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METABOLISM OF A NOVEL NITROSOUREA, TAUROMUSTINE, IN THE RAT

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Abstract—A novel nitrosourea, 1-(2-chloroethyl)-3-[2-(dimethylaminosulphonyl)ethyl]1-nitrosourea, tauromustine (TCNU), has been investigated for *in vitro* metabolism by different rat organs. Two kinds of reactions were seen, demethylation and denitrosation, both reactions required NADPH. These reactions were achieved by the liver microsomes and to a much lesser extent by lung microsomes. Induction of cytochrome P450 system with phenobarbital resulted in increased demethylation (10 times) and denitrosation (6 times) of tauromustine while induction with 3-methylcholanthrene did not have any significant effect on these reactions. Known inhibitors of different cytochrome P450 activities inhibited the demethylation and denitrosation of tauromustine to different levels. After oral administration of [^{14}C]tauromustine a metabolic pattern similar to that observed *in vitro* experiments, was seen in the urine. The demethylated compound, which has alkylating cytotoxic activity, could be detected in the urine up to 8 hr after oral administration.

Chloroethylnitrosoureas have been used in the treatment of cancer for at least two decades. The first nitrosourea compounds to demonstrate therapeutic activity were BCNU [1,3-bis-(2-chloroethyl)-1-nitrosourea] and CCNU [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea] [1, 2] to be followed by others [3, 4]. However, these compounds exhibit a delayed haematological toxicity, which can make it difficult to use these compounds in combination with other chemotherapeutic agents. However, considerable effort has been put into development of new nitrosoureas with special carrier groups, including sugar, nucleosides and amino acids to achieve optimal patterns of toxicity and antitumour activity.

Tauromustine, 1-(2-chloroethyl)-3-[2-(dimethylaminosulfonyl)-ethyl]-1-nitrosourea (TCNU), is a novel nitrosourea based on the endogenous amino acid taurine [5, 6], see Fig. 1. Tauromustine can be enzymatically metabolized and degraded *in vitro* and *in vivo*. This paper describes the two main routes of the metabolism of tauromustine in the rat, namely demethylation and denitrosation.

MATERIALS AND METHODS

Chemicals. [^{14}C]Tauromustine and [^{3}H]tauromustine with a specific activity of 5.8 mCi/mmol and 20.1 mCi/mmol, respectively, were synthesized at Pharmacia LEO Therapeutics AB, Helsingborg, Sweden with a radiochemical purity >95%. The compound was labelled with ^{3}H in the taurine moiety and with ^{14}C in the chloroethyl part of the molecule. A mixture or [^{14}C]- and [^{3}H]tauromustine (1.25:0.75; w/w) was used as substrate. The non-radioactive reference substances, tauromustine, LS 2715, LS 2724, LS 3114, LS 3850 and LS 3874, see Fig. 2, were also synthesized at Pharmacia LEO Therapeutics AB, Helsingborg.

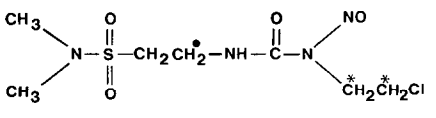

Fig. 1. Structure of tauromustine including the positions of ^{14}C (*) and ^{3}H (•).

NADPH, reduced glutathione (GSH), UDP-glucuronic acid (UDPGA), *n*-octylamine, metyrapone, 3-methylcholanthrene and Triton X-100 were obtained from the Sigma Chemical Co. (St Louis, MO). Alpha-naphthoflavone was purchased from Fluka AG Chemische Fabrik (Switzerland). Phenobarbital was purchased from Apoteksbolaget (Sweden). All other chemicals were standard commercial products of analytical grade.

Animals and preparations. Male Sprague–Dawley rats, 6 weeks old weighing about 190 g, were used. The rats were kept on a standard laboratory diet and starved overnight before being killed by decapitation. Liver, lung, kidney and testis were taken and kept in ice-cold 0.25 M sucrose at a final concentration equivalent to 1 g wet tissue per 5 mL. The microsomal fractions were resuspended in 0.25 M sucrose [7]. Groups of two animals were injected i.p. with 3-methylcholanthrene (20 mg/kg body wt in sesame oil, once daily for 4 days before being killed), phenobarbital (80 mg/kg body wt in isotonic NaCl, once daily for 4 days before being killed). Control animals were inoculated with equal volumes of sesame oil or isotonic NaCl solution. In one experiment four rats were given 3 mg/kg [^{14}C]tauromustine orally. Urine and faeces were collected 2, 4 and 8 hr after administration and acidified with acetic acid. Faeces were homogenized with methanol and evaporated before the extraction with ethyl acetate prior to analysis by HPLC.

Assay conditions. The metabolism of labelled tauromustine at 1.9 mM was determined in the microsomal fraction with a final concentration of

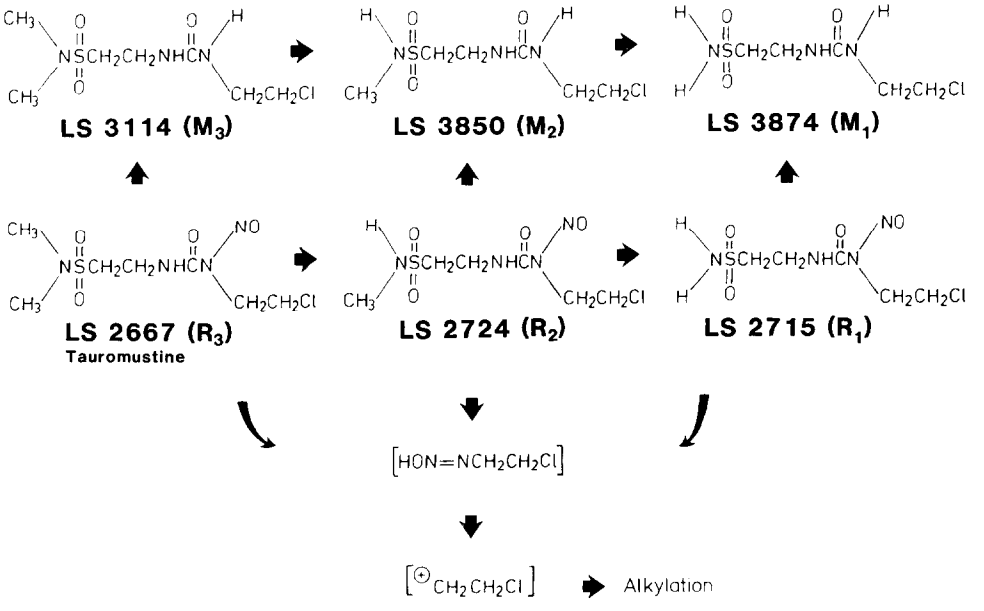

Fig. 2. Proposed metabolic pathways for tauromustine.

1 mM NADPH in a 50 mM phosphate buffer pH 7.4, for 10–120 min at 37°. The reactions were stopped by adding an equal volume of ice-cold acetone. After centrifugation the supernatant was used to determine the different metabolites by HPLC. The cytochrome P450 concentration in liver microsomes was determined according to Omura and Sato [8]. The microsomal fractions were either used immediately after preparation or stored at −70°. No detectable loss of activity could be seen after the storage of the microsomal fractions at −70° for at least a period of 2 months. Conjugation of tauromustine with reduced glutathione (GSH) was studied with a final concentration of 5 mM GSH in 50 mM phosphate buffer pH 7.4 in the cytosol. Conjugation of tauromustine with UDPGA was studied at a final concentration of 2 mM UDPGA in 50 mM phosphate buffer pH 7.4 with or without 0.05% Triton X-100 in the microsomal fraction. The protein content was assayed according to Lowry *et al.* [9] using bovine serum albumin as standard.

HPLC-system. An HPLC-system, Waters 600 Multisolvent Delivery System (Waters Division at Millipore, U.S.A.), including a Nova Pak column, was used to separate the different metabolites, with a gradient from 4:1:95 to 70:30:0 (acetonitrile: methanol: ammonium acetate buffer pH 3.5) at room temperature. The radioactive metabolites were detected by radioactive flow detector, Flo-One Beta, (Radiomatic Instruments, U.S.A.) connected to the HPLC.

Mass spectrometry. Samples from the *in vitro* experiments were analysed by HPLC and different fractions from various peaks were collected. The fractions were extracted twice with ethyl acetate and after evaporating ethyl acetate the residual were dissolved in methanol. Samples were then analysed by thermospray-tandem-mass spectrometry (TSP-MS/MS), Finnegan MAT TSQ-70 (U.S.A.). The individual samples were identified by their characteristic MS fragmentation pattern with the use of appropriate reference standard.

RESULTS

Tauromustine possesses at least two possible metabolic pathways, apart from non-enzymatic degradation. Metabolism via demethylation is one such route and denitrosation another (Fig. 2). Such reactions are known to be performed via the cytochrome P450 system [10]. Metabolism via various conjugation reactions may also occur, but this is more difficult to evaluate at this stage.

Metabolism in liver microsomes

It is concluded from *in vitro* studies (Fig. 3), that both the demethylated product LS 2724 and the denitrosated product LS 3114 are formed in the presence of NADPH. To determine the activities of demethylation and denitrosation of tauromustine, an apparent K_m for the disappearance of tauromustine was calculated to 0.95 mM. In rat liver microsomes the specific activity of demethylation was 2.4 (nmol/min/mg protein) and denitrosation was 1.2 (nmol/min/mg protein) see Table 1. The demethylation and denitrosation activities in the liver expressed in nmol/min/nmol cytochrome P450 were 4.7 and 2.2, respectively.

Whether LS 3114 is demethylated to LS 3850 or whether the demethylated product LS 2724 from tauromustine is denitrosated to LS 3850 (see Fig. 2) cannot be clarified at this stage.

Demethylation and denitrosation in extrahepatic tissues

Considerably high activities of demethylation and denitrosation of tauromustine could be detected in rat lung microsomes, 0.9 (nmol/min/mg protein) and

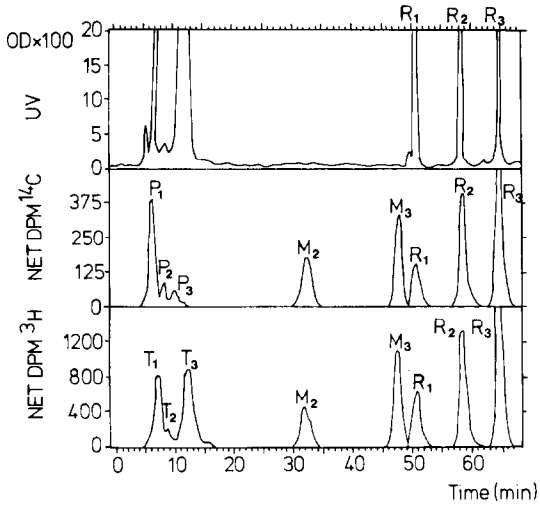

Fig. 3. HPLC-diagram of the ^{3}H- and ^{14}C-metabolites found after 10 min incubation of tauromustine and NADPH in rat liver microsomes. P_1-P_3 and T_1-T_3 are unknown polar products. M_2, M_3, R_1, R_2 and R_3 refer to LS 3850, LS 3114, LS 2715, LS 2724 and tauromustine.

Table 1. Specific activities of demethylation and denitrosation of tauromustine in microsomes from different rat organs

Organ	Type of reaction	Specific activity nmol / min × mg protein
Liver	Demethylation	2.4
Lung	Demethylation	0.9
Testis	Demethylation	<0.2
Kidney	Demethylation	<0.2
Liver	Denitrosation	1.2
Lung	Denitrosation	0.6
Testis	Denitrosation	<0.4
Kidney	Denitrosation	<0.4

The values given are the mean of 2–3 determinations, and did not differ from each other by more than 10%.

0.6, respectively (Table 1). These activities are only 50% lower than the same activities of rat liver microsomes. When kidney and testis microsomes were studied no activity could be detected. Not even an increased incubation time resulted in any detectable demethylated or denitrosated products.

Inhibition of demethylation and denitrosation reactions

Alpha-naphthoflavone, metyrapone, and *n*-octylamine are known inhibitors of different cytochrome P450 activities [10–12]. Here the demethylation and denitrosation of tauromustine were studied in the presence of these inhibitors.

It can be seen from Table 2 that metyrapone and *n*-octylamine inhibit the demethylation of tauromustine to a high degree, approximately 70 and 80%, respectively. On the other hand denitrosation was only marginally inhibited by *n*-octylamine (approx. 20%). Alpha-naphthoflavone inhibited the denitrosation of tauromustine to about 60% of the control activity while the demethylation decreased only 10 to 20%.

Induction of the metabolism of tauromustine

Phenobarbital and 3-methylcholanthrene are two well-known inducers of two different classes of cytochrome P450 isoenzymes in rat liver [13, 14]. Table 3 demonstrates that treatment of rats with 3-methylcholanthrene had no effect on the demethylation or denitrosation of tauromustine. On the other hand phenobarbital induced isoenzymes of cytochrome P450 had a considerable elevating effect on both demethylation, 996% of control, and denitrosation, 610% of control, of tauromustine.

Conjugation with GSH and UDPGA

To investigate different conjugation reactions, the cytosol and the microsomes from rat liver, lung, testis and kidney were incubated with tauromustine and GSH or UDPGA. Judged by HPLC, no additional polar peaks were seen after incubation with GSH or UDPGA. In addition, the microsomal fractions were treated with 0.05% Triton X-100 in an attempt to activate the enzyme, UDP-glucuronyltransferase. However, no additional peak could be detected after this activation.

In vivo *metabolism of tauromustine*

In order to investigate whether the metabolite pattern of tauromustine found after *in vitro* incubations could also be seen after *in vivo* treatment, rats were given [^{14}C]tauromustine orally at a dose of 3 mg/kg. The quantitative values of the different metabolites found in the urine at various times of collection can be seen in Table 4. Tauromustine could not be seen at any time. The demethylated products LS 2724 and LS 2715 could be detected up to 8 hr after administration, reaching its maximum between 2 and 6 hr.

Amongst the denitrosated products LS 3850 had the highest concentration at all times. The metabolite pattern of tauromustine found in urine 2 hr after oral administration is seen in Fig. 4. It is obvious on comparison of metabolites found *in vitro* that these are also present in the urine of rats treated with tauromustine.

The total radioactivity found in faeces was only 5% of that found in urine. After ethyl acetate extraction of homogenized faeces no detectable amounts of demethylated or denitrosated products were seen.

DISCUSSION

Tauromustine is a novel nitrosourea compound based upon taurine. The drug has shown potent activity against several experimental tumours and recently also proven its value in the treatment of colorectal cancer patients [4, 5, 15]. Tauromustine has pharmacokinetic properties which are different from those of other nitrosoureas and it has been speculated that this contributes to the antitumour activity of tauromustine [15]. In the present study the metabolism of tauromustine *in vitro* as well as *in*

Table 2. Effect of various compounds on the demethylation and denitrosation of tauromustine

	Percentage of control activity			
	Demethylation		Denitrosation	
Inhibitor	1.9 mM	0.96 mM	1.9 mM	0.95 mM
None	100	100	100	100
Alpha-naphthoflavone	83	92	60	66
Metyrapone	30	40	91	92
n-Octylamine	20	29	78	94

Rat liver microsomes from untreated rats were incubated with labelled tauromustine and NADPH for 10 min at 37°. The concentrations of inhibitors used were equal to and half of the substrate concentration. The values given are the mean of 2–3 determinations, and did not differ from each other by more than 10%.

Table 3. Induction of the demethylation and the denitrosation of tauromustine in rat liver

	Specific activity (nmol/min/mg protein)	
Treatment of animals	Demethylation	Denitrosation
NaCl solution	2.4 (100)	1.0 (100)
Phenobarbital	23.9 (996)	6.1 (610)
Sesame oil	2.7 (100)	0.6 (100)
3-Methylcholanthrene	1.9 (70)	0.6 (100)

The animals were treated by i.p. injection of each substance once daily for 4 days. The figures given in each group represent the mean of two animals, and the value within parenthesis are the percentage of the controls.

Table 4. The mean urinary concentration (nmol/mL urine) of some major metabolites of tauromustine in rats after a single oral dose of 3 mg/kg

	Time (hr)		
Compound	0–2	2–4	4–8
Tauromustine	0.1	0.1	0.1
LS 2724	18.1	32.0	3.1
LS 2715	16.8	32.4	12.6
LS 3114	39.2	75.4	29.4
LS 3850	45.4	106.4	65.4
LS 3874	16.4	32.6	26.3

vivo has been studied in the rat using radiolabelled tauromustine. For the *in vitro* studies subfractions from rat liver, lung, kidney and testis were used.

Incubation of tauromustine with microsomes from rat liver and lung in the presence of NADPH resulted in the demethylated product LS 2724. Further demethylation of LS 2724 to LS 2715 could only be detected in very small quantities. The denitrosation of tauromustine to LS 3114 could also be demonstrated in the microsomal system. These metabolites were identified by TSP-MS/MS as a part of a more extensive evaluation of tauromustine metabolism *in vivo* and *in vitro* (L. Svensson *et al.*, to be published).

The microsomal fraction contains, among many other membrane bound enzymes, different isoenzymes of cytochrome P450. Many different kinds of reductive and oxidative reactions can occur via this system [10]. Demethylation and denitrosation are two such reactions. The metabolism of tauromustine was found to be inhibited by various known inhibitors [11, 12, 16] of cytochrome P450. The induction of the cytochrome P450 system with phenobarbital resulted in a 10-fold increase of demethylation activity and a six-fold enhancement of denitrosation, while no effect was seen after 3-methylcholanthrene treatment. This relation is also true for other drugs such as methamphetamine [17, 18], and nitrosamines [19]. On the basis of the above mentioned results we concluded that the cytochrome P450 system is involved in the demethylation and denitrosation of tauromustine.

The rate of demethylation and denitrosation of tauromustine in liver microsomes was much higher than the corresponding activity in the lung microsomes and no detectable activity could be seen in the microsomal fractions from testis or kidney. No conjugation of tauromustine with either GSH or UDPGA could be detected *in vitro*. The results from the *in vitro* experiments confirm that tauromustine is mainly enzymatically degraded *in vitro* via the cytochrome P450 system in the liver and to a lesser extent in the lung, together with non-enzymatic decomposition.

The pharmacokinetic properties of tauromustine have been studied in patients participating in phase I clinical trials [16]. High plasma concentrations of tauromustine were found after oral administration

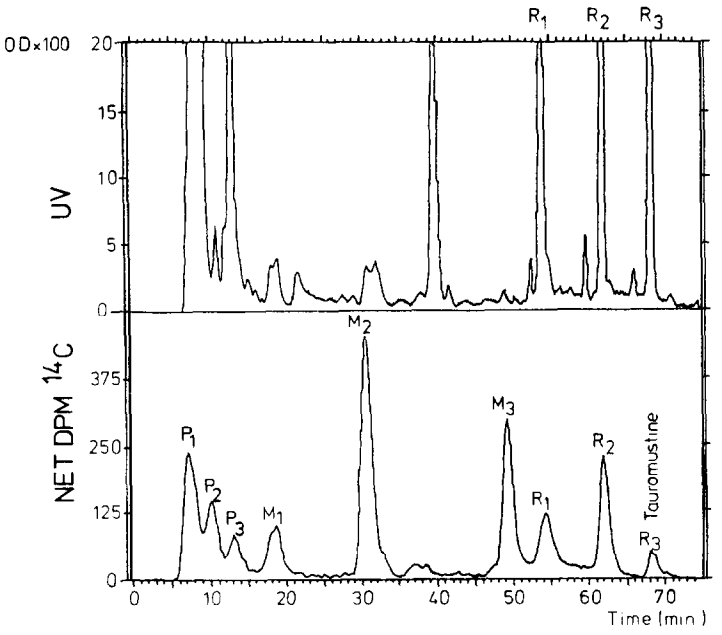

Fig. 4. HPLC-diagram of the ^{14}C-metabolites of tauromustine found in urine 2 hr after an oral dose of 3 mg/kg to rats. P_1-P_3 are unknown polar products. M_1, M_2, M_3, R_1, R_2 and R_3 refer to LS 3874, LS 3850, LS 3114, LS 2715, LS 2724 and tauromustine.

which indicated that tauromustine possesses pharmacokinetic properties which are different from those of other nitrosoureas. Our results demonstrate further interesting pharmacokinetic properties. Since the demethylated metabolites of tauromustine can exert alkylating activity [5], our results indicate that after oral administration of tauromustine a prolonged total alkylating effect could occur. The pharmacokinetic properties of tauromustine and its active metabolites may explain the good anti-tumour response to this drug.

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