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Nephrotoxicity of 5-(N-phenylcarboxamido)-2-thiobarbituric acid in the Fischer 344 rat

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Summary. In the present investigation, administration of a single i.p. dose of the anticancer drug merbarone [5-(*N*-phenylcarboxamido)-2-thiobarbituric acid] produced an acute and reversible decrease in renal function in female but not male Fischer 344 rats. The renal lesion in female rats was biochemically characterized as a decrease in *p*-aminohippuric acid accumulation by renal slices along with polyuria, glucosuria, proteinuria, and enzymuria. These functional changes were accompanied by histopathologic changes of focal tubular necrosis that was confined to the deep cortex and outer stripe of the outer medulla. The changes in these parameters were dose-dependent and were observed at doses as low as $0.2\times$ $MELD^{10}$ (12 mg/kg). This low merbarone dose increased urinary glucose and protein excretion by 26- and 9-fold, respectively, in the initial 16-h urine collection in female rats. This increase was accompanied by a 2- to 15-fold increase in the excretion of *N*-acetyl-β-D-glucosaminidase (NAG), γ-glutamyl transpeptidase (γ-GTP), and lactate dehydrogenase (LDH) activities. No significant changes in renal function were observed in male rats apart from mild enzymuria after a high dose of merbarone (36 mg/kg). The drug did not increase urea nitrogen levels in male or female rats, reflecting the focal nature of this tubular lesion. Merbarone produced small elevations in serum transaminase activities [i.e., glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT)] at doses that produced marked alterations in renal function in female rats, suggesting only mild hepatotoxicity. The present study establishes the kidney as a possible dose-limiting target organ for merbarone toxicity.

Introduction

Merbarone [5-(*N*-phenylcarboxamido)-2-thiobarbituric acid; NSC 336628] (Fig. 1) is an anticancer drug currently under investigation that was selected for clinical trials based on its curative activity in L1210 leukemia and its significant antitumor activity in several other murine tumors after i.p. or oral administration [4, 21]. The cytotoxic mechanism of merbarone is not known. However, initial studies have shown that the drug produced a dose-dependent increase in DNA single-strand breaks in L1210 cells [6]. The single-strand breaks were not accompanied by the formation of protein-DNA cross-links, and merbarone was not found to possess alkylating activity. Distribution studies in mice demonstrated that merbarone underwent extensive enterohepatic circulation and accumulated in the liver and kidney, with very low levels of drug observed in the brain [15].

Preclinical toxicologic studies in mice, rats, and dogs revealed that target organs for toxicity were the central nervous system, bone marrow, kidney, and liver [19]. The structural similarity of merbarone and CNS-depressant barbiturates along with preclinical observations demonstrating CNS toxicity suggest that the CNS may be the dose-limiting target. However, unlike most 5,5-disubstituted barbiturates, merbarone accumulates at low levels in the brain and is unlikely to exhibit significant CNS-depressant activity at therapeutic doses [15].

In contrast, the liver and kidney accumulated the greatest concentrations of merbarone, and toxicity to these organs may possibly limit the maximal therapeutic dose. More detailed analysis of the potential toxic effects of this drug is necessary, as it is currently undergoing phase I clinical trial in cancer patients. In the present study, we characterize the effect of merbarone on kidney function in Fischer 344 rats. These studies demonstrate that merbarone produced an acute and reversible renal injury that was more pronounced in female than in male rats.

Materials and methods

Animals. Fischer 344 (F344) rats (Taconic Farms, Germantown, NY) weighing 130–170 g were housed in a temperature- and light-cycle-controlled (12 h light, 12 h darkness) room and were allowed food and water ad libitum prior to the experiments.

Treatments. Merbarone was obtained from the Pharmaceutical Resources Branch of the National Cancer In-

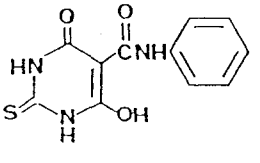

Fig. 1. Structure of merbarone [5-(*N*-phenylcarboxamido)-2-thiobarbituric acid]

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stitute and was dissolved in an *N*-methylglucamine (NMG; Chemical Dynamics Co., South Plainfield, NJ) aqueous solution in which the ratio of merbarone to NMG was 1:2. The solution was adjusted to pH 9.5 with 0.5 *N* hydrochloric acid and given in a total volume of 10 ml/kg body weight. Each group of rats received a single i.p. injection of 0 (vehicle, 72 mg NMG/10 ml), 12, 24, 36, or 50 mg/kg merbarone.

Kidney-slice studies. After their removal, the kidneys were immediately placed in ice-cold saline. Accumulation of organic anion and cation was determined by a modification of the technique of Cross and Taggart [7], as previously described [16]. Thin slices (approximately 100 mg) of the renal cortex were prepared freehand and incubated in medium containing 7.4×10^{-5} *M* *p*-aminohippuric acid (PAH), 10^{-5} *M* tetraethylammonium (TEA), [^{14}C]TEA (0.02 μCi/ml; New England Nuclear, Boston, Mass), and 10^{-4} *M* lactic acid (Sigma Chemical Co., St. Louis, Mo). The slices were incubated at 25° C under 100% oxygen for 90 min using a Dubnoff metabolic shaker. PAH was determined by the colorimetric method of Smith et al. [23] and TEA, by measuring the radioactivity present in the trichloroacetic acid extracts from the slice and medium. The accumulation of PAH or TEA in renal slices was expressed as the slice-to-medium (S/M) ratio, where S represents milligrams of PAH of dpm [^{14}C]TEA/g tissue and M represents milligrams of PAH or dpm [^{14}C]TEA/ml medium.

Serum analysis. Serum was assayed for blood urea nitrogen (BUN, Sigma Kit 535) and activities of glutaminic-oxalacetic transaminase (GOT, Sigma Kit 505) and glutamic-pyruvic transaminase (GPT, Sigma Kit 505-P).

Urinalyses. Rats were individually placed in Nalgene metabolic cages (Sybron/Nalge, Rochester, NY) with free access to water, but food was withheld during the 16-h (5 p.m.–9 a.m.) collection period. At 9 a.m., the animals were transferred to stainless-steel cages in groups of five and allowed free access to both food and water. After a 3-day period of acclimation to the cages, the animals were given merbarone. Urinalyses were conducted on individual 16-h urine specimens collected on ice. At the end of each period, urinary volume was recorded and measurements of glucose (Sigma Kit 16-UV) and protein were done [3] using commercial reagents (Bio-Rad Laboratories, Richmond, Calif). An aliquot of each urine specimen was subjected to Sephadex G-25 column chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden) to remove interfering substances and then eluted with 0.9% saline (22). This eluent was used to determine the activities of lactate dehydrogenase (LDH; [18]), *N*-acetyl-β-D-glucosaminidase (NAG; [11]), and glutamyl transpeptidase (γ-GTP; [24]).

Histology. Kidney sections were fixed in buffered formaline solution, and 6-μm paraffin sections were prepared and stained with hematoxylin and eosin.

Statistics. The mean values for each treatment group were compared with the corresponding control values using the Mann-Whitney U-test.

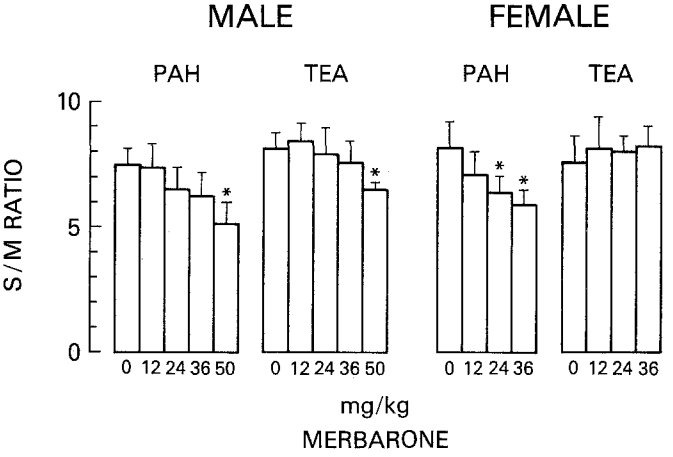

Fig. 2. Effect of in vivo merbarone treatment on PAH and TEA accumulation (slice/medium; S/M ratio) by renal slices. Rats were given a single i.p. injection of vehicle or 12, 24, 36, or 50 mg/kg merbarone, and renal slices were prepared 24 h later. Each value represents the mean ±SD from five rats. * Significantly different from control, $P < 0.05$

Results

Renal-slice accumulation of organic ions

The administration of merbarone to rats in vivo produced a decrease in the accumulation of organic anions (i.e., PAH) or cations (i.e., TEA) by the renal cortical slices in vitro (Fig. 2). PAH accumulation was decreased in a dose-dependent manner, significantly so at doses of 24 mg/kg and above in females and 50 mg/kg in males. TEA accumulation was significantly reduced only in male rats following a 50-mg/kg dose; in contrast, merbarone had no effect on TEA accumulation in female rats.

Serum biochemistry

Table 1 shows that merbarone treatment did not affect BUN levels; however, serum transaminase activities were slightly elevated. Serum GOT and GPT activities were increased in male rats receiving a dose of ≥24 mg/kg. In female rats, merbarone increased only serum GOT levels.

Table 1. Effect of merbarone on BUN and serum transaminase in male and female rats[a]

	Dose (mg/kg)	BUN (mg/dl)	GOT (units/l)	GPT (units/l)
Male	0	19.5±3.2	155±13	33.3± 6.3
	12	18.2±3.5	146± 5	33.8± 4.6
	24	16.8±1.7	183± 9*	46.7±11.1*
	36	18.1±2.7	194±22*	45.6±11.9*
	50	20.9±4.3	235±13*	63.0±13.0*
Female	0	20.9±1.1	149± 2	36.4± 4.6
	12	22.6±1.1	152± 4	36.9±13.1
	24	17.7±1.7	179±19*	34.7± 9.9
	36	17.7±3.4	188±14*	41.3±11.0

[a] 1 day after the injection of merbarone
* $P < 0.05$

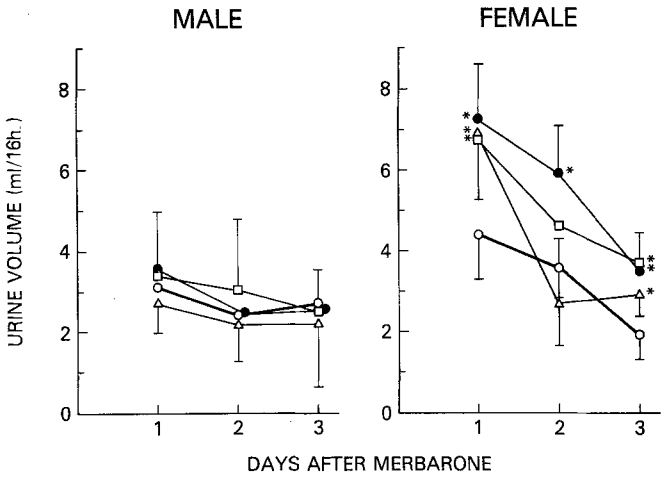

Fig. 3. Effect of merbarone on 16-h urinary volume. Rats were supplied water ad libitum and were given a single i. p. injection of merbarone (0, ○; 12, △; 24, □; or 36 mg/kg, ●) following a 3-day acclimation to the metabolic cages; thereafter, 16-h urine samples were collected at the times indicated. Each value represents the mean ±SD from five rats. * Significantly different from control, $P < 0.05$

These increases in serum transaminase were observed 1 day following drug treatment and returned to control values within 48 h (not shown). The elevation in serum transaminase activity suggests slight hepatocellular damage.

Urinalyses

Urinary volume and excretion of protein and glucose LDH, NAG, and γ-GT activities were used to assess

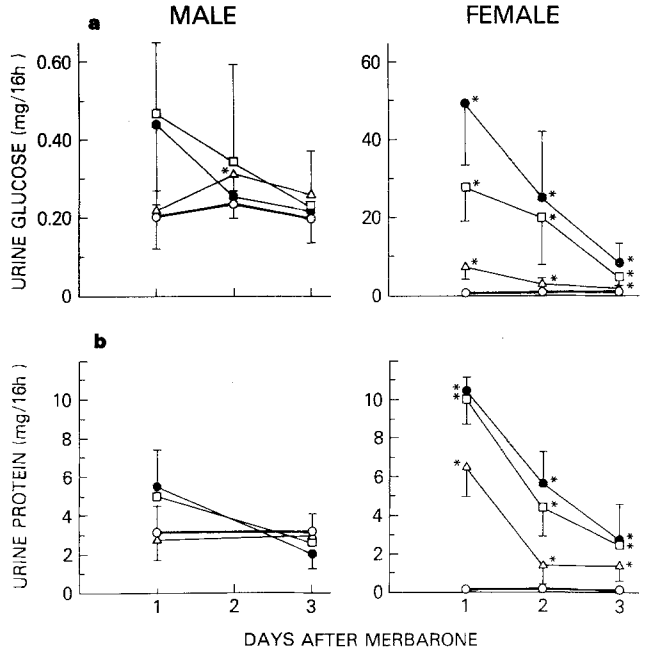

Fig. 4. Effect of merbarone on the urinary excretion of **a** glucose and **b** protein. Rats were given a single i. p. injection of merbarone (0, ○; 12, △; 24, □; or 36 mg/kg, ●), and 16-h urine samples were collected at the times indicated. Each value represents the mean ±SD from five animals. * Significantly different from control, $P < 0.05$

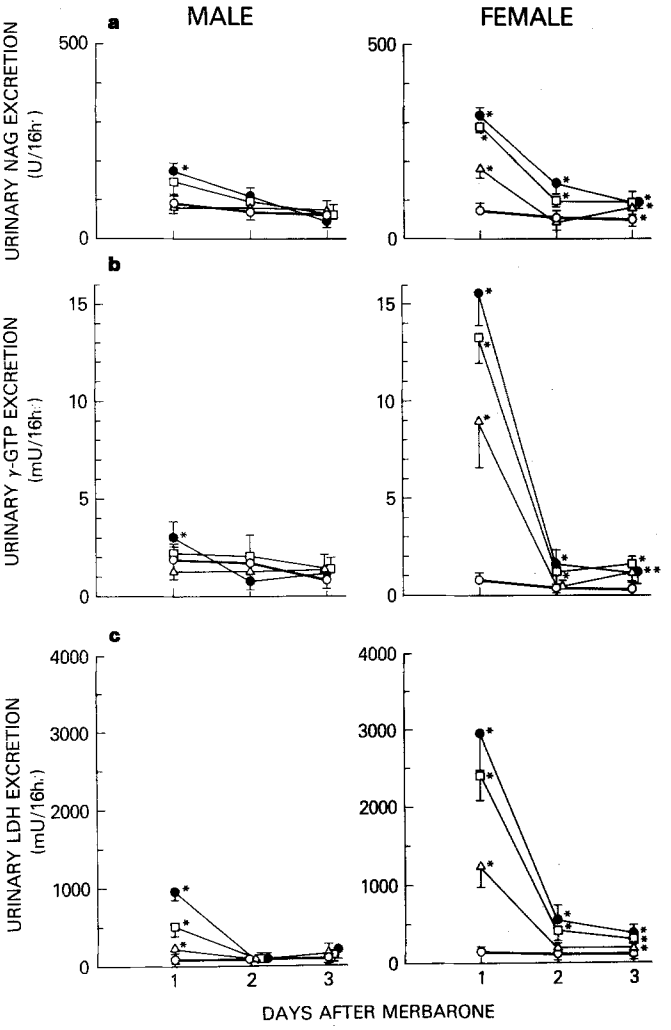

Fig. 5. Effect of merbarone on the urinary excretion of **a** NAG, **b** γ-GTP, and **c** LDH activities. Rats were given a single i. p. injection of merbarone (0, ○; 12, △; 24, □; or 36 mg/kg, ●), and 16-h urine samples were collected at the times indicated. Each value represents the mean ±SD from five animals. * Significantly different from control, $P < 0.05$

tubular function. The administration of merbarone to female rats resulted in a dose-dependent elevation in urinary volume (Fig. 3), proteinuria and glucosuria (Fig. 4), as well as enzymuria (Fig. 5). The elevation in these indices of renal injury were maximal in the initial 16-h urine specimen and had nearly returned to control levels by the 3rd day after merbarone treatment. However, on day 3, slight but statistically significant increases in these parameters were observed in all merbarone-treated female rats. In contrast, in male F344 rats merbarone resulted in no significant change in renal function apart from mild enzymuria at the highest tested dose (36 mg/kg).

Urinary LDH was found to be the most sensitive index of merbarone nephrotoxicity in male rats (Fig. 5). LDH excretion was maximal in the initial 16-h urine specimen, showing an approximately 11-fold increase over control values. This compares with the 37-fold increase in urinary LDH observed in female rats. In female animals, urinary glucose was the most sensitive index of merbarone nephrotoxicity, and its excretion was also maximal in the initial 16-h urine specimen (Fig. 4). Low-dose merbarone

(12 mg/kg) produced a 26-fold increase in urinary glucose, and high-dose merbarone (36 mg/kg) increased urinary glucose 170-fold. Drug-induced glucosuria was accompanied by marked proteinuria (Fig. 4) and a small decrease in serum glucose levels (data not shown), indicative of tubular injury. In male rats, high-dose merbarone produced only a twofold increase in urinary glucose.

Histopathology

The biochemical alterations in renal function resulting from merbarone treatment were accompanied by histopathologic changes (not shown). The renal damage to female F344 rats was characterized by multifocal tubular cell degeneration and necrosis that was confined to the deep cortex and outer stripe of the outer medulla (OSOM). The lesions produced in male rats were less striking and showed no marked pathology other than mild congestion, a slight vacuolization of proximal tubules in the deep cortex, and occasionally sclerotic glomeruli. Lesions in male and female rats were maximal 1 day after merbarone treatment and had resolved by day 3.

Discussion

The present study establishes the kidney as a possible dose-limiting target organ for merbarone toxicity. This is consistent with initial phase I clinical trials showing elevated creatinine levels [8] and renal insufficiency [17] in patients receiving the drug by continuous infusion. In our model, merbarone was nephrotoxic in female but not male F344 rats. It is not yet known whether similar sex-related differences in susceptibility to renal injury exist in patients.

The renal lesion in female rats was biochemically characterized as a slight reduction in the accumulation of PAH by renal slices along with polyuria, glucosuria, proteinuria, and enzymuria (Figs. 2–5). These functional changes were accompanied by histopathologic changes involving focal tubular necrosis that was confined to the deep cortex and OSOM (data not shown). The changes in these parameters were dose-dependent and were observed at doses as low as $0.2 \times \text{MELD}^{10}$ (i.e., 12 mg/kg). The renal injury was maximal 1 day after treatment and was readily reversible.

Merbarone produced small elevations in serum transaminase activities at nephrotoxic doses (Table 1), suggesting that it was only mildly hepatotoxic. The drug-induced elevation in SGOT activity was comparable in both male and female rats; however, SGPT activity was elevated only in male rats. Thus, in contrast to the renal lesion, merbarone-induced hepatotoxicity was not sex-related.

Sex-related differences in susceptibility to renal injury have been reported for a variety of nephrotoxicants including 4-ipomeanol [13], mercuric chloride [9], cephaloridine [1], gentamicin [2], and hexachloro-1,3-butadiene [10]. Sex-dependent differences in liver or kidney drug metabolism and detoxification mechanisms are considered to be responsible for moderating the sex-related susceptibility to renal injury [5, 14]. In this regard, merbarone was extensively metabolized in dogs and mice and was found to be highly tissue-bound [20]. Moreover, sex-related differences in the plasma pharmacokinetics of parent merbarone were observed in mice [20]. These studies established that the plasma half-life of merbarone was twice as long in male mice as in female animals (2.5 vs 1.1 h) and that renal excretion accounted for 24% and 60% of the drug in dogs and rats, respectively. Thus, the kidneys of female rats may be exposed to higher initial levels of merbarone and/or its metabolite(s) than male rat kidneys. A relationship between tissue concentration and target organ toxicity is consistent with a previous observation that the liver and kidney were the principal organs that accumulated merbarone [15].

Although tissue levels appear to correlate with target organ toxicity, the cellular mechanism for the renal injury and/or antitumor activity of merbarone remains unclear. Using electron spin resonance (ESR) and spin-trapping techniques, liver microsomes have been shown to generate free radicals in the presence of merbarone (Dr. D. Cooney, personal communication). A free-radical mechanism of antitumor activity is consistent with the merbarone-induced formation of non-protein-associated DNA strand breaks in L1210 cells [6]. Merbarone has also been shown to inhibit topoisomerase II activity in vitro by a mechanism that does not involve the formation of the covalent topoisomerase II-DNA complex [12]. This differs from the topoisomerase inhibition produced by agents such as etoposide or amscarine. It is not known whether merbarone causes renal injury by similar mechanisms. However, a further understanding of the mechanism for the sex-dependent toxicity of merbarone may provide some insight into the mechanism underlying the antitumor activity of this new chemotherapeutic agent.

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