ELSEVIER

Chemico-Biological Interactions 102 (1996) 79–92

Chemico-Biological Interactions

Formation of mammalian metabolites of cyclobenzaprine by the fungus, *Cunninghamella elegans*

Donglu Zhang, Frederick E. Evans, James P. Freeman, Yifan Yang, Joanna Deck, Carl E. Cerniglia*

National Center for Toxicological Research, US Food and Drug Administration, Jefferson, Arkansas 72079, USA

Received 23 October 1995; revised 5 August 1996; accepted 7 August 1996

Abstract

The fungus, *Cunninghamella elegans*, was used as a microbial model of mammalian drug metabolism to biotransform a tricyclic antidepressant, cyclobenzaprine. Seventy-five percent of this drug at a concentration of 1 mM was metabolized within 72 h by *C. elegans* grown on Sabouraud dextrose broth. Milligram amounts of fungal metabolites were isolated by reversed-phase high performance liquid chromatography (HPLC) and their structures were characterized by ^{1}H NMR spectroscopy, mass spectrometry, and UV spectroscopy analyses. The major fungal metabolites of cyclobenzaprine were 2-hydroxycyclobenzaprine (59%), *N*-desmethylcyclobenzaprine (21%), cyclobenzaprine *trans*-10,11-dihydrodiol (5%), *N*-desmethyl-2-hydroxy-cyclobenzaprine (3%), 3-hydroxycyclobenzaprine (3%), and cyclobenzaprine *N*-oxide (1%). These fungal metabolites were used as standards to investigate the metabolism of cyclobenzaprine by rat liver microsomes. Rat liver microsomes also biotransformed cyclobenzaprine to produce similar metabolites as the fungus. The isotope labeling of 2-hydroxycyclobenzaprine by $^{18}O_2$ and the *trans*-configuration of the dihydrodiol suggested that these reactions were catalyzed by cytochrome P-450 monooxygenases in *C. elegans*. These results also demonstrated that the fungal biotransformation system could be used to predict and synthesize the mammalian drug metabolites.

* Corresponding author. Tel.: + 1 501 5437341; fax: + 1 501 5437307; e-mail: ccerniglia@nctr.fda.gov

PII S0009-2797(96)03736-2

Keywords: Cyclobenzaprine; *Cunninghamella elegans*; Cytochrome P-450; Drug metabolism; Hydroxylation; Microbial biotransformation; *N*-Demethylation; *N*-Oxidation

1. Introduction

Microorganisms are used as biocatalyst to degrade and mineralize toxic chemicals in bioremediation [1]. In addition, microorganisms catalyze the synthesis of useful compounds such as agrichemicals, pharmaceuticals, flavors, fragrances, and other fine chemicals by biotransformations [2]. The zygomycete fungi *Cunninghamella* spp. have shown the ability to metabolize a variety of xenobiotics in ways similar to those in mammals [3–9]. Therefore, the fungal system has been proposed as a potential microbial model for mammalian drug metabolism to synthesize metabolites of various pharmaceutical drugs [3,5,7,9]. Using a microbial model for mammalian drug metabolism has the advantages of low cost, ease of handling, scale up capability, and obviation of the need to conduct complex chemical synthesis [5,10].

Previously, we have investigated the biotransformation pathways of amitriptyline by *C. elegans* [11]. This fungus produced similar metabolites of amitriptyline as the rat and human, in which the methylene hydroxylation at position C-11 was the major metabolic pathway. In the process of evaluating the fungal model for mammalian drug metabolism in response to structural changes of the substrates, cyclobenzaprine (CBP), a close structural analog of amitriptyline, was chosen as a structural probe for metabolism studies with *C. elegans*. CBP (3-(5H-dibenzo[a,d] cyclohepten-5-ylidene)-*N*,*N*-dimethyl-1-propylamine) is a widely used antidepressant. The metabolism of CBP in the rat, dog, monkey, and human have been reported [12,13]. CBP is eliminated slowly from animals with a half-life of 1–3 days, and its primary elimination mechanism is metabolism through glucuronidation. The identified phase I metabolites were derived from epoxidation, *N*-oxidation, and *N*-demethylation, aromatic hydroxylation reactions [12,13]. In this study, we report the isolation and identification of major metabolites from the metabolism of CBP by *C. elegans* with the aim of generating large amounts of metabolites for toxicological testing.

2. Materials and methods

2.1. Chemicals and enzymes

CBP HCl was purchased from Sigma (St. Louis, MO). Oxygen ($^{18}O_2$, 95–98 at.%) was purchased from Cambridge Isotope Laboratories (Andover, MA). All other chemicals were reagent grade and the highest purity available. A sample of rat liver microsome was prepared by Dr Peter Fu at National Center of Toxicological Research.

2.2. Culture conditions and isolation of metabolites

C. elegans ATCC 9245 was grown on Sabouraud dextrose agar plates for 5 days at room temperature, then stored at 4°C, and transferred every month. Standard biotransformation experiments were performed using 30 ml of Sabouraud dextrose broth and 5 ml of blended *C. elegans* mycelial suspension (two plates in 150 ml of saline solution) as an inoculum in 125 ml Erlenmeyer flasks. The cultures were incubated at 25°C with agitation at 150 rpm. CBP was dissolved in sterile water (100 mg/ml) and added to 48 h old cultures. Controls included an incubation of the fungus without the drug, and an incubation of drug added to the uninoculated medium.

For production of CBP metabolites, CBP (50 mg in 0.5 ml water) was added to 200 ml of 48 h old culture in a 1 l Erlenmeyer flask. The incubation was continued at 25°C and 150 rpm for 96 h. Methanol (100 ml) and phosphate buffer (100 ml, 100 mM) were added to the flask. The mixture was extracted with chloroform (4×300 ml). The extraction efficiency for each step was analyzed by HPLC analysis of aliquots from both organic and aqueous layers. The extracted material, after concentration, was dissolved in methanol and separated by preparative HPLC. The samples from the HPLC separation were isolated with a Sep-Pak cartridge to remove the buffer as described by the manufacturer (Waters, Milford, MA). Some of the samples were dried on a Speed-Vac evaporator and weighed. The quantities of minor metabolites were estimated by comparison of relative peak intensities.

2.3. Analytical methods

HPLC was performed on a Shimadzu model LC-600 dual pump system equipped with a photodiode array detector (Shimadzu Scientific Instruments, Columbia, MD). Samples were analyzed on a 4.6×250 mm SCD-column (5 μm, SynChrom, West Lafayette, IN). The metabolites were eluted isocratically with 80/20 (v/v) acetonitrile/10 mM phosphate buffer (pH 7.2) at a flow rate of 1 ml/min. For large scale preparations of metabolites, a preparative column (21.2×250 mm, 10 μm) was used and the metabolites were eluted at a flow rate of 7 ml/min. In some cases, the materials collected from the first purification were further purified on a semi-preparative (10×250 nm, 5 μm) Cyano-HPLC column (Beckman Instruments, San Ramon, CA). The samples were eluted with 60/40 (v/v) acetonitrile/10 mM phosphate buffer (pH 7.2) at 3 ml/min.

^{1}H NMR spectra were recorded at 500.13 MHz on a Bruker AM500 spectrometer (Bruker Instruments, Billerica, MA) at about 29°C. General conditions and procedures for acquisition of spectra have been previously reported [14]. Resonance assignments were based mainly on integration, homonuclear decoupling, nuclear overhauser effect (NOE) experiments and comparisons between spectra. Samples were dissolved in methanol-d_4 and were not degassed. Chemical shifts are reported on the δ (ppm) scale by assigning the residual proton signal of the solvent to 3.30 ppm. Coupling constants are first-order measurements with data point resolution of 0.215 Hz/pt following zero-filling.

Ammonia chemical ionization mass spectrometry (CIMS) analyses were performed on a Finnigan MAT (Finnigan MAT, San Jose, CA) model 4000 quadrupole mass spectrometer upgraded to model 4500 capabilities using a direct exposure probe. The samples were placed onto the DEP rhenium wire and analyzed as the wire current was increased from 0 to 650 mA with a linear rate of 5 mA/s. The ion sources were set at 120°C. The quadrupole was scanned from 50 to 650 da with a 1 s cycle time. Other MS analyses were performed as described previously [11].

2.4. ^{18}O-Labeling experiments

A 35 ml of 48 h old *C. elegans* culture in a 125 ml Erlenmeyer flask was dosed with CBP (10 mg), purged with N_2 four times, and filled with $^{18}O_2$. The ^{18}O content was 96 atom % at first as determined by MS and 64 atom % after 24 h of

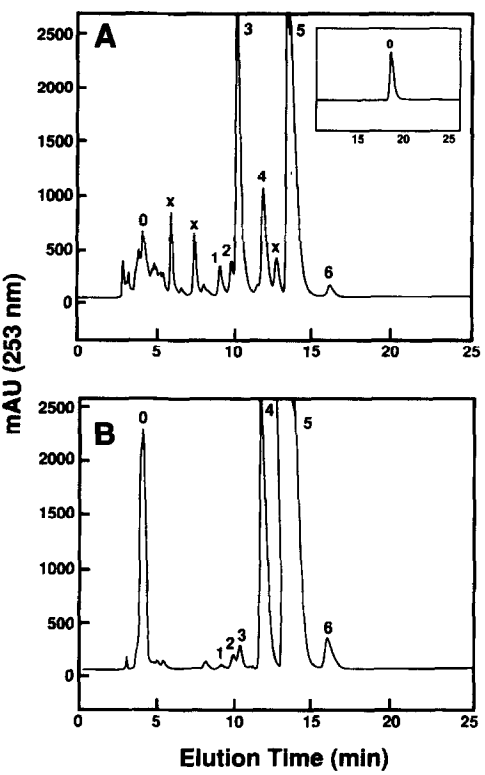

Fig. 1. HPLC chromatograms of cyclobenzaprine and its metabolites produced by (A) *C. elegans* from the preparative scale incubation (B) by rat liver microsomes. Peaks 0, 1, 2, 3, 4, 5 and 6 are cyclobenzaprine 10,11-*trans*-dihydrodiol, *N*-desmethyl-2-hydroxycyclobenzaprine, 3-hydroxycyclobenzaprine, 2-hydroxycyclobenzaprine, *N*-desmethylcyclobenzaprine, cyclobenzaprine, and cyclobenzaprine *N*-oxide, respectively. Where marked '*x*', the compounds were not fully characterized. The insert was repurification of Peak 0 in (A) by a Cyano-column.

Table 1
UV and MS analysis data of cyclobenzaprine and its metabolites by *C. elegans*

Compounds and peak no.[a]	UV λ_{max} (nm)	MW	NH_3CI m/z and %RI[b]
Cyclobenzaprine 10,11-*trans*-dihydrodiol, no.0	243, 219	309	310(100), 308(21), 292(22), 262 (12)
N-Desmethyl-2-hydroxy-cyclobenzaprine,no.1	290, 240	277	278(100)
3-Hydroxycyclobenzaprine, no.2	320, 230	291	292(100), 73(11), 58(69)
2-Hydroxycyclobenzaprine, no.3	290, 240	291	292(100), 73(6), 58(77)
N-Desmethylcyclobenzaprine, no.4	293, 244, 226	261	262(100)
Cyclobenzaprine, no.5	293, 244, 226	275	276(100)
Cyclobenzaprine *N*-oxide, no.6	293, 244, 226	291	292(100), 276(5), 262(2)

[a] Peak nos. are referred to in Fig. 1.
[b] RI, relative intensity.

incubation. Following the incubation period, methanol (25 ml) was added to each flask. The metabolites were extracted with chloroform (4 × 50 ml). The organic extracts were combined and the mixture was purified by preparative HPLC, and analyzed by EIMS and CIMS.

2.5. Metabolism of CBP by rat liver microsomes

Uninduced rat liver microsomes containing 20 mg of protein was incubated with 2 mM of CBP in 5 ml of a buffer containing 50 mM phosphate buffer (pH 7.4), 1 mM DTT, 20% glycerol, 0.5 M sucrose, and 2 mM of NADPH at 37°C for 5 h. The reaction mixture was extracted with chloroform (3 × 5 ml). After evaporation, the material was dissolved in 1 ml of methanol. Aliquots were analyzed by HPLC. The sample was also co-injected with purified metabolites from the fungal biotransformations.

3. Results

3.1. Isolation and identification of CBP metabolites

The chloroform-extracted residual CBP and metabolites formed by *C. elegans* were separated by HPLC as shown in Fig. 1A. Peak 0 was re-purified on a Cyano-column as indicated in the insert of Fig. 1A. The control samples showed no metabolism. The major fungal metabolites were collected and their structures were determined by ^1H NMR spectroscopy, MS, and UV analyses. Some of these data are listed in Table 1 and Table 2.

Peak 0 (HPLC retention time 4 min in Fig. 1A), after a second HPLC purification on a Cyano-column (insert in Fig. 1A), had UV absorptions at 219 and 243 nm and no absorption at 293 nm (Table 1), suggesting a structural change in the

Table 2
^{1}H NMR resonance assignments and spectral parameters of cyclobenzaprine and its fungal metabolites[a]

Assignment	Chemical Shift (ppm)					
	Nor-2-OH-CBP peak 1	3-OH-CBP peak 2	2-OH-CBP peak 3	Nor-CBP peak 4	CBP peak 5	CBP-NO peak 6
1	6.69	7.12	6.69	7.25–7.41	7.25–7.41	7.28–7.43
2	OH	6.73	OH	7.25–7.41	7.25–7.41	7.28–7.43
3	6.81	OH	6.81	7.25–7.41	7.25–7.41	7.28–7.43
4	7.18	6.79	7.17	7.25–7.41	7.25–7.41	7.28–7.43
6	7.23	7.23	7.23	7.25–7.41	7.25–7.41	7.28–7.43
7	7.38	7.38	7.38	7.25–7.41	7.25–7.41	7.28–7.43
8	7.31	7.32	7.30	7.25–7.41	7.25–7.41	7.28–7.43
9	7.35	7.35	7.35	7.25–7.41	7.25–7.41	7.28–7.43
10	6.85	6.71	6.84	6.88[b]	6.87[b]	6.88[b]
11	6.76	6.79	6.75	6.88[b]	6.87[b]	6.88[b]
12	5.44	5.52	5.42	5.50	5.48	5.49
13	2.43, 2.53	2.46, 2.56	2.43, 2.52	2.44, 2.53	2.42, 2.51	2.69[c]
14	2.92, 3.03	3.06, 3.13	2.95, 3.03	2.89, 3.00	2.87, 2.97	3.44, 3.62
15	2.56	2.68	2.60	2.53	2.55	3.26[d]
16	—	2.68	2.60	—	2.55	3.22[d]

[a] Samples were dissolved in methanol-d_4 and measurements were made at 500.13 MHz. Selected coupling constants that were most suitable for the first-order analysis are as follows: for 2-OH-CBP, $J_{1-3} = 2.6$; and $J_{3-4} = 8.4$; for Nor-2-OH-CBP, the same as 2-OH-CBP within experimental error; for 3-OH-CBP, $J_{1-2} = 8.4$; and $J_{3-4} = 2.6$ Hz. J_{9-10} was approximately 12.0 Hz for all compounds. It is estimated that the metabolites are about 70% protonated at the nitrogen atom (with the exception of CBP-NO).
[b] An average chemical shift is reported. The H10 and H11 resonances appear as an AB quartet with a chemical shift difference of approximately 0.01 ppm.
[c] Average chemical shift of methylene protons.
[d] No distinction between methyl groups is implied.

conjugation system. MS analysis gave a protonated molecule at m/z 310, indicating addition of two oxygen atoms (Fig. 2). Further MS analysis indicated strong fragment ions at m/z 292 and 262, corresponding to dehydration (MH^+-18) followed by loss of formaldehyde CH_2O (MH^+-18-30) (Fig. 2), which was consistent with a dihydrodiol structure. The NMR spectrum was substantially different from that of the parent compound. There was no evidence of the H10 and H11 resonances in the aromatic region (7.13–7.71 ppm). Instead, there were several doublets with chemical shifts consistent with carbinol protons, and there was resonance doubling throughout the observable spectrum that was characteristic of chemical exchange. The ratio of the subspectra was 1.0:0.6. The two doublets from the carbinol protons of the major subspectrum (5.16 and 4.47 ppm) exhibited a coupling constant of 9.5 Hz. This data was indicative of a 10,11-*trans*-dihydrodiol. With the loss of the double bond between C10 and C11, this metabolite could be considered as an amitriptyline derivative. Resonance doubling in amitriptyline has previously been reported [11] and references cited therein. The NMR spectrum of peak 0 was also complicated by the presence of possible CBP related impurities (roughly 20%) and a very large impurity resonance in the aliphatic region. Based on the MS and NMR data, peak 0 was identified as cyclobenzaprine 10,11-*trans*-dihydrodiol (CBP-diol).

Peak 1 (HPLC retention time 9 min in Fig. 1A) had similar UV and NMR spectral properties to those of 2-OH-CBP, except the methyl resonance integrated as three protons instead of six protons indicating *N*-demethylation. Some changes in chemical shifts of protons near the methyl group were also observed in the NMR spectrum. This is consistent with demethylation, but small changes in degree of ionization at nitrogen would also affect these chemical shifts. The demethylation was further confirmed by MS analysis, giving a protonated molecule (MH^+) at m/z 278. This metabolite was identified as *N*-desmethyl-2-hydroxycyclobenzaprine (Nor-2-OH-CBP).

Peak 2 (HPLC retention time 10 min in Fig. 1A) also had a protonated molecule at m/z 292 in CIMS analysis (Table 1), but showed different UV spectral properties from those of CBP and 2-OH-CBP (Table 1), supporting a different aromatic

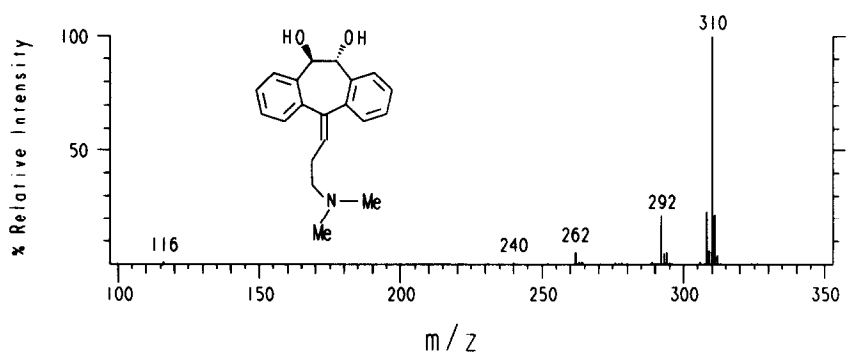

Fig. 2. CIMS spectrum of cyclobenzaprine 10,11-*trans*-dihydrodiol.

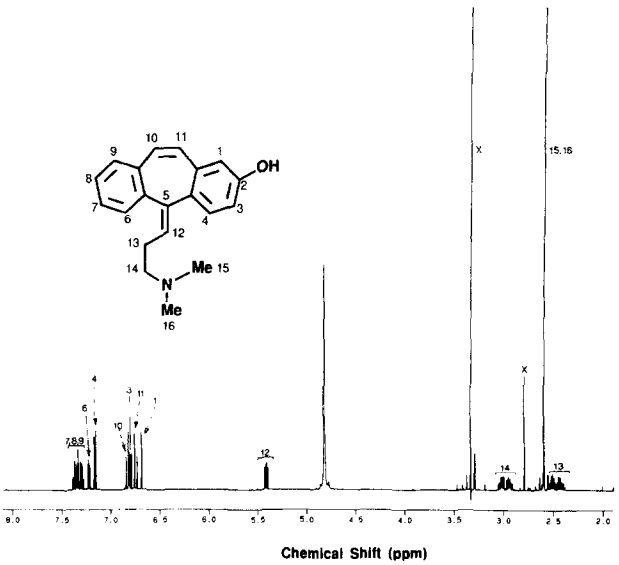

Fig. 3. ^{1}H NMR spectrum at 500.13 MHz of 2-hydroxycyclobenzaprine in methanol-d_4 with resonance assignments. The main impurity peaks are labeled by '*x*'.

hydroxyl-substitution. Peak 2 was determined by NMR spectroscopy to be 3-hydroxycyclobenzaprine (3-OH-CBP) by methods analogous to those described below for 2-OH-CBP.

Peak 3 (HPLC retention time 10.3 min in Fig. 1A) had a protonated molecule (MH^+) at m/z 292 in MS analysis (Table 1), indicating an addition of an oxygen atom to CBP. The UV spectrum of peak 3 was different from that of CBP (Table 1) which also supported aromatic hydroxylation. The NMR spectrum (Fig. 3) clearly showed a single substitution on the aromatic ring system and there were upfield shifts characteristic of hydroxyl-substitution. An NOE experiment involving saturation of the H12 resonance provided the key data for determining the site of substitution. It resulted in a relatively large enhancement of the aromatic doublet at 7.17 ppm (8%), which enabled assignment of the H4 resonance. From this assignment, the site of the substitution was readily determined as being C3 from the coupling pattern and homonuclear decoupling experiments in a manner analogous to that previously reported for amitriptyline metabolites [11]. Note that we have used the ring numbering system to define *cis* and *trans* orientations relative to the C5–C12 double bond. Thus, with this convention, the aromatic ring containing the C1–C4 atoms is defined as being *trans* to the alkyl side chain. The NMR data for the alkyl side chain revealed no further modifications. This metabolite was identified as 2-hydroxycyclobenzaprine (2-OH-CBP).

Peak 4 (HPLC retention time 12.2 min in Fig. 1A) had the same UV spectrum as CBP (Table 1), however, the methyl resonance in the NMR spectrum integrated as three protons indicating *N*-demethylation. The demethylation was further

confirmed by CIMS analysis, showing a protonated molecule (MH^+) at m/z 262. This metabolite was identified as *N*-desmethylcyclobenzaprine (Nor-CBP).

Peak 5 (HPLC retention time 14 min in Fig. 1A) was confirmed as CBP since it eluted at the same HPLC retention time and had MS spectral properties identical to authentic CBP (Table 1). The NMR data were in accord with the CBP structure. In addition, comparison of chemical shifts with authentic CBP in the hydrochloride form and in the neutral form suggest that peak 5 is 70% protonated at the nitrogen atom.

Peak 6 (HPLC retention time 16.3 min in Fig. 1A) had similar UV spectral properties to those of CBP (Table 1). Large downfield shifts were observed in the NMR spectrum for protons near the nitrogen atom indicating substitution at this site. Unlike the other *N*-dimethylated compounds, separate chemical shifts were observed for the two methyl groups. CIMS analysis showed a protonated molecule (MH^+) at m/z 292, and fragment ions at m/z 276 and 262. These 16 and 30 mass unit losses are typical for an *N*-oxide as reported for similar *N*-oxide metabolites [15]. The metabolite was identified as cyclobenzaprine *N*-oxide (CBP-NO).

3.2. Oxygen-18 experiments

The mechanism for the formation of the major metabolite, 2-OH-CBP, was investigated by an $^{18}O_2$ labeling experiment. The HPLC-isolated metabolite showed a weak molecular ion at m/z 293 by electron ionization method (Fig. 4A), then was

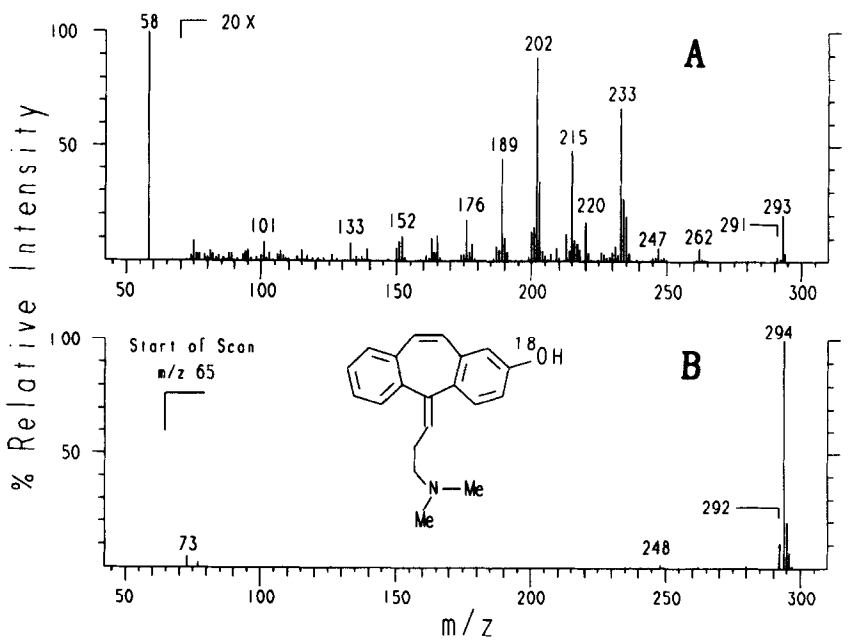

Fig. 4. Mass spectra of ^{18}O-labeled 2-hydroxycyclobenzaprine. (A) EIMS (B) CIMS.

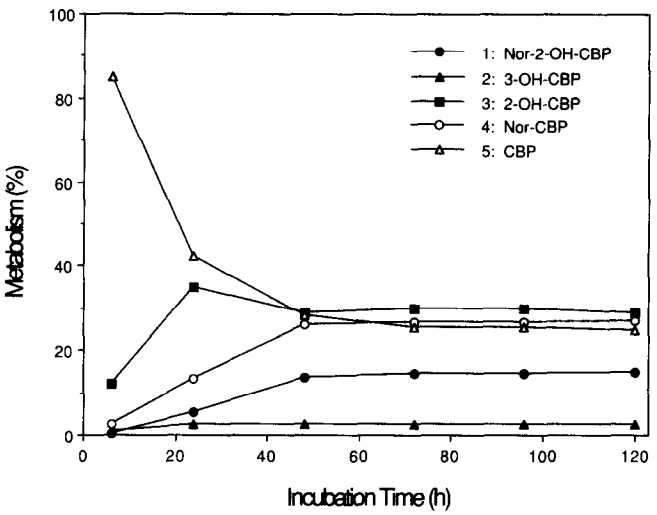

Fig. 5. Time course for biotransformation of cyclobenzaprine by *C. elegans*. Metabolism represented residual cyclobenzaprine or the metabolites generated, and is expressed as a percent of cyclobenzaprine added originally.

subsequently analyzed by CIMS, which showed a strong protonated molecule at m/z 294 (Fig. 4B). The incorporation of ^{18}O into 2-0H-CBP suggested that the formation of 2-OH-CBP was catalyzed by a monooxygenase.

3.3. Mass balance studies

Relative amounts of CBP and its major metabolites formed by *C. elegans* at different incubation times are presented in Fig. 5. As CBP decreased, Nor-CBP and 2-0H-CBP increased. About 75% of CBP was metabolized within the initial 72 h of incubation. In a typical biotransformation experiment, about 80% of the 50 mg dosed CBP was recovered as CBP (compound 5 in Fig. 1A, 25 mg) and two major metabolites, Nor-CBP (compound 4, 4 mg) and 2-OH-CBP (compound 3, 13 mg). The slowdown of the metabolism (Fig. 5) after 50 h incubation of the drug with *C. elegans* could be caused by inactivation of the metabolism enzymes and depletion of enzyme cofactors.

3.4. CBP metabolism by rat liver microsomes

For comparison to the fungal metabolism, CBP was incubated with rat liver microsomes. The chloroform-extracted material was analyzed by HPLC (Fig. 1B). The metabolites (peak 0–6) formed were chromatographically identical to the fungal incubation. These peaks were confirmed by co-injections with purified fungal metabolites and had identical UV properties and HPLC retention times. Since there were no MS and NMR analyses, these metabolites were tentatively assigned as compounds 0–6 corresponding to the numbering for the fungal metabolites.

4. Discussion

We investigated the microbial metabolism of CBP. Six metabolites were isolated and characterized from incubation of CBP with *C. elegans*. Fig. 6 shows the structures of these compounds, and proposed metabolic pathways. Six metabolites,

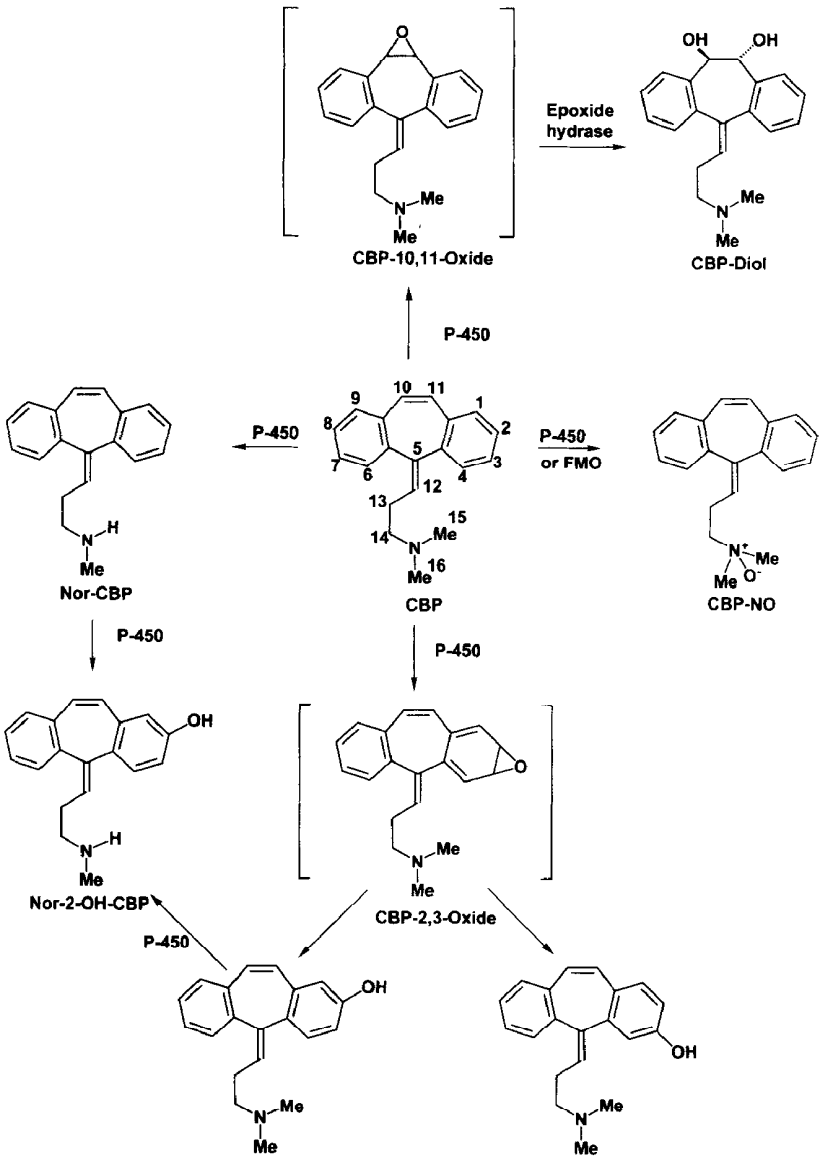

Fig. 6. Proposed biotransformation pathways of cyclobenzaprine in *C. elegans*.

CBP 10,11-*trans*-dihydrodiol, Nor-2-OH-CBP, 3-OH-CBP, 2-OH-CBP, Nor-CBP and CBP-NO were characterized, indicating that aromatic hydroxylation and *N*-demethylation were major metabolic pathways. For direct comparison, we also investigated the biotransformation of this drug by the rat liver microsomes. As shown in Fig. 1B, the rat liver microsomes qualitatively synthesized all of the six metabolites produced by *C. elegans*, except that the rat liver microsomes produced the Nor-CBP and CBP 10,11-*trans*-dihydrodiol as the major metabolites instead of 2-OH-CBP from the fungus.

About 57% of all metabolites of amitriptyline by *C. elegans* was 11-hydroxyamitriptyline, an aliphatic hydroxylation product [11]. The change of the 10,11 carbon–carbon single bond of amitriptyline to the 10,11 carbon–carbon double bond of cyclobenzaprine, caused a shift of the major reaction site from position 11 to position 2. 2-OH-CBP, an aromatic hydroxylation product, constitutes approximately 59% of all CBP metabolites produced by *C. elegans*. Major metabolites of CBP in the rat urine were reported to be phenolic compounds [13], which is similar to that in the fungus, however, it is different from rat liver microsomes incubation with CBP in this study.

CBP 10,11-*trans*-dihydrodiol was isolated from the incubation of CBP with *C. elegans*, although the absolute stereochemistry of the two chiral carbon centers in the dihydrodiol remains to be determined. Both cyclobenzaprine 10,11-epoxide and CBP 10,11-*trans*-dihydrodiol have been identified from rats and humans [12,13,16]. Isolation of the fungal *trans*-dihydrodiol provides another example of the similarity of eukaryotic organisms such as animals and fungi to oxidize double bonds in aromatic or olefinic substrates with subsequent enzymatic hydration to form *trans*-dihydrodiols, while bacteria generally oxidize these substrates to form *cis*-dihydrodiols by dioxygenases [17–20].

CBP *trans*-dihydrodiol is probably formed by a fungal cytochrome P-450-mediated formation of unidentified CBP 10,11-epoxide intermediate followed by action of an epoxide hydrase. Microsomal cytochrome P-450 monooxygenase activities and epoxide hydrase activity have been identified in *C. elegans* [21–24]. The results of the isotope labeling of 2-OH-CBP by $^{18}O_2$ and the *trans*-configuration of CBP 10,11-dihydrodiol further suggested that the initial reactions were catalyzed by cytochrome P-450 monooxygenases in *C. elegans*.

The availability of sufficient quantities of metabolites for structural characterization is often limited from animal or human sources because of low allowable doses and high expenses. In our experiments, a small scale incubation of CBP with the filamentous fungus *C. elegans* led to production of milligram amounts of metabolites that can be used for structural characterization. By comparison between fungal metabolism of amitriptyline and cyclobenzaprine, we also demonstrated that *C. elegans* respond to the structural changes of substrates to produce the correspond ing mammalian metabolites. Therefore, microbial biotransformation is very useful in initial investigations of drug metabolism to generate potential standard compounds for further animal studies and for the studies of toxicity and pharmacology of the metabolites in humans.

Acknowledgements

This research was supported in part by an appointment to the Postgraduate Research Program at the National Center for Toxicological Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the US Food and Drug Administration.

References

[1] R.M. Atlas and C.E. Cerniglia, Bioremediation of petroleum pollutants, BioScience, 45 (1995) 332–338.
[2] J.P. Rosazza, Biocatalysis, microbiology, and chemistry: the power of positive linking, ASM News, 61 (1995) 241–245.
[3] C.E. Cerniglia, Biodegradation of polycyclic aromatic hydrocarbons, Curr. Opin. Biotechnol., 4 (1993) 331–338.
[4] A.M. Clark and C.D. Hufford, Use of microorganisms for the study of drug metabolism: an update, Med. Res. Rev., 11 (1991) 473–501.
[5] P.J. Davis, Microbial models of mammalian drug metabolism, Dev. Ind. Microbiol., 29 (1988) 197–219.
[6] J.B. Reighard and J.E. Knapp, Microbial models of mammalian metabolism, Pharm. Int., (1986) 92–96.
[7] R.V. Smith and J.P. Rosazza, Microbial transformations as means of preparing mammalian drug metabolites. in: J.P. Rosazza (Ed.), Microbial Transformation of Bioactive Compounds, Vol. II, CRC Press, Boca Raton, FL, 1982, pp. 1–42.
[8] C.E. Cerniglia, E.B. Hansen, Jr., K.J. Lambert, W.A. Korfmacher and D.W. Miller, Fungal transformations of antihistamines: metabolism of methapyrilene, thenyldiamine and tripelennamine to *N*-oxide and *N*-demethylated derivatives, Xenobiotica, 18 (1988) 301–312.
[9] E.B. Hansen, Jr., C.E. Cerniglia, W.A. Korfmacher, D.W. Miller and R.H. Heflich, Microbial transformation of the antihistamine pyrilamine maleate. Formation of potential mammalian metabolites, Drug Metab. Dispos., 15 (1987) 97–106.
[10] R.J.P. Cannell, A.R. Knaggs, M.J. Dawson, G.R. Manchee, P.J. Eddershaw, I. Waterhouse, D.R. Sutherland, G.D. Bowers and P.J. Sidebottom, Microbial biotransformation of the angiotensin II antagonist GR117289 by *Streptomyces rimosus* to identify a mammalian metabolite, Drug Metab. Dispos., 23 (1995) 727–730.
[11] D.L. Zhang, F.E. Evans, J.P. Freeman, B. Duhart, Jr. and C.E. Cerniglia, Biotransformation of amitriptyline by *Cunninghamella elegans*. Drug Metab. Dispos., 23 (1995) 1417–1425.
[12] H.B. Hucker, A.J. Balletto, B.H. Arison and A.G. Zacchei, Metabolism of cyclobenzaprine in the dog, Drug Metab. Dispos., 6 (1978) 184–192.
[13] H.B. Hucker, S.C. Stauffer, A.J. Balletto, S.D. White, A.G. Zacchei and B.H. Arison, Physiological disposition and metabolism of cyclobenzaprine in the rat, dog, rhesus monkey, and man, Drug Metab. Dispos., 6 (1978) 659–672.
[14] F.E. Evans, J. Deck and P.C. Howard, Structures of phenolic isomers of 2- and 3-nitrofluoranthene studied by one- and two-dimensional ^{1}H NMR spectroscopy. Comparative analysis of mutagenicity, Chem. Res. Toxicol., 7 (1994) 352–357.
[15] W.A. Korfmacher, C.L. Holder, C.E. Cerniglia, D.W. Miller, E.B. Hansen, Jr., K.L. Lambert, A.B. Gosnell, W. Slikker, Jr., L.G. Rushing and H.C. Thompson, Jr. Desorption chemical ionization mass spectrometry of nine antihistamines and some of their metabolites, Spectros. Int. J. 4 (1985) 181–192.

[16] G. Belvedere, C. Pantarotto, V. Rovei and A. Frigerio, Identification of 10,11-epoxide and other cyclobenzaprine metabolites isolated from rat urine, J. Pharm. Sci., 65 (1976) 815–821.
[17] C.E. Cerniglia, J.B. Sutherland and S.A. Crow, Fungal metabolism of aromatic hydrocarbons, in: G. Winkelmann (Ed.), 'Microbial Degradation of Natural Products', VCH Verlagsgesellschaft, Weinhein, Germany, 1992, pp. 193–217.
[18] C.E. Cerniglia, D.T. Gibson and R.H. Dodge, Metabolism of benzo[a]anthracene by the filamentous fungus *Cunninghamella elegans*, Appl. Environ. Microbiol., 60 (1994) 3931–3938.
[19] J.B. Sutherland, Detoxification of polycyclic aromatic hydrocarbons by fungi, J. Ind. Microbiol., 9 (1992) 53–62.
[20] D.M. Jerina, P.J. van Bladeren, H. Yagi, D.T. Gibson, V. Mahadevan, A.S. Neese, M. Koreeda, N.D. Sharma and D.R. Boyd, Synthesis and absolute configuration of the bacterial *cis*-1,2-, *cis*-8,9-, and *cis*-10,11-dihydrodiol metabolites of benzo[a]anthracene formed by a strain of *Beijerinckia*, J. Org. Chem., 49 (1984) 3621–3628.
[21] C.E. Cerniglia and D.T. Gibson, Metabolism of naphthalene by cell-extract of *Cunninghamella elegans*, Arch. Biochem. Biophys., 186 (1978) 121–127.
[22] J.P. Ferris, L.H. MacDonald, M.A. Patrie and M.A. Martin, Aryl hydrocarbon hydroxylase activity in the fungus *Cunninghamella bainieri*: evidence for the presence of cytochrome P450, Arch. Biochem. Biophys., 175 (1976) 443-452.
[23] D.L. Zhang, Y. Yang, J.E.A. Leakey and C.E. Cerniglia, Phase I and Phase II enzymes produced by *Cunninghamella elegans* for the metabolism of xenobiotics, FEMS Microbiol. Lett., 138 (1996) 221–226.
[24] L.P. Wackett and G.T. Gibson, Metabolism of xenobiotic compounds by enzymes in the cell extracts of the fungus *Cunninghamella elegans*, Biochem. J. 205 (1982) 117–122.