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PRIMARY AND SECONDARY OXIDATIVE METABOLISM OF DEXTROMETHORPHAN

IN VITRO STUDIES WITH FEMALE SPRAGUE–DAWLEY AND DARK AGOUTI RAT LIVER MICROSOMES

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Abstract—The O-demethylation of dextromethorphan (DM) to dextrorphan (DR) is catalysed by the polymorphic CYP2D6 (cytochrome P4502D6) isozyme in man. DM is commonly used as a probe for phenotyping subjects as either poor or extensive metabolizers for the debrisoquine/sparteine oxidative polymorphism via CYP2D6. The enzyme kinetics of DM O- and N-demethylation, and the N- and O-demethylations of the primary metabolites DR and 3-methoxymorphinan (3MM), respectively, were studied in liver microsomes from female Dark Agouti (DA) rats, the poor metabolizer counterpart, and female Sprague–Dawley (SD) rats, the extensive metabolizer counterpart. The formation of metabolites was quantified by HPLC with fluorescence detection and kinetic parameters were calculated. The intrinsic clearance (V_{max}/K_m) of the O-demethylation of 3MM to 3-hydroxymorphinan (3OHM) was 180-fold lower in DA rats (0.11 vs 20.77 mL/hr/mg) due to a 60-fold higher K_m (108.7 vs 1.76 μM) and 3-fold lower V_{max} (11.5 vs 35.95 nmol/mg/hr). The kinetics for DR N-demethylation to 3OHM did not differ between rat strains. The Michaelis–Menten constant (K_m) for DM N-demethylation to 3MM was similar between SD and DA rats (85.04 vs 68.99 μM); however, SD rats displayed a 2-fold higher V_{max} (83.37 vs 35.49 nmol/mg/hr) and intrinsic clearance (0.96 vs 0.51 mL/hr/mg). The O-demethylation of DM to DR in SD rats showed a high and low affinity enzyme component, with the high affinity intrinsic clearance contributing 98% of the total intrinsic clearance in these rats. DM O-demethylation in DA rats was characterized by a single enzyme system. The high affinity O-demethylating enzyme in SD rats showed a 20-fold lower K_m (2.5 vs 55.6 μM) and a three-fold higher V_{max} (51.04 vs 16.84 nmol/mg/hr) resulting in a 66-fold higher intrinsic clearance (20.04 vs 0.31 mL/hr/mg) compared to DA rats. Quinine, dextropropoxyphene, (±)methadone and (±)propafenone were shown to be potent inhibitors of 3MM and DM O-demethylation but did not inhibit DR or DM N-demethylation at similar concentrations. SD and DA rats showed a clear strain difference in 3MM O-demethylation and DM O-demethylation. In contrast, DR N-demethylation and DM N-demethylation do not appear to be under genetic control in the female SD–DA rat model. Kinetic parameters and inhibition studies suggest that 3MM and DM O-demethylation pathways in the rat may be mediated by the same cytochrome P450 isozyme.

Dextromethorphan (DM§), a widely used antitussive agent is oxidatively metabolized by O- and N-demethylation (Fig. 1) [1–5]. The O-demethylation of DM to dextrorphan (DR) is catalysed by the polymorphic CYP2D6 (cytochrome P4502D6) isozyme in humans. DR is further metabolized by N-demethylation to 3-hydroxymorphinan (3OHM) and both metabolites are then conjugated. DM is also N-demethylated to 3-methoxymorphinan (3MM) which is further O-demethylated to 3OHM. Thus 3OHM can be formed from DM by two separate pathways (Fig. 1).

The genetically determined metabolism of DM has been reported to co-segregate in humans with the debrisoquine/sparteine type oxidative polymorphism [3]. DM is now commonly used as a probe to phenotype subjects as either poor or extensive metabolizers of drugs oxidized by CYP2D6. Phenotype determination can be based on the measurement of urinary concentrations of DM and DR in order to calculate a metabolic ratio [3–6].

Female Dark Agouti (DA) and Sprague–Dawley (SD) rats, animal counterparts of human poor and extensive metabolizers, respectively, have been useful animal models for investigating the CYP2D6 oxidative polymorphism [7–11]. DA rats have a deficiency in CYP2D1 (cytochrome P4502D1) which performs similar functions to the human CYP2D6 isozyme [12–14]. Furthermore, the DA rat has been shown to be a suitable model for the dextromethorphan poor metabolizer phenotype [9].

Based on *in vitro* kinetic parameters, Zysset *et al.* [9] found strain differences between SD and DA rats in the O-demethylation of DM. There are however few data on the secondary oxidative metabolism of DM. Roos *et al.* [15] reported on the O-demethylation of DR and N-demethylation of 3MM in male SD rats with biliary and micronodular cirrhosis. Their data were difficult to interpret for

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§ Abbreviations: SD, Sprague–Dawley; DA, Dark Agouti; DM, dextromethorphan; DR, dextrorphan; 3OHM, 3-hydroxymorphinan; 3MM, 3-methoxymorphinan; CYP2D6, cytochrome P4502D6; CYP2D1, cytochrome P4502D1.

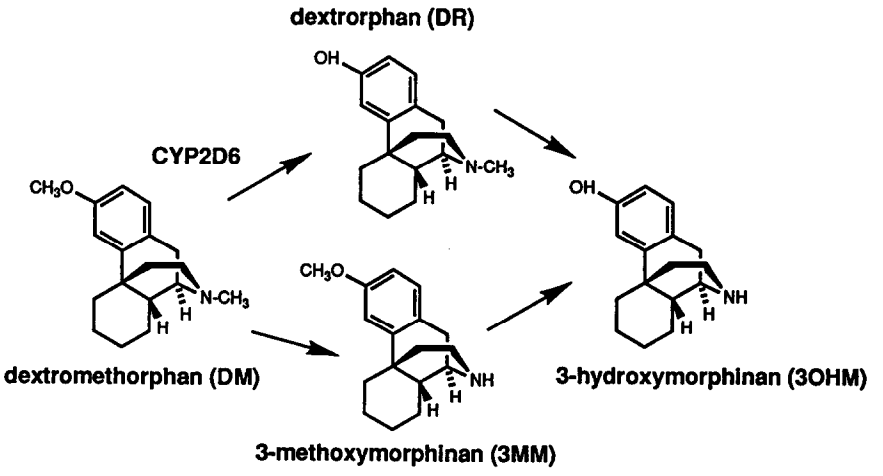

Fig. 1. Metabolic pathways of DM metabolism. The O-demethylation of DM to DR is mediated by CYP2D6 which is under genetic control in humans.

several reasons including the incorrect assignment of the reaction pathways. An *in vivo* study by Chen *et al.* [16] investigated the subsequent O- and N-demethylation of 3MM and DR in SD rats by measuring the urinary recoveries of metabolites following their intra-peritoneal administration. These findings revealed that following DR administration most of the dose was recovered as conjugated DR, whereas after 3MM administration 40% of the dose was excreted as the O-demethylated 3OHM. These *in vivo* data suggest that the 3OHM formed is primarily derived from DM N-demethylation through 3MM.

The aims of this study were to determine if (i) the two O-demethylation reactions were under the same genetic control and (ii) the O- and N-demethylation reactions were catalysed by the same isozyme.

MATERIALS AND METHODS

This study was approved by the Animal Ethics Committee of the University of Adelaide.

Chemicals. DM hydrobromide, DR tartrate, 3OHM hydrobromide and 3MM hydrobromide were all obtained from Roche Pty Ltd (Dee Why, Australia). Thebaine alkaloid powder, quinidine sulphate, quinine HCl, human albumin fraction V, cimetidine, lignocaine, isocitric dehydrogenase and DL-isocitric acid Na_3 were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Morphine HCl was obtained from Macfarlan Smith Ltd (Edinburgh, U.K.). NADP-Na_2 and (−)sparteine sulphate were obtained from E. Merck (Darmstadt, Germany). Salicylic acid, $NaH_2PO_4.2H_2O$, $Na_4P_2O_7.10H_2O$ and $MgCl_2$ were obtained from Ajax Chemicals Pty Ltd (Auburn, Australia). Phenacetin and EDTA were obtained from BDH Chemicals (Victoria, Australia) and oxymorphone from Du Pont Merck Pharmaceutical Company (Wilmington, DE, U.S.A.). (±)Propafenone HCl was obtained from Knoll AG (Ludwigshafen, Germany) and (±)methadone HCl was a gift from Dr S Pond, Dept of Medicine, University of Queensland (Australia). Dextropropoxyphene HCl was obtained from Fisons Pharmaceuticals (Thornleigh, Australia) and midazolam from F. Hoffmann-La Roche (Basel, Switzerland). All reagents were of analytical grade.

Preparation of microsomes. Livers from female SD (N = 3 or 4) and female DA (N = 3 or 4) rats were used in all experiments. Microsome fractions were prepared by differential centrifugation of liver homogenates as described previously by Meier *et al.* [17]. Microsomes were aliquoted and stored in 0.1 M $Na_4P_2O_7.10H_2O$ and 1 M EDTA buffer at −80°. Microsomal protein concentrations were measured by the method of Lowry *et al.* [18] using human serum albumin (fraction V) as the standard.

Microsomal incubation. 3MM O-demethylation, DR N-demethylation and DM O- and N-demethylation pathways were investigated *in vitro* by incubating substrates with SD and DA rat liver microsomes. The microsomal incubations were performed in a final volume of 100 μL containing an NADPH generating system (1 mM NADP, 5 mM isocitrate, 5 mM $MgCl_2$, 1 U of isocitrate dehydrogenase type IV), 0.1 M $NaH_2PO_4.2H_2O$ buffer (pH 7.4) and one of the following substrates: (1) DM (0.4–600 μM), (2) 3MM (0.4–1950 μM) and (3) DR (35–2660 μM). Following a 5 min preincubation at 37°, the reaction was initiated by the addition of microsomes.

Linearity of time and protein dependency was demonstrated under the following conditions: for 3OHM formation from 3MM, 25 min and 70 μg microsomal protein; for 3OHM formation from DR, 100 μg microsomal protein and 30 min; for DR and 3MM formation from DM, 45 min and 50 μg microsomal protein. For kinetic experiments involving DM O- and N-demethylation reactions, varying concentrations of substrate were incubated for 5 min with 50 μg microsomal protein at 37°. The microsomal

incubations with 3MM were performed for 5 min with 50 μg microsomal protein and incubations with DR were performed for 15 min with 50 μg microsomal protein. The reactions were stopped by the addition of 30 μL acetonitrile and the samples were centrifuged at 10,000 *g* for 5 min. An aliquot (80 μL) of supernatant was injected directly onto the HPLC column.

Inhibition experiments. The inhibition of 3MM and DM O-demethylation and DR N-demethylation by various compounds was conducted in SD microsomes. Inhibition experiments were performed at fixed concentrations of substrate based on the K_m values estimated from kinetic experiments. Potential inhibitors of 3MM O-demethylation were incubated with 1.95 μM 3MM and 50 μg microsomal protein for 5 min. The compounds tested for their ability to inhibit O-demethylation included: quinine, quinidine, thebaine, (±)methadone, (±)propafenone, (−)sparteine, lignocaine, dextropropoxyphene, phenacetin and salicylic acid. The five most potent inhibitors of 3MM O-demethylation were also tested for their ability to inhibit the O-demethylation of DM. These potential inhibitors of DM O-demethylation were incubated with 6 μM DM and 50 μg microsomal protein for 5 min. For inhibition studies of DR N-demethylation, various compounds were incubated with 2660 μM DR and 50 μg protein for 15 min. The following compounds were tested for their capacity to inhibit DR N-demethylation: quinine, (±)methadone, (±)propafenone, thebaine, (−)sparteine, lignocaine, phenacetin, salicyclic acid, midazolam, cimetidine, morphine and oxymorphone.

Analysis of metabolites. DR, 3MM and 3OHM formations were quantified by HPLC with fluorescence detection according to the method described by Chen *et al.* [6] with minor modifications of the mobile phase. The pH of the mobile phase for the quantification of metabolites from DR and DM was adjusted to 2.5 instead of 3.0, and for the quantification of 3OHM from 3MM, the acetonitrile composition was increased from 17% to 19% with a pH of 3.0. No extraction procedure of the samples was required prior to injection onto the HPLC column. The standard curves for 3OHM, DR and 3MM were linear between 25 and 800 ng/mL. The limit of quantification for all metabolites was 25 ng/mL and precision and accuracy experiments resulted in coefficients of variation of less than 15%. None of the drugs when incubated with microsomes interfered with metabolite concentration estimations.

Data analysis. The formation of metabolites (3OHM, DR and 3MM) was calculated as nanomoles formed per milligram of microsomal protein per hour (nmol/mg/hr). The untransformed kinetic data were fitted to the Michaelis–Menten equation for either a single enzyme system (1) or a two enzyme system (2) using an iterative non-linear least-squares regression program [19]:

$$V = \frac{V_{max} \cdot S}{K_m + S} \quad (1)$$

$$V = \frac{V_{max1} \cdot S}{K_{m1} + S} + \frac{V_{max2} \cdot S}{K_{m2} + S} \quad (2)$$

where V = formation rate of metabolite, S = substrate concentration, V_{max} = maximum formation rate of metabolite and K_m = Michaelis–Menten constant. The intrinsic clearance (CL_i) was calculated as the ratio of V_{max} to K_m. The decision as to whether the formation of metabolite was best characterized by a single or two enzyme system was based on the linearity of Eadie–Hofstee plots (V versus V/S), the goodness of the fit to the Michaelis–Menten equations and a significantly smaller sum of squared deviations for the fitted data to the Michaelis–Menten equation [10, 11].

The concentration of inhibitor causing 50% inhibition of metabolite formation (IC_{50}) was determined from plots of % control formation versus the log concentration of inhibitor. The Mann–Whitney U-test was used to evaluate the data for differences (statistical significance $P < 0.05$) in kinetic parameters for (i) single oxidation reactions between rat strains, (ii) DM and DR N-demethylation reactions within rat strains and (iii) DM and 3MM O-demethylation reactions in the SD rat strain. All data are tabulated as mean ± standard deviation.

RESULTS

3MM O-demethylation and DR N-demethylation

Eadie–Hofstee plots for 3MM O-demethylation and DR N-demethylation showed one enzyme Michaelis–Menten characteristics in both rat strains. The calculated kinetic parameters are summarized in Table 1. The Michaelis–Menten constant (K_m) for 3MM O-demethylation was 60 times lower ($P < 0.05$) and the maximum velocity of formation (V_{max}) three times higher ($P < 0.05$) in SD compared to DA rats. In addition, the intrinsic clearance (CL_i) to 3OHM was about 180-fold lower ($P < 0.05$) in DA rats. The kinetics of DR N-demethylation did not differ ($P > 0.05$) between SD and DA rats.

DM O-demethylation and N-demethylation

The Eadie–Hofstee plots for DM N-demethylation in SD and DA rats were typical of a one enzyme system. Similarly, the kinetics of DM O-demethylation in DA showed one enzyme Michaelis–Menten characteristics, whereas the kinetics of DM O-demethylation in SD rats were best described by a two enzyme system. The K_m and V_{max} of DM O- and N-demethylation in SD and DA microsomes are summarized in Table 2. The K_m values for DM N-demethylation in DA and SD rats were not significantly different ($P > 0.05$), although SD rats showed a 2-fold higher V_{max} ($P = 0.05$).

In SD rats there was a 60-fold difference in K_m values between the high and low affinity components of DM O-demethylation. In addition, the high affinity intrinsic clearance (CL_{i1}) contributed 98% of the total intrinsic clearance in SD rats (Table 2). The kinetic parameters for the low affinity O-demethylation showed large variations within the SD rat strain. The Michaelis–Menten parameters for the one enzyme system in DA rats were quite similar to those for the low affinity isozyme in SD rats with only a 3-fold difference in K_m ($P = 0.05$), a 2-fold difference in V_{max} ($P = 0.05$) and no difference in CL_i ($P > 0.05$). The high affinity O-demethylating

Table 1. Summary of *in vitro* microsomal 3MM O-demethylation and DR N-demethylation in two rat strains

Rat strain	K_m (μM)	V_{max} (nmol/mg/hr)	CL_i (mL/hr/mg)
3MM O-demethylation			
SD (N = 4)	1.76 ± 0.41	35.95 ± 9.22	20.77 ± 4.99
DA (N = 4)	108.73 ± 20.37	11.48 ± 2.78	0.11 ± 0.03
DR N-demethylation			
SD (N = 4)	1605.80 ± 157.57	39.63 ± 8.87	0.02 ± 0.01
DA (N = 4)	1354.45 ± 240.20	35.58 ± 14.13	0.03 ± 0.01

K_m = Michaelis–Menten constant, V_{max} = maximum velocity of metabolite formation and CL_i = intrinsic clearance calculated as V_{max}/K_m.
Values represent means ± SD.

Table 2. Summary of *in vitro* microsomal DM N-demethylation (one enzyme system) and DM O-demethylation (two enzyme system) in two rat strains

	1 enzyme			2 enzyme		
Rat strain	K_{m1} (μM)	V_{max1} (nmol/mg/hr)	CL_{i1} (mL/hr/mg)	K_{m2} (μM)	V_{max2} (nmol/mg/hr)	CL_{i2} (mL/hr/mg)
DM N-demethylation to 3MM						
SD (N = 3)	85.04 ± 8.31	83.37 ± 40.42	0.96 ± 0.41			
DA (N = 3)	68.99 ± 16.11	35.49 ± 0.33	0.51 ± 0.09			
DM O-demethylation to DR						
SD (N = 3)	2.50 ± 0.36	51.04 ± 16.15	20.04 ± 3.88	157.89 ± 106.83	38.72 ± 13.71	0.31 ± 0.19
DA (N = 3)	55.62 ± 17.53	16.84 ± 3.23	0.31 ± 0.05			

K_m = Michaelis–Menten constant, V_{max} = maximum velocity of metabolite formation and CL_i = intrinsic clearance calculated as V_{max}/K_m.
Values represent means ± SD.

isozyme in SD rats displayed a 20-fold lower K_m and a 3-fold higher V_{max} compared to DA rats ($P < 0.05$). The CL_i of O-demethylation was 66-fold lower ($P < 0.05$) in DA rat liver microsomes.

Comparison of the 3MM and high affinity DM O-demethylations in SD rats revealed that the enzyme kinetics (V_{max}, K_m and CL_i) were similar ($P > 0.05$). In SD and DA rats, the V_{max} values for DM and DR N-demethylation were also similar ($P > 0.05$), although the K_m and CL_i values were both significantly different for these two pathways ($P < 0.05$).

Inhibition studies

The inhibitions of O- and N-demethylation by various compounds are summarized in Table 3. Quinine, dextropropoxyphene, (±)methadone and (±)propafenone were potent inhibitors of both 3MM and DM O-demethylations, yet at similar concentrations they did not inhibit DR N-demethylation. Only very high concentrations of (±)propafenone, phenacetin and lignocaine produced 50% inhibition of DR N-demethylation. The compounds that inhibited DM O-demethylation did not inhibit DM N-demethylation. Salicylic acid (as a negative control) did not inhibit either the O- or N-demethylation pathway.

DISCUSSION

The present study has examined both the enzyme kinetics and inhibition of DM O- and N-demethylation to DR and 3MM, respectively, and the subsequent O-demethylation of 3MM and N-demethylation of DR in two different rat strains. To our knowledge, only one other study has investigated the kinetic parameters of DM O- and N-demethylation in the female SD–DA rat model [9].

The kinetic parameters of DM O-demethylation in SD rats are characteristic of a two enzyme system. This observation is in contrast to a previous study [9] which reported single enzyme Michaelis–Menten kinetic parameters for DM O-demethylation in SD rats. These authors did however report that data from some SD rats indicated that two enzymatic binding sites may be involved in DR formation, which could have been quantified with improved assay sensitivity as their lowest DM substrate concentration of 2.5 μM was equivalent to our K_m value. Regardless of this, the high affinity Michaelis–Menten constant (K_{m1} 2.5 μM) and the maximum rate of formation (V_{max} 51 nmol/mg/hr) of DM O-demethylation in the present study are similar to those reported by Zysset *et al.* [9] (K_m 4.1 μM; V_{max} 36 nmol/mg/hr). However, more recent studies by

Table 3. *In vitro* inhibition (IC_{50}) of the oxidative metabolism of DM and metabolites in SD rat liver microsomes (N = 1)

Compound	IC_{50} (μM) 3MM→3OHM (O-demethylation)	DR→3OHM (N-demethylation)	DM→DR (O-demethylation)
Quinine	0.4	>11	4
Quinidine	—	—	17
(±)Methadone	0.75	>10	7
(±)Propafenone	2.5	800	6
Thebaine	22	>730	—
(−)Sparteine	9	>80	—
Lignocaine	8	4500	—
Phenacetin	>400	2000	—
Dextropropoxyphene	6	—	12
DM	7	—	—
3MM	—	—	15
Salicylic acid	>2000	>400	—
Midazolam	—	>3636	—
Cimetidine	—	>18	—
Morphine	—	>18	—
Oxymorphone	—	>60	—

Substrate concentrations used for inhibition experiments were: 3MM 1.95 μM, DR 1240 μM and DM 6 μM.

the same group have reported markedly different kinetic parameters (K_m 36 mM; V_{max} 174 nmol/min calculated for total microsomal protein fraction) following DM incubation with liver microsomes from male SD rats [15]. The reasons for the differences were not reported by the investigators.

The K_m value of DM O-demethylation in our study is identical to the DM inhibition constant (K_i 2.53 μM) of codeine O-demethylation reported by Mikus *et al.* [10]. Moreover, these kinetic parameters describing DM O-demethylation are in agreement with previously published results involving a two enzyme system for thebaine O-demethylation to oripavine in SD rat liver microsomes [11]. These results demonstrate that DM, codeine and thebaine have similar affinities for their respective O-demethylating cytochrome P450 isozyme and are likely to be mediated by the same P450 isozyme.

DM O-demethylation to DR displayed strain differences between female DA rats and the high affinity enzyme in SD rats. The data reported in the present study showed a 64-fold diminished intrinsic clearance in DA rats compared to the high affinity component in SD rats. This is similar to the 60-fold decrease in intrinsic clearance between SD and DA rats demonstrated by Zysset *et al.* [9]. These results are in agreement with Zysset's findings that the DA rat is a good model for poor metabolizers of DM [9]. Furthermore, it could be concluded that the CYP2D1 isozyme, which is deficient in DA rats, catalyses the high affinity component of DM O-demethylation in SD rats.

Comparison of the kinetic parameters for 3MM O-demethylation also revealed large strain differences. The K_m value for the SD rat (1.76 μM) was comparable to the inhibitor concentration ($IC_{50} < 4$ μM) for 3MM inhibiting codeine O-demethylation reported in a previous study [10].

In the present study we have demonstrated a genetic polymorphism for both 3MM and DM O-demethylation in the female SD–DA rat model. Therefore, besides being a good model for poor metabolizers of DM, female DA rats are also a suitable model for distinguishing poor metabolizers of 3MM O-demethylation. The existence of a genetic polymorphism in 3MM O-demethylation in human liver microsomes is currently under investigation.

Inhibition studies of both 3MM and DM O-demethylation revealed that quinine and racemic methadone are potent inhibitors of the O-demethylating isozyme in SD rats. Previous studies by Mikus *et al.* [10, 11] have also shown that quinine and methadone are potent inhibitors of codeine and thebaine O-demethylation. Other substrates of CYP2D1 in rats and CYP2D6 in humans, racemic propafenone, sparteine and lignocaine [20–23], also inhibited the O-demethylation of 3MM. Dextropropoxyphene is a mild analgesic structurally related to methadone and has been shown to inhibit desipramine and debrisoquine hydroxylation in human liver microsomes [24, 25]. In the present study, dextropropoxyphene was a potent inhibitor of both 3MM and DM O-demethylation. These studies indicate that dextropropoxyphene could act as a substrate for CYP2D1 in rats, but as yet it is not known whether its metabolism is polymorphic in the female SD–DA rat model.

Phenacetin, whose O-deethylation to paracetamol *in vivo* was shown to be reduced by 15% in DA rats [7], did not inhibit 3MM O-demethylation at a concentration of 400 μM. This is not unexpected since recent studies using specific inhibitory monoclonal antibodies have shown that phenacetin O-deethylation is catalysed by cytochrome CYP1A2 in the rat [26–28]. Furthermore, it has been established that the 4-hydroxylation of debrisoquine and the O-

deethylation of phenacetin are catalysed by different P450 isozymes in humans [29, 30].

Inhibition of 3MM O-demethylation by DM was observed (IC_{50} 7 μM) and, in turn, DM O-demethylation was inhibited by 3MM (IC_{50} 15 μM). The IC_{50} values of the two O-demethylation pathways cannot be compared directly since different concentrations of 3MM and DM were used in the inhibition experiments. However, the rank order of potency of inhibitors is consistent for both 3MM and DM O-demethylation. Furthermore, the O-demethylation of 3MM and the high affinity O-demethylation of DM have similar enzyme affinities (3MM K_m 1.76 μM; DM K_{m1} 2.5 μM) and identical intrinsic clearances in SD rats. These kinetic parameters and inhibition studies of the two O-demethylation pathways give credence to the hypothesis that 3MM and DM O-demethylation are catalysed by the same cytochrome P450 isozyme.

DM N-demethylation to 3MM displayed similar enzyme affinities in the SD and DA rat strains (K_m 85 vs 69 μM). The K_m value in SD rats is comparable to that reported by Zysset *et al.* [9] (K_m 95 μM), although in the same study DA rats had a lower affinity for the N-demethylating enzyme (K_m 162 μM). The main difference between the two rat strains in the present study is the 2-fold lower V_{max} in DA rats, which corresponds to a 2-fold diminished CL_i. The reason for this difference is not clear but may be due to a general decrease in enzyme content in DA rats.

DR N-demethylation was inhibited *in vitro* by racemic propafenone and lignocaine. Both these compounds have multiple isozymes of cytochrome P450 involved in their metabolism [21, 23]. It follows then that propafenone and lignocaine could inhibit both CYP2D1 mediated O-demethylation and the DR N-demethylating isozyme in SD rats. Of the other compounds tested, none were able to inhibit this N-demethylation pathway potently and specifically.

Similar to DM N-demethylation, no differences in DR N-demethylation were observed within SD and DA rat strains. Interestingly, the N-demethylations of DR and DM displayed a 20-fold difference in K_m; however, V_{max} values for these pathways were similar. This disparity in enzyme affinity is probably due to the presence of a methoxyl group at position 3 of the DM structure which appears to increase the affinity of DM for the N-demethylating P450 isozyme markedly. The comparable V_{max} values give some credence to the hypothesis that the two N-demethylation pathways are catalysed by the same P450 isozyme in rats.

DM is metabolized by O- and N-demethylation to DR and 3MM, respectively. These primary metabolites are then further oxidized by O- and N-demethylation to form 3OHM. However, like Zysset *et al.* [9], we did not detect any 3OHM formation following *in vitro* incubation of DM with microsomes despite the fact that 3OHM is a major urinary metabolite following oral DM administration. Zysset *et al.* [9] concluded that the lack of 3OHM formation *in vitro* was due to an end-product inhibition mechanism. From our enzyme kinetics and inhibition experiments an alternative hypothesis may explain the absence of 3OHM formation *in vitro* with either DR or 3MM. Firstly, in SD rats, 3MM O-demethylation is potently inhibited by DM and as a result no 3OHM would be formed from the 3MM formed by DM. In addition, the low capacity and affinity of the N-demethylating enzyme involved in the formation of 3OHM from DR suggests that this pathway would not produce any 3OHM during the short incubation time.

From these results it would be predicted that the major *in vivo* metabolite of DM in SD rats is the O-demethylated compound DR, since this metabolic pathway has the highest CL_i (20.04 mL/hr/mg). It would be unlikely that DR would undergo substantial further oxidation since the intrinsic clearance of DR N-demethylation is very low in SD rats (0.02 mL/hr/mg) indicating that this is likely to be a minor pathway in the metabolism of DM. These *in vitro* data confirm previous *in vivo* results [16] which showed that only 2.5% of administered DR is N-demethylated to 3OHM.

In conclusion, we have shown a strain difference in DM and 3MM O-demethylation in the female SD–DA rat model. Kinetic parameters and inhibition studies provide evidence that these O-demethylation pathways are catalysed by the same P450 isozyme, whereas the O-demethylation and N-demethylation pathways appear to be mediated by different P450 isozymes in rats.

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