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Biotransformation of Chlorpromazine and Methdilazine by *Cunninghamella elegans*

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When tested as a microbial model for mammalian drug metabolism, the filamentous fungus *Cunninghamella elegans* metabolized chlorpromazine and methdilazine within 72 h. The metabolites were extracted by chloroform, separated by high-performance liquid chromatography, and characterized by proton nuclear magnetic resonance, mass, and UV spectroscopic analyses. The major metabolites of chlorpromazine were chlorpromazine sulfoxide (36%), *N*-desmethylchlorpromazine (11%), *N*-desmethyl-7-hydroxychlorpromazine (6%), 7-hydroxychlorpromazine sulfoxide (5%), and chlorpromazine *N*-oxide (2%), all of which have been found in animal studies. The major metabolites of methdilazine were 3-hydroxymethdilazine (35%), methdilazine sulfoxide (30%), methdilazine *N*-oxide (4%), phenothiazine (3%), and 2-hydroxymethdilazine (3%). $^{18}O_2$ labeling experiments indicated that the oxygen atoms in methdilazine sulfoxide, methdilazine *N*-oxide, and 3-hydroxymethdilazine were all derived from molecular oxygen. The production of methdilazine sulfoxide and 3-hydroxymethdilazine was inhibited by the cytochrome P-450 inhibitors metyrapone and proadifen. An enzyme activity for the sulfoxidation of methdilazine was found in microsomal preparations of *C. elegans*. These experiments suggest that the sulfoxidation and hydroxylation of methdilazine and chlorpromazine by *C. elegans* are catalyzed by cytochrome P-450.

Phenothiazines are widely used in medicine for treatment of psychoses, depression, nausea, vomiting, and pruritus (12). The antimicrobial properties of some phenothiazines have also been established (5, 6). The metabolism of chlorpromazine (CPZ), an antipsychotic phenothiazine drug, is an important factor in both its therapeutic efficacy and its toxic side effects. Among its four primary mammalian metabolites, *N*-desmethylchlorpromazine (Nor_1-CPZ) and 7-hydroxychlorpromazine (7-OH-CPZ) are pharmacologically active, chlorpromazine *N*-oxide (CPZ-NO) is potentially active, and chlorpromazine sulfoxide (CPZ-SO) is inactive (1, 27). Although the metabolism of chlorpromazine has been well studied (16, 19, 21, 25, 26), little is known about the metabolism of methdilazine (MDZ), an analgesic, antipruritic, and antimicrobial phenothiazine drug (5).

The zygomycete fungi of the genus *Cunninghamella* have been shown to have the ability to metabolize xenobiotics with various structural features to produce metabolites similar to those found in mammals (3, 7, 9, 23, 24). On the basis of these results, fungal systems have been proposed as models for mammalian drug metabolism, and the advantages of using such a microbial model include low cost, ease of handling, and scale-up capability as reviewed by Davis (9). In this study, CPZ and MDZ were used as structural probes to investigate the in vitro metabolism of N-substituted phenothiazines by *Cunninghamella elegans.*

MATERIALS AND METHODS

Chemicals. CPZ, metyrapone, NADPH, *p*-toluenesulfonyl fluoride, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, Mo.). MDZ was from Mead Johnson & Company (Evansville, Ind.). Proadifen (SKF 525-A) was from SmithKline Beecham (Philadelphia, Pa.). Sodium hydrosulfite was from Aldrich Chemical Co. (Milwaukee, Wis.). Oxygen ($^{18}O_2$, 95 to 98 atom%) was from Cambridge Isotope Laboratories (Andover, Mass.).

Microorganism and fermentation conditions. *C. elegans* ATCC 9245 was grown on Sabouraud dextrose agar plates for 5 days and then stored at 4°C. Standard incubations were performed in 30 ml of Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.). The medium was inoculated with 5 ml of a blended *C. elegans* mycelial suspension (two plates in 150 ml of saline solution) in 125-ml Erlenmeyer flasks and incubated at 25°C with agitation at 150 rpm. For biotransformation experiments, each drug was dissolved in *N*,*N*-dimethylformamide (100 mg/ml) and added to 48-h-old cultures. Control experiments included an incubation of the fungus without drugs and an incubation of each drug added to an uninoculated medium.

Analytical methods. High-performance liquid chromatography (HPLC) was performed with a model LC-600 dual-pump system equipped with a photodiode array detector (Shimadzu Scientific Instruments, Columbia, Md.). Samples were analyzed on a Synchropak SCD-100 reversed-phase column (4.6 by 250 mm; particle size, 5 μm; SynChrom, West Lafayette, Ind.). The metabolites were eluted isocratically with acetonitrile–10 mM phosphate buffer (pH 7.0 to 7.2; 80/20 [vol/vol]) at a flow rate of 1 ml/min. For large-scale purifications of metabolites, a semipreparative column (21.2 by 250 mm; particle size, 10 μm) was used and the flow rate was 5 to 7 ml/min.

Electron ionization and ammonia chemical ionization (CI) mass spectrometry (MS) were performed on a model 4000 quadrupole mass spectrometer upgraded to model 4500 capabilities (Finnigan MAT, San Jose, Calif.). For positive-ion CI analyses, the reagent was 10% ammonia in nitrogen, with the ion source pressure 2.8 torrs (ca. 373 Pa) and the ion temperature 120°C. For electron ionization analyses, the ion source temperature was 150°C. Samples were analyzed with a direct-exposure probe, with a linear current ramp of 5 mA/s. The quadrupole was scanned from 40 to 650 Da with a 1-s cycle time.

^{1}H nuclear magnetic resonance (NMR) analyses were performed at 300 MHz on a Gemini 300 NMR spectrometer (Varian Instruments, Palo Alto, Calif.) at room temperature. ^{1}H NMR assignments were based on chemical shifts, integration, and homonuclear decoupling measurements. Samples were dissolved in deuterated methanol, and chemical shifts are reported on the δ (parts per million) scale by assigning the methyl resonance to 3.35 ppm.

Isolation of CPZ and MDZ metabolites. Four flasks, each containing 35 ml of a 48-h-old *C. elegans* culture, dosed with 10 mg of CPZ or MDZ, were incubated at 25°C for 96 h. Methanol (25 ml) was added to each flask, and the combined mixture was extracted with chloroform (four times, 200 ml each). The organic and aqueous layers from each extraction were analyzed by HPLC. More than 95% of the chloroform-extractable material was recovered after four extractions. The extracted material, after evaporation of solvent, was dissolved in methanol

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and separated by semipreparative HPLC. Samples from the HPLC separations were passed through a C_{18} Sep-Pak cartridge (Waters Associates, Milford, Mass.) to remove the buffer and subjected to structural analysis.

$^{18}O_2$ labeling experiments. A 35-ml culture of *C. elegans* in a 125-ml Erlenmeyer flask was dosed with 10 mg of MDZ. The flask was purged with N_2 four times and once with $^{18}O_2$ and finally filled with $^{18}O_2$. The ^{18}O content in each flask, as determined by MS, was 96.5 atom% at first and 68 atom% after 24 h of incubation. After the addition of 25 ml of methanol, the metabolites were extracted with chloroform (four times, 50 ml each), purified by semipreparative HPLC, and analyzed by electron ionization MS.

Inhibition studies. Duplicate experiments were performed. Metyrapone (2 mM) or SKF 525-A (1.5 mM) in 0.05 ml of dimethylformamide was added to 48-h-old 35-ml cultures (28). After 15 min of incubation, MDZ (1 mM) was added. At 0, 7, 24, 48, and 72 h, 1-ml aliquots were removed and frozen at −20°C. The samples were thawed, vortexed, and centrifuged before HPLC analysis of the supernatants.

Cell-free homogenate studies. A cell-free homogenate of *C. elegans* was prepared as described by Cerniglia and Gibson (4) in 50 mM phosphate buffer (pH 7.4) containing 1 mM dithiothreitol, 20% glycerol, and 2 mM *p*-toluenesulfonyl fluoride. The homogenate was centrifuged at 12,000 × *g* for 15 min, and the microsome fraction was pelleted by centrifugation at 120,000 × *g* for 90 min. The protein concentration was determined with a Coomassie blue protein assay reagent kit (Pierce, Rockford, Ill.). The reduced carbon monoxide difference spectrum was determined as described by Omura and Sato (20).

Reaction mixtures contained 1.5 mg of protein (cell-free homogenate, supernatant, microsomes, or boiled-protein fractions), 50 mM phosphate buffer (pH 7.4), 10% glycerol, 1 mM MDZ, 1 mM dithiothreitol, and 1 mM NADPH in a final volume of 0.5 ml and were incubated at 30°C for 1 h. In inhibition experiments, either 1 mM SKF 525-A was included in the reaction mixture or the microsomes were bubbled with carbon monoxide for 20 s. Samples from reaction mixtures were analyzed by HPLC. The amounts of the metabolites were estimated by integration of HPLC peaks and comparison with those of metabolites isolated from cell cultures.

RESULTS

Fungal metabolites. The metabolites of CPZ and MDZ were separated by HPLC, as shown in Fig. 1A and B, respectively. The peaks marked "X" in Fig. 1 were not identified. HPLC analysis of control experiments showed that the compounds were stable under the conditions used. The major metabolites were collected and characterized by MS and UV analyses (Table 1) and by ^{1}H NMR (Table 2) when sufficient quantities were available.

CPZ. The material in peak 6 (Fig. 1A) showed a molecular ion M^+ at *m/z* 320 (Table 1), consistent with both N demethylation and addition of an oxygen atom. ^{1}H NMR analysis showed that the compound had one methyl group and six aromatic protons, suggesting that the additional oxygen was on one of the aromatic rings. H6 showed only *meta* coupling to H8 (J = 2.7 Hz). H8 showed *ortho* coupling to H9 (J = 8.4 Hz), in addition to *meta* coupling to H6, suggesting that position 7 had been hydroxylated (Table 2). This metabolite was identified as *N*-desmethyl-7-hydroxychlorpromazine (Nor_1-7-OH-CPZ).

Peak 5 (Fig. 1A) had a molecular ion M^+ at *m/z* 351, indicating an additional two oxygen atoms. The MS analysis also showed a fragment ion at *m/z* 335 due to the loss of one oxygen atom (Table 1). ^{1}H NMR analysis showed six aromatic protons, suggesting that one oxygen atom was attached to an aromatic ring. The coupling pattern of the aromatic protons was the same as those in Nor_1-7-OH-CPZ (Table 2). The UV spectral shifts from the spectrum of CPZ, with absorption peaks at 285, 314, and 361 nm, suggested the addition of an oxygen atom to the sulfur at position 5 to give the sulfoxide. On the basis of UV, MS, and NMR spectral properties, this metabolite was identified as 7-hydroxychlorpromazine sulfoxide (7-OH-CPZ-SO).

The major metabolite of CPZ (Fig. 1A, peak 4) showed UV absorption at 343 nm (Table 1), suggesting a delocalization of the conjugation system. MS analysis, which showed a molecular ion M^+ at *m/z* 334 and an oxygen loss ion at *m/z* 318 (Table 1) was consistent with sulfoxidation. ^{1}H NMR analysis showed

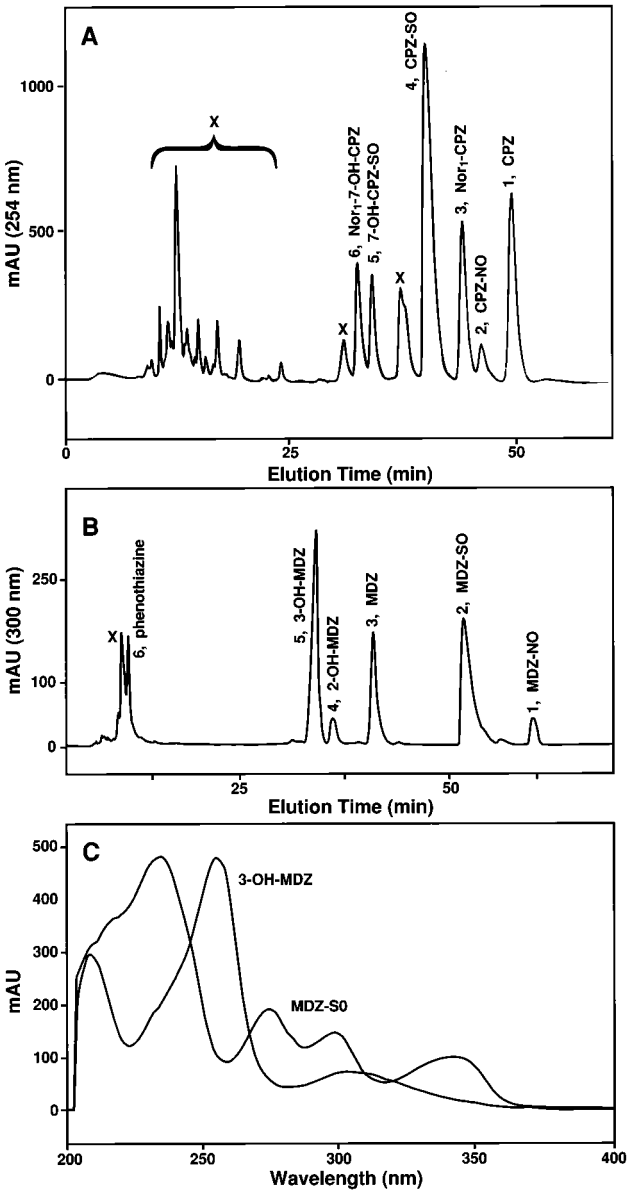

FIG. 1. (A) HPLC chromatogram of CPZ and its fungal metabolites; (B) HPLC chromatogram of MDZ and its fungal metabolites; (C) UV spectra of 3-OH-MDZ and MDZ-SO. AU, absorbance units.

seven aromatic protons (Table 2). This metabolite had MS and UV spectral properties identical to those of synthetic CPZ-SO (2).

Peak 3 (Fig. 1A) had UV and ^{1}H NMR spectral properties identical to those of CPZ, except that the methyl protons were integrated as three (Tables 1 and 2), indicating an N-demethylated product. The N demethylation was confirmed by MS analysis, which showed an M^+ at *m/z* 304 (Table 1). This metabolite was identified as Nor_1-CPZ.

Peak 2 (Fig. 1A) had the same UV spectrum as that of CPZ. MS analysis showed an M^+ at *m/z* 334 and M^+-16, M^+-30, and M^+-60 fragment ions (Table 1), characteristic of an *N*-oxide (18). Therefore, this metabolite was identified as CPZ-NO.

Peak 1 (Fig. 1A) was the parent compound CPZ, as confirmed by coelution with an authentic sample of CPZ and by MS and ^{1}H NMR analyses (Tables 1 and 2).

TABLE 1. UV and MS analysis data of CPZ, MDZ, and their metabolites by *C. elegans*

Compound	UV λ_{max} (nm)	Peak no.[a]	Mass spectrum [*m/z* (% relative intensity)]
Nor_1-7-OH-CPZ	257, 316	6 (320)	322 (24), 321 (12), 320 (67, [M^+]), 290 (7), 289 (6), 288 (22), 264 (6), 263 (5), 262 (17), 251 (18), 250 (22), 249 (49), 248 (44), 232 (4), 230 (9), 75 (15), 72 (100), 71 (20)
7-OH-CPZ-SO	245, 261, 285, 314, 361	5 (350)	NH_3 CI: 354 (8), 353 (41), 352 (22), 351 (100, [MH^+]), 337 (21), 336 (16), 335 (66), 316 (15), 301 (15), 250 (10), 248 (6), 179 (13), 161 (45), 114 (10), 99 (29), 85 (14), 84 (31), 79 (19), 73 (37), 72 (57), 71 (10), 70 (42), 69 (14)
CPZ-SO	243, 278, 300, 343	4 (334)	336 (2), 334 (4, [M^+]), 319 (2), 318 (1), 317 (4), 274 (1), 273 (1), 272 (4), 259 (1), 248 (14), 246 (36), 216 (4), 214 (12), 84 (12), 74 (10), 58 (100)
Nor_1-CPZ	259, 313	3 (304)	306 (31), 305 (13), 304 (79, [M^+]), 274 (13), 273 (11), 272 (35), 248 (8), 247 (9), 246 (21), 235 (25), 234 (28), 233 (75), 232 (54), 216 (9), 214 (31), 198 (14), 197 (22), 196 (21), 153 (11), 152 (11), 72 (100), 71 (38), 70 (14), 69 (16), 58 (25), 57 (12), 56 (12)
CPZ-NO	259, 313	2 (334)	334 (5, [M^+]), 320 (12), 319 (5), 318 (30), 306 (4), 304 (13), 275 (11), 274 (10), 273 (28), 272 (19), 246 (8), 235 (14), 234 (31), 233 (34), 232 (100), 214 (7), 198 (7), 197 (10), 196 (9), 86 (34), 85 (10), 84 (8), 72 (12), 61 (7)
CPZ	259, 313	1 (318)	320 (7), 318 (20, [M^+]), 272 (10), 86 (28), 85 (12), 58 (100)
Phenothiazine	252–255, 318	6 (199)	200 (14), 199 (100, [M^+]), 198 (15), 167 (32), 166 (11), 99 (9)
3-OH-MDZ	254, 310	5 (312)	314 (15), 312 (100, [M^+]), 229 (11), 228 (37), 216 (7), 215 (92), 196 (16), 98 (54), 97 (56), 96 (34), 83 (13), 82 (25), 55 (33)
2-OH-MDZ	252, 313	4 (312)	314 (15), 312 (58, [M^+]), 128 (31), 215 (100), 196 (13), 182 (10), 103 (26), 98 (40), 97 (43), 96 (27), 93 (11), 83 (11), 82 (19), 75 (46), 59 (27), 57 (27), 55 (27)
MDZ	251, 297	3 (296)	298 (5), 297 (14), 296 (78, [M^+]), 213 (17), 212 (38), 200 (8), 199 (60), 198 (32), 180 (29), 179 (5), 154 (5), 152 (5), 98 (65), 97 (100), 96 (52), 84 (11), 83 (14), 82 (40), 70 (8), 58 (5), 55 (46)
^{18}O-MDZ-SO	235, 275, 299, 343	2 (314)	315 (1), 314 (8, [M^+]), 312 (2), 297 (5), 295 (17), 293 (5), 251 (7), 214 (5), 213 (14), 212 (100), 199 (7), 198 (10), 180 (23), 96 (39), 82 (13), 59 (19), 58 (42), 44 (11), 42 (11) NH_3 CI: 317 (4), 316 (18), 315 (100, [MH^+]), 313 (26), 299 (4), 298 (14), 297 (68), 296 (4), 295 (9), 212 (7), 98 (4), 97 (4), 96 (7), 94 (4)
MDZ-NO	251, 297	1 (312)	312 (10, [M^+]), 296 (12), 295 (25), 213 (16), 212 (100), 199 (38), 198 (16), 186 (10), 180 (31), 167 (14), 98 (10), 97 (17), 96 (44), 82 (18), 57 (16), 55 (19) NH_3 CI: 313 (7, [MH^+]), 299 (5), 298 (18), 297 (100), 296 (8), 295 (12), 283 (5), 217 (5), 201 (5), 200 (35), 199 (7), 114 (6), 98 (6), 97 (6), 96 (9), 94 (11), 82 (5)

[a] The peak numbers for the first six compounds correspond to those in Fig. 1A; the peak numbers for the last six compounds correspond to those in Fig. 1B. Values in parentheses in this column are molecular weights.

MDZ. The mass spectrum of a polar metabolite of MDZ (Fig. 1B, peak 6) showed a molecular ion at *m/z* 199 (Table 1). An MS library search gave a 99% fit to 10H-phenothiazine.

One major metabolite of MDZ, peak 5 (Fig. 1B), had an M^+ at *m/z* 312 (Table 1), indicating the addition of an oxygen atom. MS analysis of the ^{18}O-labeled metabolite (Fig. 2A) showed a 2-mass-unit increase of the molecular ion and the major fragment ions at *m/z* 230 and 217 (Table 1). ^{1}H NMR analysis (Table 2) showed that the resonances of the three protons on the substituted ring had been shifted upfield, while those of the four protons on the unsubstituted ring had not changed. H2 had *ortho* coupling to H1 (J = 7.8 Hz) in addition to *meta* coupling to H4 (J = 2.7 Hz). H4 was coupled only to H2. This pattern is consistent with a hydroxyl group at position 3. This metabolite was identified as 3-hydroxymethdilazine (3-OH-MDZ).

TABLE 2. ^{1}H NMR assignments for CPZ, MDZ, and their fungal metabolites

Compound	^{1}H NMR assignments
CPZ-SO	8.01 (1H, d, J = 7.8 Hz, H4), 7.98 (1H, d, J = 8.6 Hz, H6), 7.82 (1H, J = 1.8 Hz, H1), 7.8–7.77 (2H, brd, H8 and H9), 7.38 (1H, dd, J = 7.6, 6.5 Hz, H7), 7.36 (1H, dd, J = 7.8, 1.8 Hz, H3), 4.63–4.54 (2H, dt, J = 7.2 Hz, H11), 3.12–3.04 (2H, t, J = 7.2 Hz, H13), 2.75 (3H, s, H14), 2.68 (3H, s, H15), 2.2 (2H, m, H12)
Nor_1-CPZ	7.28–6.95 (7H, m, H1, H3, H4, and H6–9, 4.10–4.02 (2H, t, J = 7.2 Hz, H11), 3.10–3.01 (2H, dt, J = 7.2 Hz, H13), 2.80 (3H, s, H14), 2.18–2.10 (2H, m, H12)
Nor_1-7-OH-CPZ	7.12 (1H, d, J = 7.5 Hz, H4), 7.0 (1H, d, J = 1.8 Hz, H1), 6.94 (1H, dd, J = 7.5, 1.8 Hz, H3), 6.89 (1H, d, J = 8.4 Hz, H9), 6.67 (1H, dd, J = 8.4, 2.7 Hz, H8), 6.64 (1H, d, J = 2.7 Hz, H6), 4.0 (2H, t, J = 7.4 Hz, H11), 3.05 (2H, m, H13), 2.6 (3H, s, H14), 2.45 (2H, t, J = 6.7 Hz, H12)
7-OH-CPZ-SO	7.92 (1H, d, J = 8.4 Hz, H4), 7.72 (1H, d, J = 2.4 Hz, H1), 7.63 (1H, d, J = 8.4 Hz, H3), 7.34 (1H, d, J = 2.7 Hz, H6), 7.29–7.24 (1H, dd, J = 8.2, 1.6 Hz, H9), 7.23–7.20 (1H, dd, J = 8.2, 2.7 Hz, H8), 4.5 (2H, t, J = 7.4 Hz, H11), 2.95 (2H, m, H13), 2.6 (3H, s, H14), 2.3 (2H, m, H12)
CPZ	7.3–6.95 (7H, m, H1, H3, H4, and H6–9), 4.10–4.02 (2H, t, J = 7.2 Hz, H11), 3.15–3.10 (2H, dt, J = 7.3 Hz, H13), 2.85 (3H, s, H14), 2.70 (3H, S, H15), 2.2–2.1 (2H, dt, H12)
MDZ-SO	7.9–7.85 (2H, d, J = 7.5 Hz, H1 and H9), 7.7–7.6 (4H, m, H2, H3, H7 and H8), 7.3–7.2 (2H, dd, J = 7.2, 7.4 Hz, H4 and H6), 4.6–4.45 (2H, m, H11), 3.3–2.9 (4H, m, H13 and H15), 2.7 (3H, s, H14), 2.0 (2H, m, H16)
3-OH-MDZ	7.2–6.95 (2H, m, H7 and H8), 6.94–6.86 (1H, d, J = 7.9 Hz, H9), 6.85–6.75 (1H, d, J = 7.5 Hz, H6), 6.75–6.7 (1H, d, J = 7.8 Hz, H1), 6.55 (1H, dd, J = 7.8, 2.7 Hz, H2), 6.5 (1H, s, H4), 3.95–3.75 (2H, ddd, J = 6.3, H11), 3.3–2.9 (4H, m, H13 and H15), 3.7 (3H, ss, H14), 2.0 (2H, m, H16)
MDZ	7.18–7.02 (4H, m, H2, H3, H7 and H8), 7.0–6.9 (2H, d, J = 7.9 Hz, H1 and H9), 6.89–6.8 (2H, dd, J = 7.4, 7.7 Hz, H4 and H6), 4.1–3.85 (2H, m, H11), 3.3–2.9 (4H, m, H13 and H15), 2.7 (3H, s, H14), 2.0 (2H, m, H16)

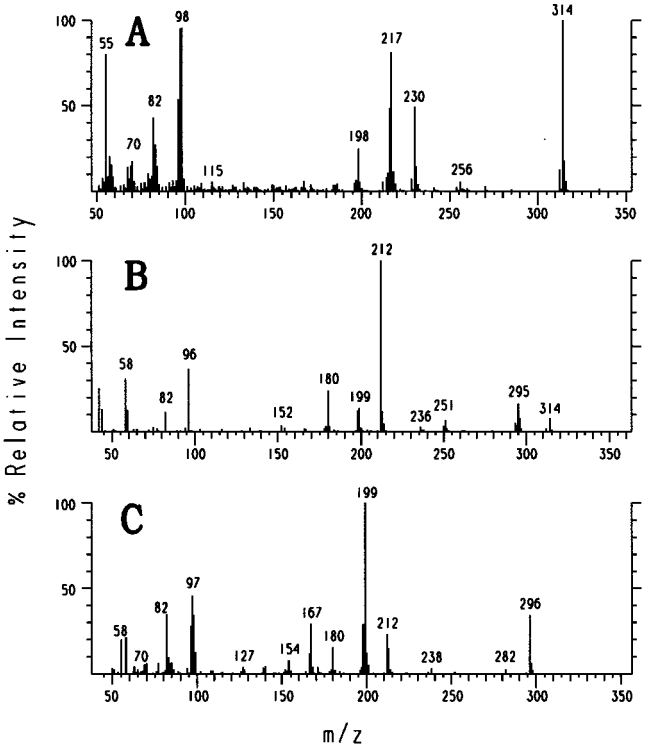

FIG. 2. Electron ionization mass spectra of ^{18}O-labeled 3-OH-MDZ (A), MDZ-SO (B), and MDZ-NO (C) produced from the metabolism of MDZ by *C. elegans.*

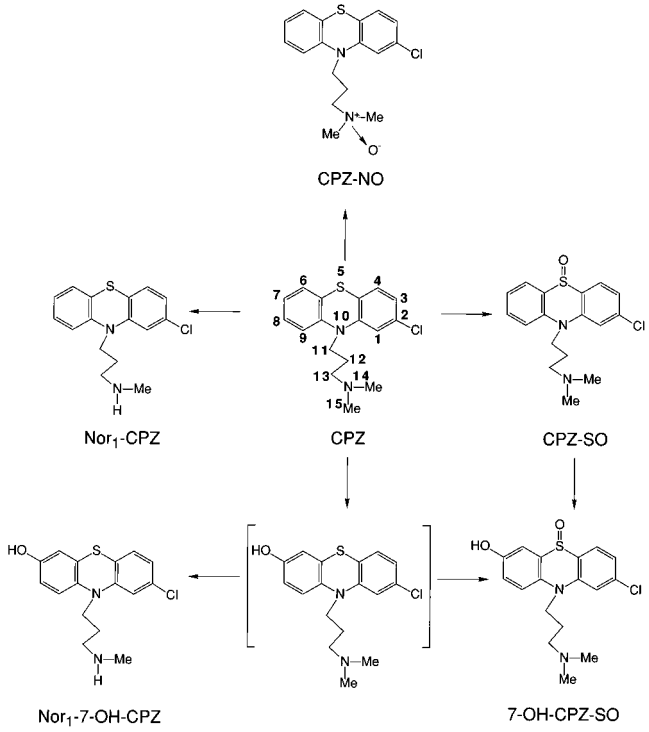

FIG. 3. Metabolic pathways of CPZ by *C. elegans*. The compound in brackets was not isolated.

A minor peak (Fig. 1B, peak 4) had a retention time and MS fragmentation pattern similar to those of 3-OH-MDZ. This metabolite was tentatively identified as 2-hydroxymethdilazine (2-OH-MDZ), a positional isomer of 3-OH-MDZ.

Peak 3 (Fig. 1B) was identified as residual MDZ by coinjection with an authentic sample of MDZ and by MS analysis (Table 1).

Another major metabolite of MDZ was peak 2 (Fig. 1B). The ^{18}O-labeled metabolite gave a molecular ion M^+ at *m/z* 314 and an oxygen loss ion at *m/z* 295 (Table 1 and Fig. 2B), suggesting the addition of an oxygen atom to the sulfur. This was confirmed by characteristic UV absorption peaks (Fig. 1C). ^1H NMR analysis also showed that eight aromatic protons had been shifted downfield relative to MDZ and that the resonances of other protons were similar to those of MDZ (Table 2). The metabolite was identified as MDZ sulfoxide (MDZ-SO).

A minor peak (Fig. 1B, peak 1) showed an M^+ at *m/z* 312 and characteristic M^+-16 and M^+-30 fragment ions. This is typical of an *N*-oxide (18). Figure 2C shows an MS spectrum of this compound from an ^{18}O labeling experiment. Fragment ions at *m/z* 282 and 296 are oxygen (M^+-18) and formaldehyde (M^+-32) losses from molecular ion M^+ at *m/z* 315 (data not shown). This metabolite was identified as MDZ *N*-oxide (MDZ-NO).

Comparison of metabolites. Thus, five metabolites produced when *C. elegans* was incubated with either CPZ (Fig. 3) or MDZ (Fig. 4) had the following common spectroscopic features: (i) all three-ring associated fragments of CPZ gave chlorine isotope (^{37}Cl) ions at 30% of the ^{35}Cl intensity; (ii) the sulfoxides and *N*-oxides generated strong characteristic oxygen loss ions at *m/z* $[M-16]^+$; (iii) ^1H NMR resonances of the aliphatic regions of all compounds showed broadening and splitting probably as a result of the presence of different conformers, although no doubling was observed in the aromatic regions; (iv) sulfoxidation caused the resonances of the aro-

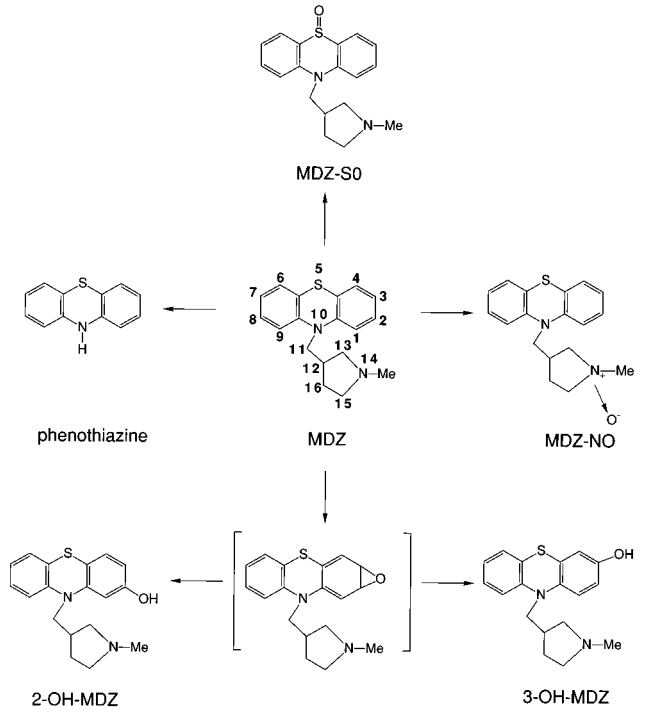

FIG. 4. Metabolic pathways of MDZ by *C. elegans*. The intermediate in brackets was not isolated.

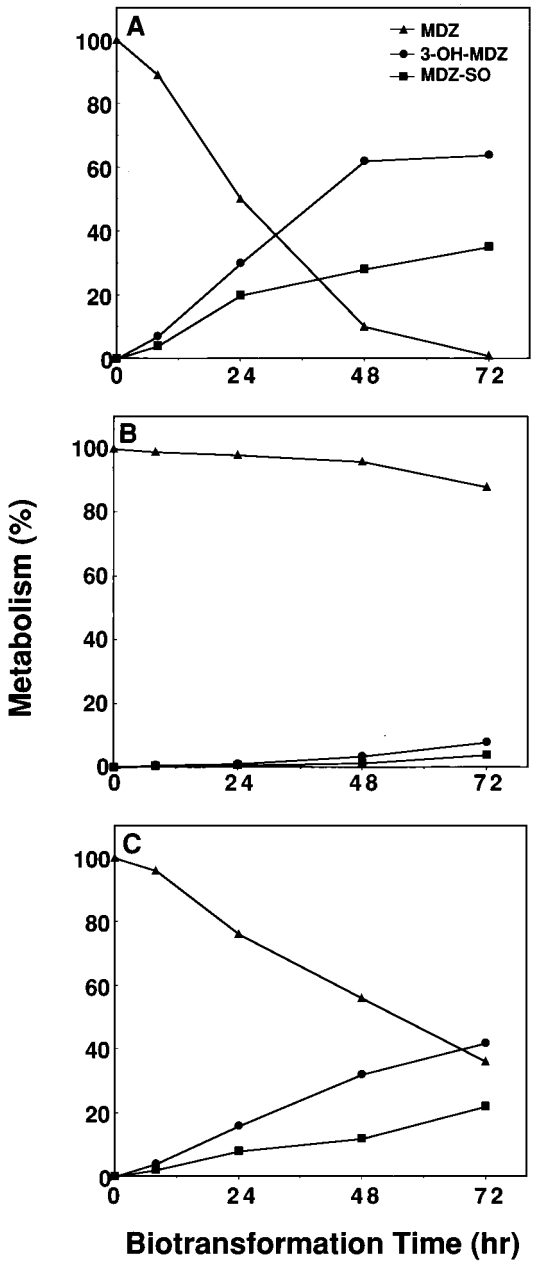

FIG. 5. Inhibition of biotransformation of MDZ (1.0 mM) by metyrapone (2 mM) (B) and SKF 525-A (1.5 mM) (C). The control is shown in panel A. Metabolism is expressed as a percentage of residual MDZ.

matic protons and C-11 methylene protons to shift downfield by about 0.5 ppm relative to the parent compounds; and (v) sulfoxidation caused significant characteristic changes in the UV spectra.

Catalytic mechanism. The reduced CO difference spectrum of the microsomal fraction had an absorption peak at 450 nm, but it also had a major peak at 420 nm that indicated the instability of the microsomal cytochrome P-450. The cytochrome P-450 inhibitors SKF 525-A and metyrapone inhibited both the sulfoxidation and the 3-hydroxylation reactions of MDZ; SKF 525-A inhibited both reactions by 50% (Fig. 5B), and metyrapone inhibited them by 95% (Fig. 5C). The oxygen atoms in the 3-OH-MDZ and MDZ-SO were derived from molecular oxygen (Fig. 2A and B). The enzyme activity for MDZ sulfoxidation was found in the microsomal fraction and had a specific activity of 9 nmol h^{-1} mg of protein^{-1}. The specific activities were 4 and 3 nmol h^{-1} mg of protein^{-1}, respectively, when 1 mM SKF 525-A or CO was included in the reaction mixture. No such activity was found in the supernatant fraction. These data suggest that the hydroxylation and sulfoxidation of MDZ are mediated by cytochrome P-450 enzymes.

DISCUSSION

The major metabolites of CPZ from *C. elegans* were Nor_1-CPZ, CPZ-SO, CPZ-NO, Nor_1-7-OH-CPZ, and 7-OH-CPZ-SO, which were produced by N demethylation, sulfoxidation, N oxidation, and aromatic hydroxylation reactions (Fig. 3). MDZ, a structural analog of CPZ, was also metabolized via metabolic pathways (Fig. 4) similar to those of CPZ, although the relative quantities of the metabolites were different. Similarly, in humans, the major metabolites of CPZ are produced by the same four types of metabolic reactions (13, 16, 19). The hydroxylated metabolites may be formed from a cytochrome P-450-mediated epoxide intermediate followed by a nonenzymatic rearrangement (17). The mechanism for the formation of the N-demethylated metabolites may be similar to the N demethylation of codeine, which involves hydroxylation of a methyl carbon (11). Formation of the sulfoxides can be explained by a heteroatom oxidation with heme-bound oxygen by a cytochrome P-450 monooxygenase (15).

Our experiments with cytochrome P-450 inhibitors along with ^{18}O labeling, determination of the cell-free enzyme activity, and P-450 measurement suggest that sulfoxidation and 3-hydroxylation reactions of MDZ in *C. elegans* are catalyzed by cytochrome P-450 monooxygenases. These inhibitors also inhibited the metabolism of methylcarbazole by *Cunninghamella echinulata* (28). The sulfoxidation of dibenzothiophene by *C. elegans* was also attributed to catalysis by a cytochrome P-450 enzyme (22). Other P-450 enzymes identified in *C. elegans* include naphthalene hydroxylase and anisole aryl hydroxylase (4, 10). The mechanism of CPZ oxidative metabolism has been studied in microsomal preparations from humans, rats, guinea pigs, cattle, and dogs (8, 16, 21, 25). SKF 525-A, metyrapone, and CO inhibited the metabolism of CPZ in human microsomes (14, 16). Our experiments show that a microsomal cytochrome P-450 isozyme from *C. elegans* catalyzed the sulfoxidation of MDZ, which was inhibited by treatment with SKF 525-A or CO.

The catalytic sulfoxidation of phenothiazines by nitric oxide, which was discovered recently, has been proposed to occur via radical cation intermediates by Bosch and Kochi (2). Similarly, cytochrome P-450 monooxygenase-mediated heteroatom oxidations also produce radical cation intermediates (15). A flavin-containing monooxygenase (29) may also partially contribute to the sulfoxidation of phenothiazines, as was discussed previously for the sulfoxidation of dibenzothiophene (22).

In summary, *C. elegans* biotransformed CPZ and MDZ to potential mammalian metabolites. The diverse fungal metabolic pathways included cytochrome P-450 mechanisms similar to those of mammalian systems.

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