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Thiourea toxicity in mouse C3H/10T½ cells expressing human flavin-dependent monooxygenase 3☆

P. Blaise Smith[a,*], Charles Crespi[b]

[a]*Department of Biochemistry, School of Medicine, Wake Forest University, Medical Center Boulevard, Winston-Salem, NC 27157-1019, USA*
[b]*Gentest, Woburn, MA 01801, USA*

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Abstract

Human flavin-dependent monooxygenase (FMO) isoforms 1 and 3 were expressed by retroviral gene transfer in mouse C3H/10T½ cells. FMO function was determined by the sulfoxidation of *p*-tolylmethylsulfide (TMS). Enzyme activity ranged from 4 to 30 nmol *p*-tolylmethylsulfoxide (TMSO)/30 min/mg cell protein for FMO 3 clones; for FMO 1 clones, the range was 1–6 nmol TMSO/30 min/mg. Cytotoxicity in these clones after exposure to thiocarbamate compounds was assessed by clonogenic assay. Thiourea (TU), phenylthiourea (PTU), and α-naphthylthiourea (ANTU) were toxic to FMO 3 cells but not to parental and FMO 1 clones; 50% toxicity was attained at 1×10^{-4} M TU, 5×10^{-6} M PTU, and 1×10^{-6} M ANTU. Toxicity was observed after a minimum exposure time of 6 hr. Parental cells were resistant to toxicity for exposure times spanning the entire clonogenic assay period (10 days). Ethylene thiourea (ETU) was not toxic to FMO 3 cells, but preincubation with 1×10^{-3} M ETU blocked TU toxicity. Reducing GSH levels by preincubation with 1×10^{-5} M buthionine sulfoxime (BSO) increased TU sensitivity in FMO 3 cells from 1×10^{-4} to 1×10^{-6} M to achieve 50% toxicity. BSO also increased the sensitivity of ''low expressor'' FMO 3 clones to TU, but did not alter the refractoriness of either parental or FMO 1 expressing cells to TU. *N*-Acetylcysteine afforded modest protection to TU toxicity by shifting 50% cytotoxicity for TU from 5×10^{-5} to 1×10^{-3} M. TU mutagenicity was assayed by the development of ouabain resistance in parental and FMO 3 C3H/10T½ cells. Exposure to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG, direct acting mutagen) and TU was executed with and without prior sensitization with BSO. The mutation frequency for MNNG was $76/1 \times 10^6$ surviving cells, whereas no mutants were observed for TU-exposed cultures. The results of this study show that, in isolation, the major human hepatic form of FMO is capable of promoting thiocarbamate toxicity. Consistent with the known reactivity of thiocarbamate intermediates with GSH, treatments that alter GSH levels also altered toxicity in either the protective or sensitizing direction. These cell lines expressing variable levels of FMO 3 and TU sensitivity should prove useful as *in vitro* systems for dissecting the thiocarbamate toxicity pathway.

Keywords: Thiourea; C3H/10T½ cell; huFMO 1; huFMO 3; Cytotoxicity

1. Introduction

Metabolic capability is the capacity of a cell to modify upon exposure the structure of a foreign chemical. The modification may facilitate inactivation of any intrinsic toxicity of the compound and hasten its elimination; alternatively, it may create intermediates that escape elimination and react with cellular constituents to initiate cell death, mutation, and carcinogenesis. A major determinant of metabolic capability is the isozyme composition and expression level of monooxygenases [1]. Monooxygenases catalyze the NADPH-dependent introduction of oxygen from O_2 into substrate and water, the first step in xenobiotic metabolism. Two families, the cytochromes P450 and the FMOs, mediate this reaction [2,3]. Both monooxygenase families share the characteristics of diverse substrate specificity, multiple isozyme subtypes, variable levels of inducibility, and types of chemical inducers [4]. Mammalian cells express combinations of CYP P450 and FMO isozymes that give each cell type a unique substrate

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* Corresponding author. Tel.: +1-336-716-4676; fax: +1-336-716-7671.
E-mail address: pbsmith@invader.bgsm.wfu.edu (P.B. Smith).

Abbreviations: FMO, flavin-dependent monoxygenase; huFMO 1, human flavin-dependent monooxygenase 1; huFMO 3, human flavin-dependent monooxygenase 3; BME, Basal Modified Medium; DMEM, Dulbecco's Modified Essential Medium; FBS, fetal bovine serum; TU, thiourea; ANTU, α-naphthylthiourea; ETU, ethylene thiourea; PTU, phenylthiourea; BSO, buthionine sulfoxime; TMS, *p*-tolylmethylsulfide; TMSO, *p*-tolylmethylsulfoxide; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; and RDRV, replication defective retrovirus.

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preference for xenobiotic metabolism and, as a consequence, susceptibility to the toxic effects of their metabolites [5].

TU is the parent compound for numerous thiocarbamate-based therapeutic drugs, agrichemicals, and chemicals used in the rubber, adhesive, and mining industry. TU is considered genotoxic based on the development of thyroid [6,7] and liver [8,9] tumors in rats exposed to it via their drinking water. Derivatives of TU, most notably ANTU, cause pulmonary edema via damage to airway endothelial cells [10]. TU used in the vulcanization process can in susceptible individuals induce contact dermatitis when they encounter rubber products [11]. *In vitro* cytotoxicity and genotoxicity evaluations of the thiocarbamates have been done in a number of cell lines that display different toxic endpoints. TU exposure in the range of 10^{-2} to 10^{-3} M has been claimed to result in chromosomal damage as evidenced by increased micronuclei formation and frequency of single-strand DNA scissions and intrachromosomal deletions [12]. TU is not mutagenic in the microsomal activation/Salmonella test system, and either feeble or negative TU mutagenicity has been reported by different laboratories for the HPRT locus in the V79 mammalian genotoxicity test system [13].

The metabolic activation of TU is believed to occur via *S*-oxidation of the thionocarbonyl functional group. FMO has been shown to catalyze this reaction for TU and derivatives [14,15]. These observations served as the basis for the present study in which human isoforms of FMO were singly expressed in C3H/10T½ cells to create a new capability for thiocarbamate metabolism.

2. Materials and methods

Mouse C3H/10T½ cells were purchased from the ATCC. The retroviral packaging cell line (ecotropic) GP + E-86 was provided by Dr. Arthur Bank, Department of Genetics and Development, Columbia University. The pMV7 vector DNA was provided by Dr. I.B. Weinstein of Columbia University. Tissue culture supplies, including BME and DMEM powder, penicillin/streptomycin (100× solution), FBS, and Geneticin (G418) were purchased from Life Technologies GIBCO/BRL. TU, PTU, and ANTU were purchased from the Aldrich Chemical Co. All other chemicals were of reagent grade and obtained from the usual commercial sources.

2.1. Construction of huFMO 1 and huFMO 3: pMV7 retroviral vectors

Human liver FMO 1 in the pACUW 42 vector was provided by Gentest, and human liver FMO 3 in pBluescript was obtained from Dr. Richard Philpot of the NIEHS. FMO 1 was released as a 1729 bp fragment after *Bgl*II digestion of the plasmid; FMO 3 was released as a 1785 bp fragment by *Acc*I digestion. Both retroviral vectors were constructed by blunt end ligation of these respective fragments into the *Eco*RI subcloning site of the retroviral vector designated pMV7. Both vector constructs were screened for successful insertions by restriction enzyme mapping to confirm a 5′ → 3′ orientation of each huFMO. Integrity of the 5′ and 3′ end, as well as orientation, was confirmed by DNA sequencing 250 bp bracketing the 5′ and 3′ ends of the subcloning site.

2.2. Production of RDRV

The pMV7:huFMO 3 vector was converted into an RDRV by calcium phosphate transfection into the GP + E-86 cell line as previously described for the cytochromes P450 [16]. GP + E-86 cells were seeded at 5×10^5 cells/100 mm dish in DMEM + 10% FBS. Twenty-four hours later, a calcium phosphate precipitate containing 14 μg vector DNA in 400 μL was prepared [17] and added to each 100 mm dish. The incubation with the precipitate was for 6 hr at 37°/6% CO_2 after which time the cells were washed twice with fresh medium. Selection was initiated by the addition of Geneticin (G418) at either 400 or 800 μg/mL. Medium with G418 was changed at 3-day intervals. By day 12 there were no survivors in the untransfected cell control. Surviving cells from the vector-transfected plates were grown to confluence with G418 selection at 800 μg/mL. To obtain infectious supernatant containing shed RDRV, G418-resistant cells were first grown without G418 for two passages in harvest medium (DMEM + 10% FBS + 1 μM hydrocortisone + 0.1 U/mL of insulin). Cells (5×10^6) were seeded in a T-75 flask, and 24 hr later medium was replaced with 3 mL of harvest medium and incubated for 16 hr. The medium was isolated and centrifuged at 1000 *g* for 5 min at 4° and served as the source of RDRV bearing the RNA copy of the pMV7: huFMO 3 vector.

2.3. Infection of mouse C3H/10T½ cells with RDRV

C3H/10T½ cells were seeded at a density of 2.5×10^5 cells/60 mm dish in BME + 10% FBS. Twenty-four hours later, medium was replaced with 1 mL BME (minus serum) containing polybrene (20 μg/mL) plus 1 mL of infectious harvest medium and incubated for 24 hr at 37°/6% CO_2. Then the cells were trypsinized with 2 mL trypsin/EDTA for 5 min and seeded at either 100 or 200 cells/100 mm dish in BME + 10% FBS. G418 selection (400 μg/mL of medium) was initiated the following day and changed on day 8. Individual clones were large enough by day 13 for ring isolation. Isolated clones were expanded and frozen at 5×10^6 cells/0.5 mL medium plus 10% DMSO, in liquid nitrogen.

2.4. TMS activity

Homogenates of individual clones were prepared by sonicating 5×10^6 cells in 1 mL of medium consisting

of 50 mM sucrose, 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM benzamidine, 1 mM diisopropylfluorophosphate, and 1 mM phenylmethylsulphonyl fluoride. To measure the sulfoxidation of TMS, a 0.25-mL reaction mixture containing 50 μg protein, 0.065 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL of magnesium chloride, and 2.0 mM TMS in 0.2 M glycine (pH 9.5) was incubated at 37° for 10 min. After incubation, the reaction was stopped by the addition of 75 μL acetonitrile and centrifuged (10,000 *g*) for 5 min at room temperature. One hundred microliters of the supernatant was injected into a 4.6 × 250 mm 5 μm C18 HPLC column and separated at 45° with a mobile phase initially of 46% methanol increasing to 55% methanol over 7 min (the substrate was then eluted with 100% methanol) at a flow rate of 1 mL/min. The product was detected by its absorbance at 237 nm and quantitated by comparing the absorbance to a standard curve of TMSO [18].

2.5. Cytotoxicity

The cytotoxicity of TU and derivatives was assessed by a clonogenic assay. In preparation for a typical study, parental and FMO 3 expressing C3H/10T½ cells were grown to ~80% confluency in BME + 10% FBS. Cells were trypsinized, immediately diluted 1:10 in medium, and counted. A stock suspension of cells (200–500 cells/7 mL) was prepared and used to seed (7 mL) multiple T-25 flasks. After 24 hr at 37°/6% CO_2, test chemicals were added. TU was prepared in water, whereas PTU and ANTU were prepared in acetone. Chemicals were added to the T-25 flasks in volumes not exceeding 25 μL; controls with vehicle only were included. Acetone at these levels (0.3%) had no deleterious effects on C3H/10T½ cloning efficiency (typically around 18%). After addition of the chemical, flasks were sealed tightly and incubated for 48 hr. After the exposure period, medium was removed, cells were washed, and the medium was replaced for a further 9-day incubation to allow development of clones (~5 mm diameter). Clones were stained by a 4-min treatment with 1% crystal violet (in 50% methanol) followed by three rinses with deionized H_2O. Each point represents the average of triplicate flasks.

2.6. Mutagenesis

Mutation of the ouabain binding site of the Na^+, K^+ ATPase was determined by the clonal growth of C3H/10T½ cells in the presence of 1×10^{-3} M ouabain [19]. Test cells (5×10^5) were plated in T-75 flasks with BME + 10% FBS. For the positive mutation control, cells were incubated with either 2×10^{-6} or 8×10^{-6} M MNNG for 12 hr. For GSH depletion, cells were incubated with 1×10^{-5} M BSO for 18 hr. Next, the medium was replaced, and TU was added to a final concentration of either 1×10^{-4} or 1×10^{-5} M. After a 48-hr exposure to TU, medium was replaced and the incubation continued for 48 hr to allow expression of the mutation. At the end of this expression period, monolayers from each exposure condition were trypsinized, and 1×10^5 cells were plated in 60-mm plastic dishes. Twenty-four hours later medium was changed to that containing 1×10^{-3} M ouabain. Medium (containing ouabain) was changed at 6-day intervals for the 18-day duration of selection. Ouabain-resistant clones were stained with 1% crystal violet and counted.

3. Results

The initial characterization of the G418-resistant clones was based on their ability to oxidize TMS and their susceptibility to TU toxicity. TMS is a model substrate for the assessment of enzymatically competent FMO [20,21]. FMO catalyzes the sulfoxidation of TMS to yield TMSO. G418-resistant clones were expanded, and sonicated cell suspensions were prepared for the TMSO assay. Clones exhibited TMSO activity ranging between 3890 and ~30,000 pmol/30 min/mg, whereas in the parental (vector infected) cells activity was only 224 pmol/30 min/mg (Table 1, column 2). Each clone was prepared for clonogenic assay to test for TU cytotoxicity (Table 1, columns 3 and 4). Cells (500/T-25 flask) were exposed to 1×10^{-3} M TU for 48 hr. Parental cells were resistant to the toxic action of TU, but FMO 3 clone 7, with the highest TMSO activity, was sensitive to TU toxicity to the extent of about 14% survival under this condition. The other clones did not show this degree of toxicity; clones 9 and 11 had ~88% survival and the lowest TMSO expressing clones, 8 and 10 (~4000–5000 pmol/30 min/mg), were unaffected by exposure to TU.

The exposure limits of TU toxicity were assessed by incubating cells with a range of TU concentrations and an exposure period of 3 hr to 10 days. The survival curve for various concentrations of TU was virtually unchanged for

Table 1
Characterization of FMO 3 expressing clones of C3H/10T½

Clone	TMSO activity (pmol/30 min/mg)	TU toxicity		
		Number surviving		% Survival
		−TU	+TU	
Parental	224	55 ± 5	58 ± 3	100
7	29,750	65 ± 7	9 ± 2	14
8	4,802	44 ± 3	45 ± 6	100
9	6,496	42 ± 6	37 ± 3	88
10	3,890	53 ± 6	51 ± 8	100
11	6,416	47 ± 4	42 ± 6	89

The assay of FMO 3 enzymatic activity was performed on whole cell sonicates of parental and G418-resistant clones of RDRV-infected C3H/10T½ cells. The method for assessing the conversion of TMS to TMSO is described under Section 2. The initial screen for TU toxicity was performed by exposing cells at cloning density (500 cells/T-25 flask) to 1×10^{-3} M TU for 48 hr followed by 10 days of growth in fresh medium to allow clones to form. Values represent the means ± SEM, derived in triplicate, from 3 separate experiments.

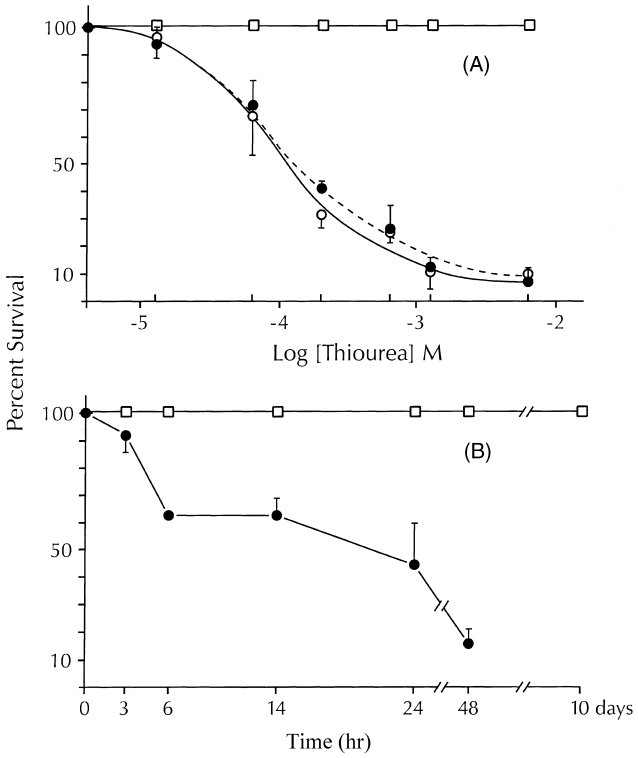


Fig. 1. TU cytotoxicity: concentration and exposure time dependence. C3H/10T½ parental (□) and FMO 3 clone7 (○,●) cells. (A) Cells were exposed to the indicated concentrations of TU for 48 hr (●) and 96 hr (○). (B) Cells were exposed to 1×10^{-3} M TU for the indicated time periods. After each exposure, the medium was changed to allow clonal growth to proceed. Clones were stained and counted 9–10 days later. Each value represents the mean ± SEM derived from triplicate determinations in 3 separate experiments.

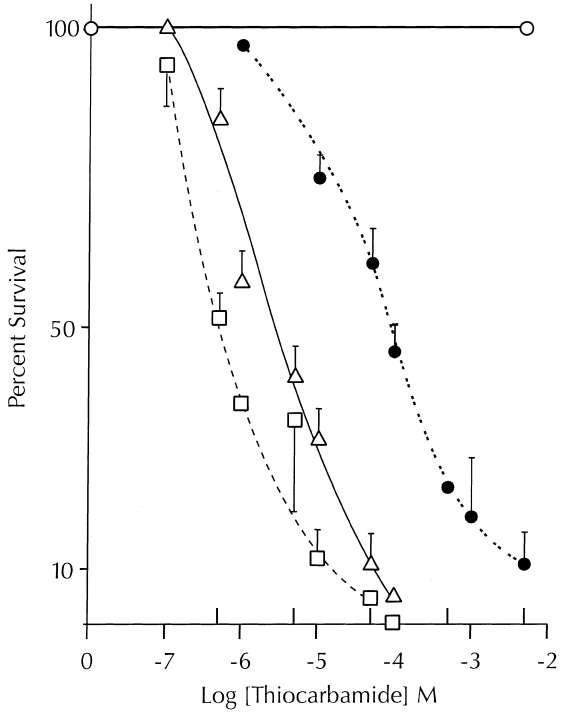


Fig. 2. Cytotoxicity of TU and derivatives. C3H/10T½ FMO 3 clone 7 cells were exposed for 48 hr to the indicated concentrations of TU (●), PTU (△), and ANTU (□), and a clonogenic assay was performed; control parental C3H/10T½ cells (○). Each value represents the mean ± SEM derived from triplicate determinations in 3 separate experiments.

either a 48- or 96-hr exposure with 50% survival at 1×10^{-4} M TU (Fig. 1A). The toxic effect of TU on FMO 3 clone 7 was exerted as early as 6 hr of exposure (Fig. 1B) and reached a maximum at the 48-hr interval. Parental cells (Fig. 1B) were resistant with the extreme condition of continuous exposure to 1×10^{-3} M TU over the entire 10-day cloning period.

The substrate preference for cytotoxicity was tested by exposing FMO 3 clone 7 cells to a variety of TU derivatives. The choice of these compounds was made based on earlier work with the purified enzyme in which the oxidation of NADPH and/or the substrate-dependent oxidation of thiocholine was used to assess thiocarbamate substrate specificity [22,23]. The concentration-dependent effects of TU and the rodenticides PTU and ANTU on survival of parental and FMO 3 clone 7 are shown in Fig. 2. The toxic potency of these thiocarbamates was determined by the concentration required to cause 50% cytotoxicity after a 48-hr exposure period. The potency series derived from this experiment was ANTU 5×10^{-7}–1×10^{-6} M > PTU 5×10^{-6} M > TU 1×10^{-4} M. Parental cells were resistant to TU but did exhibit minor toxicity to ANTU (80% survival at 1×10^{-4} M). The fungicide ETU was not toxic (data not shown).

TU toxicity was altered by treatments that affected either (a) the level of glutathione in the cell, or (b) the operation of FMO 3. Two approaches were used to compare the degree of toxicity exerted by TU and GSH status (Fig. 3A and B). First, cells were preincubated for 18 hr with BSO, an inhibitor of glutathione synthesis, at concentrations from 0.5 to 2×10^{-5} M [24]. BSO was removed, and the cells were exposed to a concentration range of TU. BSO treatment made FMO 3 clone 7 considerably more sensitive to the cytotoxicity of TU, shifting the 50% survival figure from 1×10^{-4} to 1×10^{-6} M (Fig. 3A). The optimal condition for observing the effect was 1×10^{-5} M BSO/18 hr; BSO concentrations in excess of 2×10^{-5} M resulted in 40–50% toxicity. Incubation of cells with *N*-acetylcysteine facilitates raising glutathione levels by providing more cysteine substrate for the γ-glutamyl cysteine synthase reaction [25]. FMO 3 cells were incubated with 1, 5 (Fig. 3B), and 10×10^{-3} M *N*-acetylcysteine continuously over the TU exposure period (48 hr). *N*-Acetylcysteine blunted the sensitivity to TU, shifting 50% survival from 5×10^{-5} to 1×10^{-3} M and increased the survival of FMO 3 clone 7 cells at each concentration of TU tested.

The thiocarbamate specificity of FMO was originally delineated by the ability of a spectrum of these compounds to support NADPH oxidation in the presence of purified FMO 1 [22]. In this and subsequent studies, it was shown that the kinetic characteristics for TU (V_{max} = 1180

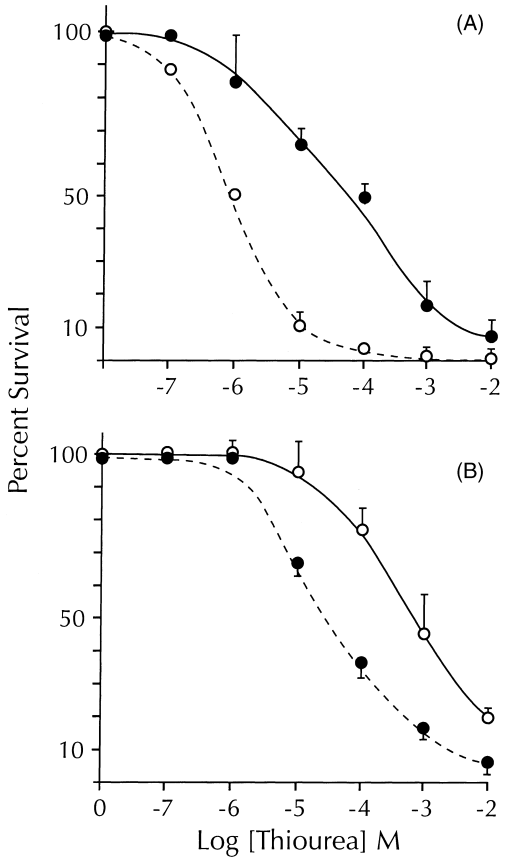


Fig. 3. Effect of (A) BSO and (B) *N*-acetylcysteine on TU cytotoxicity. (A) Forty-eight hours after initial plating, C3H/10T½ FMO 3 clone 7 cells were incubated with 1×10^{-5} M BSO for 18 hr. Medium was changed, and cells were exposed to the indicated concentrations of TU for 48 hr after which time the medium was changed and clonal growth was allowed to proceed. FMO 3 clone 7 control (●) and + BSO treatment (○). (B) Forty-eight hours after initial plating, cells were incubated with 5 mM *N*-acetylcysteine for 9 hr followed by addition of TU at the indicated concentrations. Exposure was continued for 48 hr, and clonal growth was assessed. FMO 3 clone 7 control (●) and + *N*-acetylcysteine treatment (○). Each value represents the mean ± SEM derived from triplicate determinations in 3 separate experiments.

nmol/min/mg, $K_m = 4$ μM) were similar to ETU ($V_{max} =$ 1060 nmol/min/mg, $K_m = 99$ μM), and PTU and ANTU [15,26]. When ETU was tested for cytotoxicity on parental and FMO 3 clone 7, it proved negative over a concentration range of 10^{-6} to 10^{-3} M. It was reasoned that if ETU is a nontoxic substrate for FMO 3 and it is gaining access to FMO 3 in these *in vitro* experiments, then preincubation with ETU followed by challenge with TU should block the binding of TU to FMO 3 and the formation of cytotoxic metabolites. Table 2 presents the results of such a study. FMO 3 cells were preincubated with 1×10^{-3} M ETU for 8 hr followed by the addition of either 1×10^{-4} or 1×10^{-3} M TU. ETU by itself showed only slight toxicity (83% survival). Preincubation of cells with ETU blocked TU toxicity and increased survival from 10 to 76% for 1×10^{-3} M TU and from 32 to 83% for 1×10^{-4} M TU.

The isozyme specificity and enzyme concentration dependence were determined in the presence of

Table 2
ETU blockade of TU toxicity

Treatment	Number of survivors	% Survival
Control, FMO 3 cells, minus TU	59 ± 3	100
+ 10^{-3} M TU	6 ± 3	10
+ 10^{-4} M TU	19 ± 7	32
+ 10^{-3} M ETU	49 ± 6	83
+ ETU (10^{-3} M) + TU (10^{-3} M)	45 ± 15	76
+ ETU (10^{-3} M) + TU (10^{-4} M)	49 ± 7	83

Cells were plated at cloning density (500 cells/T-25 flask) and grown for 24 hr. Next, the medium was changed, and the designated cultures were preincubated with 1×10^{-3} M ETU for 8 hr followed by a 48-hr exposure to 1×10^{-4} or 1×10^{-3} M TU. After exposure, the medium was changed, and clonal growth was continued for 10 days. Values represent the mean ± SEM, derived in triplicate, from 3 separate experiments.

1×10^{-5} M BSO, which is the condition that gave maximum sensitivity to TU cytotoxicity. In the initial screening (Table 1), only slight toxicity was observed in "low expressor" clones after exposure to 1×10^{-3} M TU. These clones, 8 and 9, as well as parental and FMO 3 clone 7 were subjected to BSO treatment. Additionally, an FMO 1 clone with TMSO activity (~5000 pmol TMSO/30 min/mg) comparable to that of the "low expressor" FMO 3 was tested (Fig. 4). With BSO, clones 8 and 9 exhibited TU cytotoxicity with 50% survival at 1×10^{-4} M and only 20% survival at

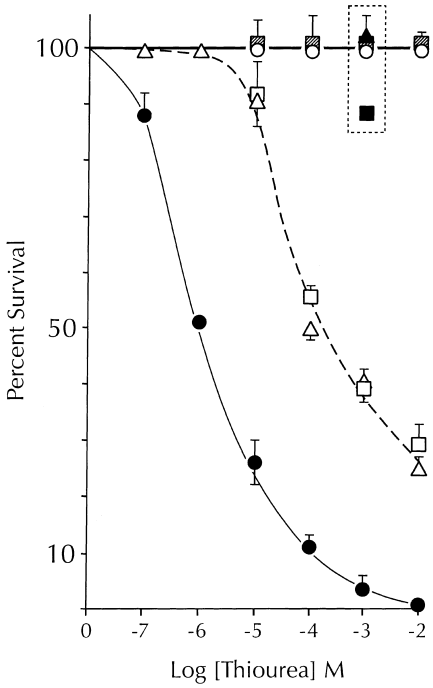


Fig. 4. Effect of BSO on TU toxicity in "low expressor" FMO 3 clones. Cells were incubated with 1×10^{-5} M BSO for 18 hr. The medium was changed and cells were exposed to the indicated concentrations of TU for 48 hr. Key: clone 7 (closed circle), clone 8 (open square), clone 9 (open triangle), clone 8 minus BSO (closed square), clone 9 minus BSO (closed triangle), FMO 1 expressor (hatched square), and parental C3H/10T½ (open circle). Each value represents the mean ± SEM derived from triplicate determinations in 3 separate experiments.

Table 3
Mutation assay

Treatment	Number of ouabain-resistant clones/10^6 cells	
	Parental	FMO 3 clone 7
Control	0	0
MNNG (2×10^{-6} M)	28 ± 7	16 ± 3
MNNG (8×10^{-6} M)	76 ± 12	70 ± 14
BSO (1×10^{-5} M) + TU (1×10^{-4} M)	0	0
BSO (1×10^{-5} M) + TU (1×10^{-5} M)	0	0

Induction of ouabain-resistant growth by a direct acting mutagen, MNNG, and TU was performed as described under Section 2 and in ref. [19]. Cells (500,000/T-75) were incubated with BSO (1×10^{-5} M) for 18 hr. Medium was changed to remove BSO, and TU was added (1×10^{-4} M or 1×10^{-5} M) for an exposure period of 48 hr. Medium was changed, and the incubation, was continued for 48 hr after which time the cells from each exposure condition were trypsinized and seeded at 1×10^{-5}/60-mm dish (18 dishes in duplicate). Ouabain selection was begun 24 hr later by adding 3 mM ouabain to the growth medium. Medium was changed three times over the 20-day selection period. Values represent the mean ± SEM, derived in triplicate, from 3 separate experiments.

1×10^{-3} M. The FMO 1-like parental C3H/10T½ cell line was resistant to TU in either the presence or absence of BSO.

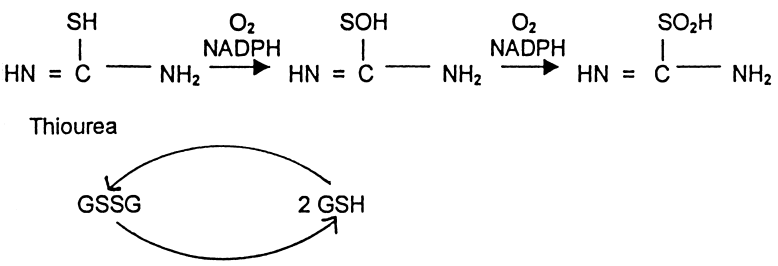
TU was not mutagenic to FMO 3 clone 7 (Table 3). Exposure of cells under maximum toxic sensitivity conditions, 1×10^{-5} M BSO plus 1×10^{-4} or 1×10^{-5} M TU, did not yield any mutants after mutant selection in the presence of 1×10^{-3} M ouabain. The positive control, MNNG, a direct acting mutagen that does not require metabolic activation for mutagenesis, yielded a mutant frequency, 76×10^{-6}, which is consistent for this locus in the C3H/10T½ cells [19].

4. Discussion

In this study, a new metabolic phenotype for the selective toxicity of thiocarbamates was conferred upon C3H/10T½ cells by the expression of FMO 3, the major hepatic isozyme of the human FMO family. What occurs in FMO 3 cells in terms of metabolic activation must be based on earlier work delineating the metabolite profile of TU resulting from FMO catalysis. *In vitro* work employing purified pig liver FMO showed that TU and its phenyl and ethylene derivatives underwent *S*-oxygenation of the thionocarbonyl group commencing with formamidine sulfenate acid formation followed by oxygenation to the corresponding sulfinate [14]:

In the presence of GSH, the sulfenate was reduced to the parent TU. *In vivo* experiments demonstrated that the same sulfenate:sulfinate oxygenation sequence operates in the hepatocyte after perfusion of the liver with either TU or PTU [27]. In accord with the *in vitro* work, liver perfused with TU increased the biliary efflux of GSSG. Efflux occurred only when the perfusion was performed with substrates liable to *S*-oxygenation by FMO to a sulfenate derivative. Inhibition of cytochrome P450 had no effect on the extent of GSSG efflux, demonstrating the specificity for FMO in thiocarbamate metabolism and GSH utilization. Additional studies on thiocarbamate toxicity in rodent species support the chemoprotective function of GSH [28–30]. Depletion of GSH by either BSO or diethylmaleate has been shown to enhance the toxicity of a number of TU derivatives. In mice, GSH depletion amplified thiocarbamate hepatotoxicity as determined by the release of alanine transaminase and lactate dehydrogenase [29,30]. Derivatives lacking the thionocarbonyl functional group were not toxic. In rats, exposure to ANTU after GSH depletion resulted in increased ANTU protein adduct formation in the lungs and pulmonary edema [30]. Raising GSH levels in the lungs protected against ANTU pulmonary toxicity and mortality [31]. Glutathione is probably chemoprotective by preventing chemical alterations in protein sulfhydryls. In favor of this idea, incubation of rat liver microsomes with ETU resulted in covalent binding of ETU and inactivation of cytochrome P450. This effect required FMO activity and was blunted by elevated levels of GSH. Covalently bound to ETU, P450 was released by the addition of GSH, indicating the presence of the mixed disulfide between ETU and the P450 protein sulfhydryl group [32–34].

The cytotoxic response of FMO 3 cells to thiocarbamates and the modification of this toxicity by agents that alter GSH status is consistent with results from these earlier findings. For the "high expressor" clone (#7) the sensitivity to TU toxicity was enhanced ~100-fold by prior treatment with the GSH depleting agent BSO. Conversely, preincubation of these cells with *N*-acetylcysteine to preserve intracellular GSH afforded a modest protection from toxicity. For the "low expressor" clones (<6000 pmol TMSO/30 min/mg) TU toxicity was slight (Table 1), but with BSO treatment these cells acquired a TU toxicity comparable to that of the "high expressor" clone. The simplest explanation is that low expressors do not create TU metabolites sufficient to cause toxicity because these



metabolites are produced at a rate that can be detoxified by GSH without exhausting GSH levels in the cell.

The structural preference of the purified FMO does not match the cytotoxic potency series observed here for the same set of TU derivatives [15]. Comparisons between TU and a series of phenyl substituted derivatives showed that TU and PTU were equivalent preferred substrates for FMO. Presumably the larger the hydrophobic substituent, the less access and binding of the thiocarbamide to the FMO active site and/or FMO microsomal domain. In the present study, hydrophobic substituents increased the sensitivity of FMO 3 clone 7 cells to thiocarbamate toxicity such that ANTU > PTU > TU. Multiple factors in addition to substrate access to FMO 3 are in play. First is cellular uptake, which, in turn, would influence intracellular pools of the thiocarbamide and presentation to microsomal FMO. The naphthyl and phenyl group would favor increased passage across the plasma membrane and entry into the microsomal domain of FMO. Second, the reactive sulfenate metabolites of ANTU and PTU may have greater access to protein sulfhydryls in a hydrophobic environment of the protein. If this adduction creates a mixed disulfide that affects protein function adversely, then toxicity would ensue. Also, the adduct could be longer lived if the naphthyl group also shields the disulfide from GSH entry to the adduct site, thus preventing reduction of the mixed disulfide and repair of the adduct.

The issue of TU mutagenic potential and genotoxicity is not resolved. Mutagenicity assays employing bacterial indicators are uniformly negative whether a liver microsomal membrane fraction (S9) or co-cultivation with primary hepatocytes is used for activation [35]. A small positive mutagenic effect (5×10^{-6} mutant frequency at 1×10^{-2} M TU) was reported for the V79 cell HPRT locus and a slight increase in micronuclei formation [12]. TU mutagenicity becomes more puzzling in the consideration of studies demonstrating that TU reduces (a) cisplatin mutagenicity, toxicity, sister chromatid exchange, and DNA crosslinks in V79 cells [36], and (b) the frequency of transformed liver foci in rats exposed to hepatocarcinogens (diethylnitrosamine, chlorinated biphenyls) [37]. It remains, however, that chronic exposure (2 years) to TU causes thyroid and liver adenomas [6,8]. Therefore, short-term TU exposures used in the aforementioned work [37] may not be optimal for observing a TU carcinogenic effect. The present study adds to the borderline status of TU as a mutagen. Unlike all previous *in vitro* work, the FMO 3 cell line controls for the variability of TU metabolic activation since FMO is considered to be the primary if not the exclusive catalyst on TU to form reactive sulfenic acids. Conditions that favored a cytotoxic response did not yield any mutants as measured by ouabain resistance. Possibly TU mutagenesis requires reactions distal to sulfoxidation by FMO, and C3H/10T½ cells are lacking this metabolic capacity. Additionally, the ouabain locus may not be as sensitive for mutant selection as the HPRT locus of V79. Expression of FMO 3 in V79 could control for this possibility. Also, use of FMO 3 microsomes from this cell line for the S9 fraction in the bacterial mutagenesis assay would permit control at the level of activation, a factor that may have been lacking in the early initial screening of thiocarbamates.

In summary, C3H/10T½ cell lines expressing the major human hepatic form of FMO exhibit selective toxicity for the thiocarbamates. Because of the GSH depletion effect, high and low expressor phenotypes were defined that showed excellent concordance between FMO 3 activity and the degree of thiocarbamate toxicity. The fact that low expressors only evinced toxicity after GSH depletion provides an *in vitro* system for exploring how the balance between GSH and a chemical stress is struck to protect the cell from toxicity.

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