POTENTIALLY REACTIVE CYCLIC CARBAMATE METABOLITE OF THE ANTEPILEPTIC DRUG FELBAMATE PRODUCED BY HUMAN LIVER TISSUE IN VITRO

IZET M. KAPETANOVIC, CYNTHIA D. TORCHIN, CHARLES D. THOMPSON, THOMAS A. MILLER, PATRICK J. MCNEILLY, TIMOTHY L. MACDONALD, HARVEY J. KUPERBERG, JAMES L. PERHACH, R. DUANE SOFIA, AND JOHN M. STRONG

Epilepsy Branch, National Institute of Neurological Disorders and Stroke (I.M.K., C.D.To., H.J.K.), Chemistry Department, University of Virginia (C.D.Th., T.A.M., T.L.M.), Laboratory of Clinical Pharmacology, Center for Drug Evaluation and Research, United States Food and Drug Administration (P.J.M., J.M.S.), and Wallace Laboratories, Research and Development (J.L.P., R.D.S.)

(Received April 7, 1998; accepted June 23, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

Felbamate (FBM) is a novel antiepileptic drug that was approved in 1993 for treatment of several forms of epilepsy. After its introduction, toxic reactions (aplastic anemia and hepatotoxicity) associated with its use were reported. It is unknown whether FBM or one of its metabolites is responsible for these idiosyncratic adverse reactions. Although the metabolism of FBM has not been fully characterized, three primary metabolites of FBM have been identified, i.e. 2-hydroxy, p-hydroxy, and monocarbamate metabolites. In addition, the monocarbamate metabolite leads to a carboxylic acid, which is the major metabolite of FBM in humans. Formation of the hydroxylated products of FBM involves cytochrome P450 enzymes, but the enzymes involved in the formation and further metabolism of the monocarbamate have not yet been elucidated.

Recently, mercapturate metabolites of FBM have been identified in human urine, and a metabolic scheme involving reactive aldehyde metabolite formation from the monocarbamate metabolite has been proposed. The present study confirmed the formation of the proposed metabolites using human liver tissue in vitro. The aldehyde intermediates were trapped as oxime derivatives, and the cyclic equilibrium product (proposed as a storage and transport form for the aldehydes) was monitored directly by HPLC or GC/MS. Formation of putative toxic aldehyde intermediates and the major carboxylic acid metabolite of FBM was differentially effected with the cofactors NADP⁺ and NAD⁺. It is possible that the cofactors may influence the relative metabolism via activation and inactivation pathways.

FBM1 (Felbatol; 2-phenyl-1,3-propanediol dicarbamate) is a broad-spectrum antiepileptic drug. It was approved in 1993 by the United States Food and Drug Administration for the treatment of several forms of epilepsy, including Lennox-Gastaut syndrome and related disorders, for which there have been very few therapeutic options. After its release, several cases of severe idiosyncratic adverse reactions (aplastic anemia and hepatotoxicity) with the use of FBM were reported. This prompted a recommendation by the United States Food and Drug Administration that FBM therapy be discontinued unless the benefit of seizure control outweighs the risk of toxicity. It is estimated that approximately 15,000 patients worldwide are still receiving FBM therapy. In the neurological community, FBM is thought to be too important to be discarded as a therapeutic agent, but it should primarily be used for patients with severe epilepsy that is refractory to other therapy (Pellock and Brodie, 1997).

The reasons for these idiosyncratic untoward reactions remain unclear. The possibility of metabolic activation to toxic products has been proposed (Thompson et al., 1996). Initially, four metabolites of FBM, i.e. pOH-FBM, 2OH-FBM, MCF, and CPPA, were identified in humans (Adusumalli et al., 1993) and experimental animals (Yang et al., 1992; Romanyszyn et al., 1993). None of these compounds is thought to have significant toxicological potential. More recently, mercapturic acid metabolites of FBM were identified in human and rat urine (Thompson et al., 1997). This was suggestive of reactive metabolite formation, and a metabolic scheme for the disposition of FBM (fig. 1) was proposed (Thompson et al., 1997).

A reactive aldehyde was proposed as an intermediate to the mercapturates. MCF could be oxidized to CBMA, which could undergo reversible cyclization to CCMF, oxidation to CPPA (the major metabolite in humans in vivo), or β-elimination (spontaneous or catalyzed) to ATPAL. ATPAL, an α,β-unsaturated aldehyde, is a potent electrophile that undergoes rapid conjugation with glutathione and could represent the toxic reactive metabolite of FBM (Thompson et al., 1996). CCMF, on the other hand, is relatively stable and could represent a metabolic depot and part of a mechanism of transport from the liver to distal sites for the putative ultimate reactive metabolite, ATPAL. The present study was designed to investigate the validity of the proposed pathways leading to reactive metabolites in humans, starting with human liver tissue in vitro. These studies were performed...
and the addition of 2 ml of ethyl acetate (with the addition of 100 μl of 1 N hydrochloric acid necessary for the extraction of CPPA). For analysis, the ethyl acetate extract was evaporated to dryness under nitrogen at room temperature and reconstituted in 50–100 μl of the mobile phase for HPLC or 50–100 μl of acetonitrile for GC/MS analysis.

Analysis. HPLC (Hewlett Packard Series 1100 system) separation was achieved using a Waters NovaPak C₁₈ column (3.9 × 150 mm, 4 μm), at 40°C. The mobile phase was composed of 30 mM monobasic potassium phosphate/acetonitrile/methanol (82:12:6), at a flow rate of 0.5 ml/min. Monitoring was performed using a diode-array detector, at 214 and 220 nm.

GC/MS (Hewlett Packard 5989B) analysis was performed with a HP-5MS cross-linked capillary column (5% phenylmethyl siloxane; 30 m × 0.25 mm, 0.25 μm), using electron impact and positive- and negative-ion ionization modes (methane reagent gas). Injections (1 μl) were performed using the splitless mode, and helium was used as the carrier gas. Quantitation was performed using SIM in the chemical ionization mode (with methane). SIM was performed at m/z 133 for CCMF ([M – 60]⁺), m/z 328 for the CBMA adduct ([M – 60]⁺), and m/z 328 for the ATPAL adduct (quasimolecular ion).

Trapping of aldehyde metabolites was achieved using PFBB, yielding the corresponding adduct. Briefly, at the end of the incubation, the microsomal solution was immediately transferred to a tube containing 1 ml of methanol, 2 ml of acetonitrile, 1 ml of 2 M ammonium phosphate, pH 4.6, and 250 μl of 50 mg/ml PFBB in methanol. The tube was capped, and the contents were mixed thoroughly and incubated for 2 hr at 65°C, followed by maintenance at room temperature overnight. On the next day, the contents were mixed with a vortex-mixer and centrifuged at 1000 g for 5 min. The supernatant was transferred to a clean tube and extracted with 1 ml of chloroform. The chloroform extract (bottom layer) was evaporated to dryness under nitrogen at room temperature. The residue was reconstituted in 50–100 μl of acetonitrile for the GC/MS analysis.

Results

Metabolism of MCF. The formation of both CCMF and CPPA from MCF by human liver S9 fractions and microsomes was demonstrated after incubation of MCF in the presence of both NADP⁺ and NAD⁺ (fig. 2). HPLC chromatograms for a S9 incubation medium extract (fig. 2, top) revealed two metabolic products, with retention times of 7.061 and 9.398 min, that coeluted with synthetic reference CCMF and CPPA, respectively, and were not present in the postreaction control medium (fig. 2, bottom). Similarly, HPLC analysis of medium extracts from CCMF S9 incubations confirmed the production of CPPA from CCMF (fig. 3).

GC/MS analysis of MCF S9 incubations was used to provide additional evidence to support formation of the proposed FBM cyclic carbamate metabolite, CCMF. GC/MS analysis of synthetic CCMF and ATPAL indicated that the two compounds coeluted from the GC column at 8.6 min and yielded similar spectra with both electron impact and methane positive-ion chemical ionization. With methane positive-ion chemical ionization, no protonated molecular ion at m/z 194 was observed for CCMF. The base peak at m/z 133 corresponds to the loss of a carbamoyl (CO₂NH₂) moiety, which is characteristic for FBM and its metabolites. However, the ion observed at m/z 133 also corresponds to that of the quasimolecular ion of ATPAL. HPLC elution times for CCMF and ATPAL were 7 and 50 min, respectively. Based on the combined HPLC and GC/MS data, we conclude that CCMF is most likely decarbamoylated in the GC injection port. These results preclude the separate identification of ATPAL and CCMF by GC/MS analysis. However, under the experimental conditions, ATPAL most likely does not contribute to the GC/MS peak for CCMF, because enzymatically generated ATPAL was not detected by HPLC.

Therefore, based on these assumptions, the ion at m/z 133 was used for subsequent SIM of CCMF. After MCF incubation with micro-

primarily with the intermediate metabolite MCF, because neither the identity nor the tissue and subcellular distribution of the enzymes responsible for its formation are known.

Materials and Methods

Chemicals. FBM, MCF, 2OH-FBM, pOH-FBM, and CPPA were provided by Wallace Laboratories (Cranbury, NJ), and CCMF and ATPAL were synthesized as described previously (Thompson et al., 1996). Methanol and acetonitrile were obtained from Burdick & Jackson (Muskegon, MI). EDTA (disodium salt), D-glucose-6-phosphate (monosodium salt), glucose-6-phosphate dehydrogenase (from Torula yeast), NADP⁺ (sodium salt), 4MP, and DDC were purchased from Sigma Chemical Co. (St. Louis, MO), PFBB from Aldrich Chemical Co. (Milwaukee, WI), and NAD⁺ from Fluka Chemie AG (Buchs, Switzerland).

Liver Tissue. Human liver specimens (medically unsuitable for transplantation) were acquired under the auspices of the Washington Regional Transplant Consortium (Washington, DC) and were stored at −80°C until use. S9 fractions and microsomes were prepared by tissue homogenization and differential centrifugation as described previously (Jamis-Dow et al., 1995), divided into aliquots, and stored at −80°C.

Metabolic Incubations. Incubations were carried out in 1 ml of phosphate buffer (31 mM monobasic potassium phosphate, 69 mM dibasic sodium phosphate, 5 mM magnesium chloride, 1 mM EDTA, pH 7.4) at 37°C for 60 min, in the presence of human liver S9 fractions or microsomes (1 mg of protein). FBM (250 μM) or MCF (250 μM) was added to the medium. For the inhibition experiments, MCF (250 μM) in the presence of either DDC (100 μM) or 4MP (100 μM) was used for the incubations. After a 3-min pre-equilibration at 37°C, the reactions were started by the addition of an NADPH-generating system (100 μM D-glucose-6-phosphate, 1 Sigma unit of glucose-6-phosphate dehydrogenase, and 10 μM NAD⁺). In some cases (as indicated), 10 μM NAD⁺ was also added because it is the commonly preferred cofactor for alcohol and aldehyde dehydrogenases (Sladek et al., 1989), which are expected to be involved in the metabolism of FBM. Control blank samples were incubated in the absence of one of the following: microsomes or S9 fraction, substrate, or NAD⁺. The omitted component was added at the end of the reaction, before the extraction. With the exception of trapping experiments (see below), reactions were terminated by the placement of tubes on ice.
somes in the presence of NAD$^+$ and NADP$^+$, the production of CCMF was confirmed by GC/MS analysis of the medium extract (fig. 4). CCMF was produced by microsomes prepared from each of the four human donor livers tested and showed an approximately 2-fold range in the amount produced.

**Cofactor Dependence.** MCF was incubated with human liver S9 fractions in the absence or presence of NADP$^+$ or NAD$^+$. GC/MS SIM or HPLC analyses of the medium extracts were used to monitor the formation of CCMF from MCF in the presence of NADP$^+$ and/or NAD$^+$. The amount of CCMF in the S9 incubation extracts, as analyzed by HPLC or GC/MS, was approximately 2-fold greater in the presence of NADP$^+$ than in the presence of NAD$^+$ and was intermediate between these values when both cofactors were added (table 1). The difference between NADP$^+$ and NAD$^+$ was even more pronounced (approximately 10-fold) with microsomes instead of S9 fractions. This finding was independently confirmed with trapping experiments (see below). On the other hand, the opposite observation was made for CPPA. The amount of CPPA detected was approximately 20-fold greater after incubation in the presence of NAD$^+$ than in the presence of NADP$^+$ and was intermediate between these values when both cofactors were present (table 1).

**Adduct Formation.** The proposed aldehyde metabolites (CBMA and ATPAL) may be reactive intermediates requiring a trapping procedure for identification. Therefore, we used a method analogous to that described for 4-hydroxycyclophosphamide/aldophosphamide (Anderson et al., 1995). The proposed aldehyde intermediate metabolites were reacted with PFBH to yield relatively stable Schiff base adducts (oxime derivatives), according to the scheme shown in fig. 5.

Total-ion chromatograms and positive-ion chemical ionization mass spectra of the ATPAL and CBMA adducts prepared from the corresponding synthetic aldehyde intermediates are shown in figs. 6 and 7, respectively. As was the case with cyclophosphamide/aldophosphamide derivatives, two peaks (A and B), representing the $E$- and $Z$-isomers, were observed for the adducts. No attempts were made to assign $E$- or $Z$-conformations to the individual adducts but, based on the expected greater steric favorability of the $E$-isomer (Anderson et al., 1995), one would expect the larger peaks shown in figs. 6 and 7, eluting at 10.6 min and 13.3 min, respectively, to represent the $E$-isomers. The GC elution times for the ATPAL and CBMA adducts differed by approximately 3 min. Positive-ion chemical ionization spectra of the two ATPAL adduct isomers of CBMA were also similar but did not show a protonated molecular ion at $m/z$ 389 (fig. 7, middle and bottom). Instead, the most abundant fragment ion corresponded to loss of a carbamoyl moiety at $m/z$ 328. After incubation of MCF with human liver microsomes, the trapping solution containing PFBH was added to the reaction mixture, as described in Materials and Methods. Resultant samples were analyzed by GC/MS, and the SIM chromatogram is shown in fig. 8 (top). Peaks corresponding to both ATPAL and CBMA adduct $E$- and $Z$-isomers were observed. The retention times and mass spectra for these peaks were
in good agreement with those of the corresponding reference adducts (figs. 6 and 7). The formation of these adducts required the presence of biological material, substrate, and cofactors. However, although both CBMA and ATPAL adducts were seen, the relative amount of enzymatically generated ATPAL adduct is uncertain, because of the apparent thermal degradation of the CBMA adduct to the ATPAL adduct (via loss of the labile carbamoyl moiety) under the GC/MS conditions.

**Metabolism of FBM.** The formation of adducts from FBM itself could also be shown, although to a much lesser extent than from MCF (fig. 8). Both ATPAL and CBMA adducts were seen, with the ATPAL adduct peaks being larger than the CBMA adduct peaks. This adduct peak ratio is opposite that seen with MCF as the substrate. However, as discussed above, the relative amount of the ATPAL adduct resulting from an enzymatic reaction is unclear.

**Inhibition.** The effects of several P450 inhibitors on adduct formation from MCF were assessed. The most pronounced effect was seen in the presence of the P4502E1-selective inhibitors DDC and 4MP. An approximately 90% decrease in the CBMA adduct level was observed in the presence of 100 μM DDC or 4MP (fig. 9). Ketoconazole (3 μM) (P4503A4) or sulfaphenazole (100 μM) (P4502C9) did not show significant effects, whereas coumarin (100 μM) (P4502A6) caused an approximately 40% decrease in the CBMA adduct level.

**Discussion**

Although FBM was marketed in 1993, its metabolic profile and the enzymes responsible for its metabolism have not been fully elucidated. The only enzymes clearly implicated in FBM metabolism are P4503A4 and P4502E1, which are involved in the formation of the hydroxy metabolites pOH-FBM and 2OH-FBM (Racha et al., 1996; Glue et al., 1997). Other enzymes possibly involved in FBM metabolism, especially via the MCF route, are esterases, amidases, hydrolases, and alcohol and aldehyde dehydrogenases. However, their identities and tissue and subcellular localization remain to be determined.
Recently, microsomal carboxylesterase (hydrolase A) was implicated in the metabolism of another carbamate, ethyl carbamate (Lee et al., 1998). In general, P450 enzymes have been the focus of attention, and the roles of other enzymes in drug metabolism have not been investigated as extensively. Recently, mercapturic acid metabolites of FBM were demonstrated in humans in vivo (Thompson et al., 1997). Their presence is suggestive of reactive metabolite formation and has led to the postulated metabolic scheme (fig. 1). This study concentrated on further elucidating the metabolic pathways involving the proposed reactive metabolites (i.e., CBMA and ATPAL) in humans in vitro.

Formation of the proposed cyclic carbamate intermediate metabolite CCMF from MCF and its metabolism to CPPA were demonstrated using human liver S9 fractions and microsomes. This provides the first direct evidence for the proposed metabolic scheme and has led to the postulated metabolic scheme (fig. 1). This study concentrated on further elucidating the metabolic pathways involving the proposed reactive metabolites (i.e., CBMA and ATPAL) in humans in vivo. 

Also in analogy to cyclophosphamide biotransformation, relatively unstable aldehyde intermediates were trapped by formation of stable oxime derivatives. These Schiff base adducts of CBMA (the open form of CCMF) and ATPAL were demonstrated in microsomal reactions with FBM as a substrate. Adduct formation was also seen in preliminary human liver slice experiments (data not shown). Subsequent experiments also showed adduct formation in the microsomal reactions with FBM as the substrate. However, the adduct formation from FBM was much less than that from MCF. This could be because MCF formation might be a rate-limiting step for the subsequent metabolism and/or extramicrosomal enzymes might be involved. It should be pointed out that there is some decomposition of the CBMA adduct to the ATPAL adduct, via a typical loss of the relatively labile carbamoyl moiety, under the GC/MS conditions. This currently makes it difficult to distinguish between metabolic and degradative contributions to ATPAL adduct levels. However, because nonenzymatic β-elimination can readily take place, the pathway for ATPAL production is available and would be expected to play a role, as was the case for cyclophosphamide; this is supported by the identification of mercapturic acids derived from ATPAL in vivo.

It is interesting to note that P4502E1-selective inhibitors were very effective in inhibiting adduct formation. This P450 isoform is known to oxidize ethanol (Itoya et al., 1998; Ingelman-Sundberg et al., 1994) and has also been implicated in the metabolism of other smaller carbamates, i.e., ethyl carbamate and vinyl carbamate (Guengerich and Kim, 1991). Our results shown in fig. 9 are consistent with the
Microsomal reactions were performed using MCF (top) or FBM (bottom) as the substrate, in the presence of an NADPH-generating system, NADP⁺, and NAD⁺. Samples were analyzed using GC/MS in the chemical ionization mode, with methane as the reagent gas.

involvement of P4502E1 in the conversion of MCF to CBMA. However, although these two inhibitors are selective for P4502E1, they have other biological activities. DDC inhibits P4502E1 and is an aldehyde dehydrogenase inhibitor, and 4MP is an alcohol dehydrogenase inhibitor; both of these enzymes could be involved in the metabolism of MCF.

Another interesting observation concerns the selectivity of metabolic pathways for the cofactors NAD⁺ and NADP⁺ (table 1). Because the conversion of MCF to CBMA and that of CBMA to CPPA preferentially utilize the cofactors NADP⁺ and NAD⁺, respectively, the NAD⁺/NAD⁺ ratio would be expected to affect the relative amounts of ATPAL and CPPA formed. This selective cofactor dependence may be an important determinant for the occurrence of toxicity, because CCMF can serve as a precursor to the putative reactive species CBMA, which is spontaneously converted to ATPAL. Factors within the body that alter the balance between these two pathways could affect the expression of toxicity. Numerous examples that demonstrate that drugs or xenobiotics can affect the NAD⁺/NAD⁺ energy state are available in the literature. For example, Ramasamy et al. (1997) reported that zopolrestat maintains a lower cytosolic NAD⁺/NAD⁺ ratio in diabetic animal models and markedly protects both nondiabetic and diabetic hearts during ischemia and reperfusion. The elevated NAD⁺/NAD⁺ ratio resulting from ethanol consumption has been implicated as contributing to hepatic toxicity (Seitz and Csomos, 1992).

Further understanding of putative toxic metabolic pathways may provide means for minimizing their contributions or may suggest structural drug modifications to preclude them. In addition to investigations of possible toxic pathways of FBM metabolism, these approaches may have more general applicability. The use of adduct formation to trap reactive metabolites may be practical for other drugs, as was shown for cyclophosphamide and FBM. The use of data from human in vitro systems to predict the in vivo generation of reactive metabolites in humans also warrants further exploration.

References


