked at 180 min after administration of the high (lethal) dose. After 180 min, TASO after the highest dose did not differ from TASO concentrations seen after the 300 mg/kg dose until the 12-h time point. With regard to the low dose, although hepatic TASO levels remained elevated until 60 min, there was a steep decline at 180 min.

Urinary Excretion of TA and TASO. Urinary excretion is represented as percentage of dose excreted in the form of unchanged TA and TASO, 12 and 24 h after TA administration (Fig. 3). Most of the urinary excretion of TA and TASO occurred by 12 h regardless of the dose, since there was no significant difference between 12 and 24 h excretion. Whereas the cumulative amount of TA excreted as a percentage of dose remained similar after 12-fold increase in TA dose (50–600 mg/kg), excretion of TASO increased 3-fold, suggesting that the metabolism of TASO is less efficient than the metabolism of TA after the higher doses. Consequently, TASO formed after the high dose is largely excreted. We have not examined the status of the sulfate conjugate and acetamide, which account for the remaining urinary metabolites of TA.

Covalent Binding of 14C-TA to Liver Macromolecules. TASO cannot be measured because it is highly reactive and unstable. Covalent binding of 14C-TA-derived radiolabel to liver protein, used as an indirect method to measure the reactive intermediate TASO, showed a less than dose-proportional increase. At 50 mg TA/kg dose, covalent binding was rapid and remained relatively constant at peak level through 12 h (Fig. 4A). After a 12-fold higher dose (600 mg TA/kg), covalent binding also remained constant near the peak level until 12 h, but it was considerably lower than the expected 12-fold increase (Fig.

<table>
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<tr>
<th>Time</th>
<th>50 mg/kg</th>
<th>300 mg/kg</th>
<th>600 mg/kg</th>
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<tr>
<td>5 min</td>
<td>0.15 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.004</td>
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<td>15 min</td>
<td>0.3 ± 0.05</td>
<td>0.08 ± 0.02</td>
<td>0.02 ± 0.003</td>
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<td>30 min</td>
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<td>0.16 ± 0.01</td>
<td>0.04 ± 0.003</td>
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<td>45 min</td>
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<td>60 min</td>
<td>7.41 ± 0.89</td>
<td>0.22 ± 0.07</td>
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The binding of TASO2 to liver tissue, which is less than dose-proportional, covalent binding. These results confirm the in vivo data on covalent injury with the lethal dose of TA (Mangipudy et al., 1995).

The lack of dose-response for the initial bioactivation-based liver second step (formation of reactive TASO2), therefore contributing to seen after the 600 mg/kg dose. This result indicates saturation at the same dose. This result implies saturation at the same dose. The calculated parameter for the concentration of TA incubated with rat liver microsomes was increased only by 57-fold (Fig. 4A). An 8-fold increase in AUC of covalent binding (Fig. 4B) was seen after the 600 mg/kg dose. This result indicates saturation at the second step (formation of reactive TASO2), therefore contributing to the lack of dose-response for the initial bioactivation-based liver injury with the lethal dose of TA (Mangipudy et al., 1995).

Covalent Binding of 14C-TA to Liver Microsomes. When the concentration of TA incubated with rat liver microsomes was increased from 0.05 to 5 mM (100-fold), covalent binding of 14C-TA-derived radiolabel to rat liver microsomes increased only by 57-fold (1.42 ± 0.09 to 80.0 ± 6.5 mg/mg protein). A 10-fold increase in concentration of TA (0.05–0.5 mM) resulted in a 7-fold increase in covalent binding (1.42 ± 0.09 to 10.11 ± 1.5 mg/mg protein). A further 10-fold increase in concentration (0.5–5 mM) resulted in a 7.9-fold increase (10.11 ± 1.5 to 80.0 ± 6.5 mg/mg protein) in covalent binding. These results confirm the in vivo data on covalent binding of TASO2 to liver tissue, which is less than dose-proportional, suggesting saturation of TA bioactivation to TASO2.

Microsomal Metabolism of Increasing Concentrations of TA. TA was incubated with rat liver microsomes in standardized and optimized incubation conditions for 10 min with increasing concentrations of TA. The calculated $K_m$ and $V_{max}$ values for metabolism of TA to TASO by rat liver microsomes are 0.1 mM and 0.624 mmol/min, respectively. Increasing concentrations of TA (0.01–10 mM) incubated with microsomes containing NADPH-regenerating system revealed a decreasing percentage of TA metabolized to TASO (Fig. 5A), which remained constant from 0.5 mM onward. Control incubations did not produce any detectable levels of TASO. Disappearance of TASO was complete at 0.01 to 0.05 mM TA concentrations, indicating very efficient metabolism of TA at this low and narrow range of concentrations. Only 60% of TA was detectable as TASO at these two concentrations. Because TA was not detected in these incubations after 10 min (Fig. 5B), the remaining portion of TASO must have been further metabolized to TASO2, the ultimate reactive metabolite, and presumably to acetamide, a nontoxic metabolite of TA. At TA concentrations above 0.5 mM, the percentage of TA detected as TASO remained constant irrespective of the increase in TA concentration. Increasing the concentration of TA from 0.5 mM to 10 mM led progressively to higher proportions of the parent compound to remain in the incubations, although the same percentage of TASO was produced. This observation suggests that the metabolism of TA to acetamide may also be saturated, resulting in a progressively greater proportion of TA remaining in these incubations (Fig. 5B).

Microsomal Metabolism of TA with Increasing Time. To confirm that saturation of TA metabolism to TASO observed in the microsomal incubations is indeed due to zero-order kinetics, time-dependent metabolism of TA was examined. If the enzyme saturation is due to zero-order kinetics, the product formation rate should remain constant regardless of substrate concentration, and the amount of product formed should increase in direct proportion to incubation time. In this experiment, we incubated 0.5 mM TA with rat liver microsomes under the same conditions used for the experiments described above, except the length of time. The reactions were stopped at 10, 20, and 30 min. We observed increasing amounts of TASO production, and for each 10-min increment, the amount of additional TASO formed was the same (data not shown).

Microsomal Metabolism of Increasing Concentrations of TASO. TASO was incubated with microsomes to compare the relative efficiency of metabolism of TASO and under the same incubation conditions used earlier for TA. At the end of 10 min, 50% of TASO was left behind from the lowest concentration of TASO (0.01 mM) incubated (Fig. 6). The calculated $K_m$ and $V_{max}$ values for TASO using the substrate depletion in the rat liver microsomal incubations are 0.62 mM and 17.47 mmol/min, respectively. This result suggests that the second step of TA to its reactive metabolite TASO2 is 6-fold less efficient than the first step (0.1 mM for TA → TASO). Indeed, the metabolism of TASO (Fig. 6) is less efficient than that of TA (Fig. 5B). It should be noted that metabolism of TASO, which includes bioactivation to TASO2, was completely inhibited at 0.1 mM and higher concentrations. These findings are consistent with the sustained high plasma levels of TASO after the higher doses of TA (Fig. 2B).

### Table 2

<table>
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<th>Parameter</th>
<th>50 mg/kg</th>
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<th>600 mg/kg</th>
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<td>TA</td>
<td>TASO</td>
<td>TA</td>
<td>TASO</td>
</tr>
<tr>
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<td>5</td>
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<tr>
<td>$C_{max}$ (µg/ml)</td>
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<td>36 ± 1.2</td>
<td>74.5 ± 4</td>
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<td>Plasma Clearance (l/kg·h)</td>
<td>1.63 ± 0.21</td>
<td>0.82 ± 0.06</td>
<td>0.30 ± 0.01</td>
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TA Incubations with cDNA-Expressed CYP2E1 Supersomes. These incubation experiments were aimed at two objectives: first to confirm that CYP2E1 is the major enzyme that mediates metabolism of TA; and, second, that if metabolism of TA is saturated, it should be more readily apparent with incubations containing only CYP2E1 and should occur at lower concentrations of TA. We used not only rat but also human CYP2E1 Supersomes because CYP2E1 is the major enzyme known to bioactivate several commonly encountered toxins (Gonzalez et al., 1991; Ingelman-Sundberg et al., 1994). Control incubations for rat and human Supersome experiments did not produce any detectable levels of TASO. Increasing concentrations of TA (0.01–10 mM) were incubated for 10 min with CYP2E1 (Supersomes; BD Gentest) in incubations containing the NADPH-regenerating system; at 0.01 mM TA concentration, TA was no longer detectable after 10 min incubation (Fig. 7A), and the highest proportion of TA was found as TASO (70% of the product was TASO) (Fig. 7B) when rat CYP2E1 was used, suggesting that CYP2E1 is the major isozyme that metabolizes TA to TASO. Saturation of sulfoxide production was evident at and above 0.5 mM TA, similar to that observed in rat liver microsomal incubations. The calculated $K_m$ and $V_{max}$ values for TA to TASO conversion with rat CYP2E1 are 5.78 mM and 1.47 mmol/min, respectively. It should be noted that metabolism of TA reached zero-order process at a much lower concentration of TA (0.05 mM) when incubated with Supersomes as compared with microsomal incubations (Fig. 5). Presumably, the difference is due to nonspecific TA binding to microsomal proteins.

When incubated with human CYP2E1 Supersomes, the results were almost the same as for the rat Supersomes in terms of the TA concentration at which saturation was evident, i.e., 0.5 mM (Fig. 8). However, the human CYP2E1 enzyme seemed to be more efficient in TASO production at 0.05 and 0.1 mM initial TA concentration (Fig. 8B) than did the rat CYP2E1 (Fig. 7B).
Discussion

The present data confirm that TA bioactivation is saturated at higher doses and suggest that it is likely to be the mechanism for the observed absence of a simple dose-response relationship for bioactivation-based liver injury observed with TA in previous studies (Mangipudy et al., 1995; Ramaiah et al., 1998). Thioacetamide is extensively used as a model hepatotoxin in acute and chronic studies, and thus, characterization of toxicokinetics of this compound is likely to be highly useful.

Several lines of evidence suggest that the first step of TA bioactivation (TA → TASO) is saturable. First, the ratio of TASO to TA decreased with increasing doses of TA, consistent with saturable metabolism of TA to TASO, and, therefore, reduced efficiency of metabolism to TASO, the penultimate reactive metabolite, at the higher doses. Plasma protein binding of TA is not a confounding factor in this assessment. Binding of TA to plasma proteins is relatively low (9.93 ± 1.16%) and did not differ, with a 20-fold increase in TA concentration. Second, higher doses of TA resulted in more than dose-proportional increases in plasma AUC of TA. Third, an increase in dose of TA resulted in an increase in time to reach peak plasma concentration of TASO and is accompanied by an increase in apparent TA elimination half-lives. Fourth, the liver levels of TA and TASO showed a profile similar to that of the plasma. Fifth, results of the in vitro rat liver microsomal and rat and human supersomal incubations also revealed saturation of TA metabolism. Furthermore, bioactivation of TASO to the ultimate reactive metabolite TASO₂ is also saturable and less efficient than the first step. From the present study, three lines of evidence suggest that the efficiency of the second step of TA bioactivation (TASO → TASO₂) is even lower than the first step (TA → TASO). First, after reaching peak concentrations of TASO in plasma, TASO concentration is sustained for nearly 3.8 h after a 300 mg/kg dose and for 9 h after the lethal dose (600 mg/kg). Moreover, the dose-normalized AUC of TASO increased with higher doses of TA. This is highly suggestive of saturation of TASO to TASO₂ conversion. Second, a lower than expected increase in covalent binding of 14C-TA-derived radiolabel to liver proteins was observed. The initiation of the hepatotoxicity of TA is due to covalent binding of highly reactive TASO₂ to liver macromolecules (Miller and Miller, 1974; Hunter et al., 1977; Porter et al., 1979). In the present study, we evaluated both dose- and time-dependent covalent binding of TA to liver tissue in rats. These findings are consistent with and explain why initiation of bioactivation-mediated liver injury did not show a dose-response (Mangipudy et al., 1995). This observation was further confirmed in rat liver microsomal incubations of ¹⁴C-TA. Covalent binding of ¹⁴C-TA-derived radiolabel to microsomal proteins was only increased 57-fold when ¹⁴C-TA concentration was increased 100-fold. Third, additional in vitro microsomal incubation studies also support this conclusion. In rat liver microsomal incubations of TA and TASO, the calculated Kₘ value for conversion of TASO to TASO₂ (0.62 mM) is 6-fold higher than the Kₘ value for
conversion of TA to TASO (0.1 mM), indicating that the second step of TA bioactivation is 6-fold less efficient. This is consistent with previous findings about the affinity of these compounds to microsomes. The \( K_a \) value of TA metabolism is reported to be lower (0.06 mM) than that of TASO metabolism (0.4 mM) in microsomes (Porter and Neal, 1978).

In our study, we chose to measure urinary excretion of TA and TASO at 12 and 24 h at two different doses of TA based on previous studies reporting that most of the TA and TASO is eliminated in the urine by 24 h (Nygaard et al., 1954). The Nygaard study reported about 20% elimination as TA and 60% as sulfate using a single dose of 6 mg of TA per rat after subcutaneous administration. We found less than 4% of the dose in the urine as the parent compound. The urinary elimination profile of TASO, the proximate reactive metabolite, showed 3-fold higher excretion of TASO at the high dose than after the low dose. This finding is the fourth line of evidence which suggests that the metabolism of TASO is slower and more saturable than the metabolism of TA, due to which more TASO is excreted unmetabolized relative to TA, further supporting the above conclusion. There is a possibility that the excretion of TASO is higher at the high dose because of higher efficiency of transporters. Since there is not much known about the role of transporters in excretion of TA or TASO, this hypothesis remains speculative at this point.

In vitro studies were conducted to confirm the in vivo results of saturation of TA metabolism by CYP2E1. The range of TA concentrations chosen for these experiments included those comparable to the concentrations found in plasma and liver upon i.p. administration. The plasma TA levels far exceeded the Michaelis-Menten constant (\( K_m = 0.1 \text{ mM} \) or 7.5 \text{ mg/ml}) after i.p. administration of 300 and 600 mg/kg doses for a prolonged time. It should be recalled that plasma protein binding of TA is only 9.93\% and does not change with increased concentration of TA. In hepatic microsomal incubations, at higher concentrations of TA, the remaining percentage of TA is presumably metabolized to the non-toxic acetamide, and as TASO\(_2\), the latter being covalently bound to the microsomes. One may expect that some of the TA could be lost to nonspecific binding in the microsomal incubations. Experiments in which increasing concentrations of TA were incubated with liver microsomes confirmed that TASO metabolism also follows saturable kinetics.

Incubations of TA with cDNA-expressed Supersomes should be helpful in minimizing alternative effects due, for example, to nonspecific binding or metabolism by other enzymes. These experiments are also useful to confirm that TA metabolism is mediated by CYP2E1. Moreover, Supersomes are devoid of other proteins that would be present in liver microsomes, and therefore, saturation of TA metabolism should occur at lower concentrations of TA. Therefore, TA was incubated with rat liver-specific cDNA-expressed CYP2E1 Supersomes. In these incubations, no TA was detected at the end of 10-min incubations containing 0.01 mM TA, and at 50-fold higher concentration, the enzyme was saturated. It is apparent that CYP2E1 catalyzes at least 75\% conversion of TA to TASO at 0.01 mM. Since CYP2E1 is a toxicologically significant enzyme because it bioactivates many drugs, industrial solvents, and carcinogens, making it of direct relevance to humans, we also incubated TA with the human cDNA-expressed CYP2E1 enzyme Supersomes. The results confirm that saturation and the zero-order process occur at TA concentrations of 0.5 mM and upwards, similar to the rat Supersomes. At lower concentrations of 0.01 and 0.05 mM, 60\% of TA was converted to TASO. The decision to use only CYP2E1 Supersomes in these experiments is based on the previous work establishing that CYP2E1 is the primary enzyme that mediates TA bioactivation (Wang et al., 2000). Other isoforms of CYP450 such as CYP1A1 and CYP2B1/2 are unlikely to be involved in its bioactivation: El-Hawari and Plaa (1983) reported that neither induction by 3-methylcholanthrene (CYP1A1) nor by phenobarbital (CYP2B1/2) increased liver injury of TA. Although flavin monooxygenase has been implicated in the bioactivation of TA, it was not found to be involved in the bioactivation of TA (Wang et al., 2000; Ramaiah et al., 2001). However, the potential contribution of these enzymes in the metabolism of TA has not been studied in Supersomes or pure isozymes.

In conclusion, our study shows that TA exhibits dose-dependent metabolism. Our results reveal saturation of CYP2E1-catalyzed TA metabolism to TASO and metabolism of TASO to TASO\(_2\) in vivo as well as in vitro. The results of toxicokinetic studies, enzyme kinetic studies, and covalent binding experiments suggest lower efficiency at the second step, which is the rate-limiting step. In conclusion, the present study has revealed that TA bioactivation follows saturable kinetics. Metabolic saturation is thus responsible for the lack of dose-response, i.e., initiation of injury increasing in proportion to the dose, for liver injury observed with TA.

References


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