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CODEINE O-DEMETHYLATION: RAT STRAIN DIFFERENCES AND THE EFFECTS OF INHIBITORS

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Abstract—The oxidative metabolism of more than 20 drugs (e.g. sparteine, debrisoquine, dextromethorphan) is mediated by cytochrome P450IID6. Codeine O-demethylation to morphine was recently demonstrated to co-segregate with the polymorphic metabolism of debrisoquine and dextromethorphan. The female Dark–Agouti rat (DA) is an animal model for the poor metabolizer phenotype (PM) using debrisoquine or dextromethorphan as substrates. Studies were carried out to evaluate codeine metabolism in liver microsomes from female DA and Sprague–Dawley (SD) rats. The intrinsic clearance of codeine to morphine was 10-fold lower in DA rats due to a 5-fold higher K_m (287 vs 49 μM) and a 2-fold lower V_{max} (48 vs 94 nmol/mg/hr). Nineteen drugs were tested for inhibition of codeine O-demethylation. The four most potent competitive inhibitors were dextromethorphan (K_i = 2.53 μM), propafenone (K_i = 0.58 μM), racemic methadone (K_i = 0.3 μM) and quinine (K_i = 0.07 μM). The differences in morphine formation from codeine between SD and DA rats and the inhibition results show that this animal model appears to be a suitable model for the human EM and PM phenotypes, respectively. These strains could be used to study the pharmacodynamic consequences of the genetic polymorphism in codeine O-demethylation, and the effects of metabolic inhibitors. The outcome of these studies could impact on the therapy of pain control.

Codeine is widely used as an analgesic drug for the relief of mild to moderate pain [1], for the relief of unproductive cough and as an anti-diarrhoeal agent. Its mechanism of action is still not clearly understood although it is generally accepted, but unproven, that the pharmacological effect(s) of codeine, especially analgesia, are mediated by morphine which is formed in the liver by O-demethylation and then crosses the blood–brain–barrier to interact with various receptors in the central nervous system [1]. Codeine metabolism (Fig. 1) involves glucuronidation to codeine-6-glucuronide, O-demethylation to morphine and N-demethylation to norcodeine [2]. Morphine and norcodeine are either glucuronidated (morphine-3- and -6-glucuronide, norcodeine-6-glucuronide) or N- or O-demethylated to normorphine, which is then glucuronidated [3]. It has been established that more than 20 drugs are metabolized in man by cytochrome P450IID6 and 6–10% of the caucasian population have a defect in the metabolism of these drugs [4, 5] due to the almost complete absence of detectable cytochrome P450IID6 [6]. Recently it was reported that the O-demethylation of codeine to morphine is under this type of genetic control and co-segregates with the sparteine/debrisoquine type polymorphism [7–9]. Furthermore, the partial metabolic clearance of codeine to morphine in a poor metabolizer (PM) was only 5% of the clearance observed in extensive metabolizers (EM) [10]. As a consequence of the impaired codeine O-demethylation to morphine in PM subjects, one might anticipate that these subjects do not obtain pain relief when given codeine due to an impaired or absent formation of both morphine and morphine-6-glucuronide which are the pharmacologically active substances.

The female Dark–Agouti (DA) rat has been used as an animal counterpart of the human PM [11–15]. The cytochromes P450IID6 and P450IID1 subserve similar functions in man and rat, respectively [16, 17]. It has been shown that in the female DA rat, the absence of the gene expression for cytochrome P450IID1 leads to the genetic deficiency, whereas in the Sprague–Dawley (SD) rat this isozyme is mainly responsible for the metabolism of substances affected by the sparteine/debrisoquine type polymorphism [17]. We are not aware that codeine O-demethylation has been studied in the DA rat or its EM counterpart, the SD rat. The aims of this study were to compare codeine O-demethylation in liver microsomes from DA and SD rats, and to screen drugs for their ability to inhibit this metabolic pathway. This latter aim could reveal the potential for *in vivo* drug interactions, and the possible involvement of cytochrome P450IID1 in the metabolism of other drugs previously not investigated.

MATERIALS AND METHODS

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

Chemicals. Codeine phosphate was obtained from the Pharmacy Department of the Royal Adelaide Hospital, morphine HCl was purchased from Macfarlan Smith Ltd (Edinburgh, U.K.). Thebaine alkaloid powder, oxycodone HCl, hydrocodone

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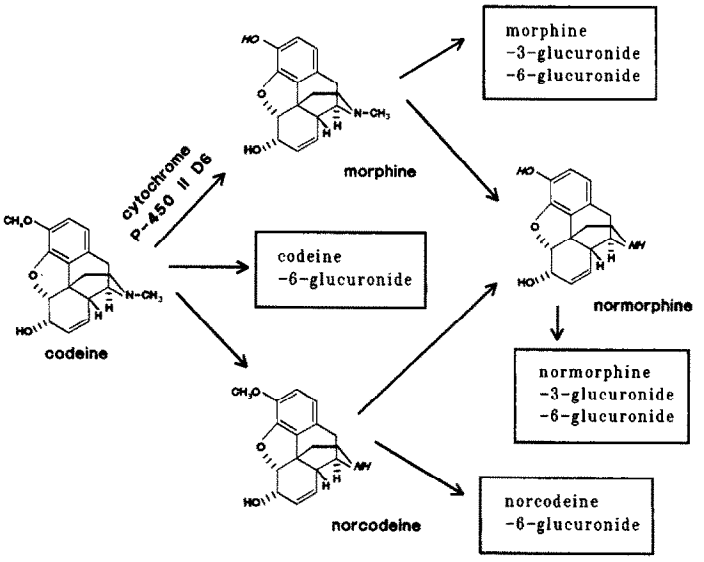

Fig. 1. Metabolic pathways of codeine. The O-demethylation of codeine to morphine is mediated by cytochrome P450IID6 which is under genetic control in humans.

bitartrate, papaverine HCl, noscapine HCl, quinidine sulphate, quinine HCl, cimetidine, yohimbine HCl, isocitric dehydrogenase, DL-isocitric acid Na_3, were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Norcodeine HCl was obtained from Makor Chemicals (Jerusalem, Israel). Pholcodine BP was purchased from Faulding Pharmaceuticals (Salisbury, Australia), salicylic acid from Ajax Chemicals Pty Ltd (Auburn, Australia). NADP-Na_2 and sparteine sulphate were from E. Merck (Darmstadt, F.R.G.). Propafenone HCl (Knoll AG, Ludwigshafen, F.R.G.), flecainide acetate (Kettelhack-Riker, Borken, F.R.G.), dihydrocodeine (Riker Laboratories Australia Pty Ltd, Sydney, Australia), dextromethorphan hydrobromide, 3-methoxymorphinan hydrobromide and debrisoquine sulphate (Roche Products Pty Ltd, Australia) were generously provided. (±)-Methadone HCl was a gift from Dr S. Pond, Dept. of Medicine, University of Queensland (Australia).

Preparation of microsomes. Female Sprague–Dawley (N = 3) and female Dark-Agouti (N = 3) rats weighing 200–300 g were obtained from the University of Adelaide Animal House. They had free access to water and food until 2 hr before use. Rats were killed by decapitation, the livers were immediately removed and microsomes were prepared from each liver by differential centrifugation as described previously [18] and stored in 200-μL aliquots at −80°. Protein concentrations were measured by the method of Lowry *et al.* [19] using bovine serum albumin (fraction V) as a standard.

Incubation conditions. The microsomal incubations were carried out in a final volume of 100 μL at 37° with a NADPH regenerating system (1 mM NADP, 5 mM isocitrate, 5 mM $MgCl_2$, 1 unit of isocitrate dehydrogenase type IV). All incubations were performed in duplicate. The reaction was started by addition of the microsomes in all experiments. Time dependency of morphine formation was determined by incubating 20 μg microsomal protein with 200 μM codeine for up to 50 min. Protein dependency was assessed by incubating 200 μM codeine for 15 min with protein amounts ranging between 2 and 100 μg. Both time and protein dependency were linear under the described conditions. For kinetic experiments, codeine (0.08–800 μM) was added to 20 μg microsomal protein and the reaction was stopped after 15 min incubation by adding 30 μL acetonitrile followed by immediate centrifugation at 8000 *g* for 4 min. A portion (10 μL) of the supernatant was injected onto a HPLC column for morphine estimation.

At a fixed codeine concentration of 200 μM, the following substances were tested for their ability to inhibit morphine formation: cimetidine, debrisoquine, pholcodine, sparteine, flecainide, propafenone, yohimbine, salicylic acid, quinine, quinidine, dextromethorphan, 3-methoxymorphinan, thebaine, noscapine, papaverine, (±)-methadone, norcodeine, hydrocodone, dihydrocodeine and oxycodone. The four most potent inhibitors, quinine (1–10 μM), (±)-methadone (1–10 μM), propafenone (1–20 μM) and dextromethorphan (10–100 μM), were added to different codeine concentrations (10–200 μM) in order to determine the type of inhibition and the inhibition constant (K_i).

Morphine determination. Morphine concentrations were estimated by high performance liquid chromatography with electrochemical detection [20] with minor modifications. These were the mobile phase (2% acetonitrile, 3% methanol, 0.02% triethylamine), flow rate (1.2 mL/min), HPLC column (Spherisorb 5 μ ODS2) and working electrode (+0.75 V). No extraction procedure of the samples was needed. Standard curves were linear between 3 and 2000 ng/mL morphine. At 3 ng/mL intraday

precision was 19% (N = 8) and at 2000 ng/mL it was 2%, and interday precisions were 16 and 2%, respectively (N = 8). Intraday accuracy was 86% at 3 ng/mL and 98% at 2000 ng/mL. Interday accuracy was 114% at 3 ng/mL and 111% at 2000 ng/mL. The limit of quantification was 3 ng/mL. None of the drugs when incubated with microsomes interfered with morphine estimation.

Data analysis. The formation of morphine from codeine was calculated as nanomoles formed per milligram of microsomal protein per hour. The untransformed kinetic data were fitted to the Michaelis–Menten equation (1) using an iterative nonlinear least squares regression program [21]:

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

where V = formation rate of morphine, C = codeine concentration, V_{max} = maximum velocity of morphine formation, K_m = Michaelis–Menten constant. The intrinsic clearance (CL_i) was calculated as V_{max}/K_m. The concentration of inhibitor causing 50% inhibition of morphine formation (IC_{50}) was determined from the plot of per cent of control activity versus log of inhibitor concentration. To determine the K_i of the four most potent inhibitors the untransformed inhibition data were fitted to different types of inhibition models (competitive (2)/ noncompetitive (3)/uncompetitive (4)):

$$V = \frac{V_{max} \cdot C}{(K_m \cdot (1 + I/K_i)) + C} \quad (2)$$

$$V = \frac{V_{max} \cdot C}{(K_m \cdot (1 + I/K_i)) + (C \cdot (1 + I/K_i))} \quad (3)$$

$$V = \frac{V_{max} \cdot C}{K_m + (C \cdot (1 + I/K_i))} \quad (4)$$

where I = inhibitor concentration and K_i = inhibition constant. The choice of model was based on visual inspection of the goodness of fit of the observed data to those predicted, a significant reduction in the weighted sum of squared deviations, and random distribution of the scatter of observed data points about the fitted curve.

RESULTS

The formation of morphine in liver microsomes of Sprague–Dawley and Dark–Agouti rats showed Michaelis–Menten characteristics (Fig. 2) and the derived kinetic parameters are listed in Table 1. The maximum velocity (V_{max}) to O-demethylate codeine was reduced about two-fold and the Michaelis–Menten constant (K_m) was increased five-fold in DA rats in comparison to SD rats. The intrinsic clearance was on average 10-fold higher in SD rat liver microsomes.

No inhibition of codeine O-demethylation was observed with salicylic acid (up to 3 mM). The degree of inhibition varied from almost no inhibition (pholcodine) up to nearly complete inhibition of the enzyme activity (Table 2). The IC_{50} values for the opioid substances norcodeine, hydrocodone, dihydrocodeine and oxycodone were of similar

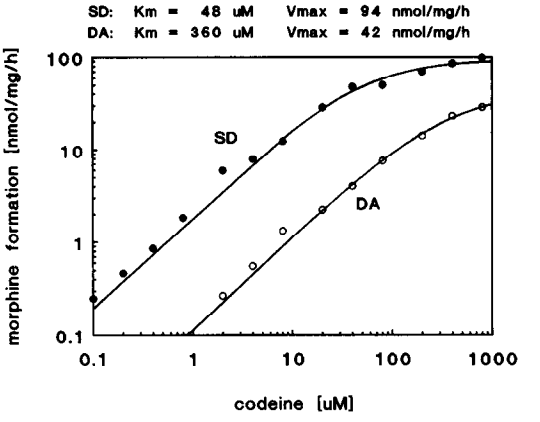

Fig. 2. Strain differences in morphine formation from codeine in rat liver microsomes. The data points show the activity for one SD and one DA rat. The lines represent the result of the computer fit to the data based on the Michaelis–Menten equation.

magnitude. The type of inhibition and the inhibition constant (K_i) were determined for the four most potent inhibitors, quinine, propafenone, dextromethorphan and (±)-methadone. Figure 3 shows the Dixon plot of the inhibition of codeine O-demethylation by (±)-methadone. All four substances showed competitive inhibition with very low inhibition constants. The K_i values were 2.53 μM (dextromethorphan), 0.58 μM (propafenone), 0.30 μM (methadone) and 0.07 μM (quinine), respectively.

DISCUSSION

Codeine O-demethylation to morphine in liver microsomes of female SD and female DA rats were monophasic and showed differences with respect to the Michaelis–Menten constant (K_m) and the maximum velocity of the morphine formation (V_{max}). Similar differences were seen between DA and Fischer 344 rats with respect to bufuralol 1′-hydroxylation where the intrinsic clearance was five-fold lower in the DA rat [14]. The data reported in the present study showed a 10-fold diminished intrinsic clearance in DA rats. This is similar to the 18-fold lower partial clearance of codeine to morphine in a PM subject compared with EM subjects [10]. Therefore, the female DA and SD rats are appropriate models for the human polymorphic codeine O-demethylation, in agreement with previously published data for dextromethorphan [12], debrisoquine [11, 13, 22] and bufuralol [14].

It was not surprising that dextromethorphan and propafenone were potent competitive inhibitors of codeine O-demethylation as these substances are substrates of cytochrome P450IID6 in humans [23, 24]. The inhibition constant for dextromethorphan (2.53 μM) is similar to the Michaelis–Menten constant K_m of dextromethorphan O-demethylation in SD rats (4.1 μM) [12] and is in the same range as that reported in microsomes from

Table 1. Michaelis–Menten kinetics (mean ± SD; N = 3) of codeine O-demethylation in rat liver microsomes. The intrinsic clearance (CL_i) was calculated as V_{max}/K_m

Rat strain	V_{max} (nmol/mg/hr)	K_m (μM)	CL_i (mL/min/mg)
Sprague–Dawley	94.2 ± 14.7	49.0 ± 14.8	122.3 ± 32.4
Dark–Agouti	47.9 ± 6.9	287 ± 109	12.0 ± 7.6

Table 2. Effects of various drugs on codeine O-demethylation in SD rat liver microsomes (N = 1). A codeine concentration of 200 μM was used

Compound	Concentration (μM)	% of control formation	IC_{50} (μM)
Cimetidine	100	49	—
Debrisoquine	100	40	—
Pholcodine	100	92	—
Sparteine	100	36	—
Flecainide	100	14	—
Yohimbine	100	60	—
Quinidine	10	36	—
3-Methoxymorphinan	4–400	42–2	<4
Thebaine	2–200	58–11	5.5
Noscapine	10–1000	47–9	<10
Papaverine	10–1000	67–5	62
Norcodeine	10–1000	65–25	110
Hydrocodone	10–1000	74–15	140
Dihydrocodeine	10–1000	88–28	210
Oxycodone	10–1000	71–38	300
Dextromethorphan	10–100	40–13	<10
(±)-Methadone	1–10	47–30	<1
Propafenone	1–20	85–17	3.2
Quinine	1–10	32–12	<1

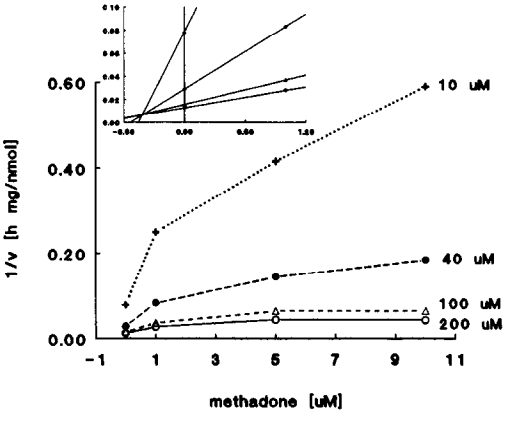

Fig. 3. Competitive inhibition of codeine O-demethylation (Dixon plot) by methadone. The symbols represent the different codeine concentrations as indicated. The smaller diagram shows in detail the intercept of the lines which is an estimate for the inhibition constant (K_i).

human extensive metabolizers (2.3–5.3 μM) [23]. Therefore, our results are in good agreement with the previously published data on dextromethorphan. Propafenone, a newer class Ic antiarrhythmic agent, was reported to be a substrate of cytochrome P450IID6 with a K_m in human liver microsomes for propafenone 5-hydroxylation of 2 μM [24]. In the rat the propafenone K_i of codeine O-demethylation (0.58 μM) was lower as compared to its K_m in humans. Based on this K_i value propafenone is a potent inhibitor of cytochrome P450IID1 in the SD rat. Flecainide was recently shown to be under the polymorphic control of cytochrome P450IID6 [25, 26] and was a very potent inhibitor of bufuralol 1′-hydroxylation in human liver microsomes [27]. Our results show that it is also an inhibitor of codeine O-demethylation in the SD rat. Other substrates of cytochrome P450IID6 and P450IID1 such as sparteine and debrisoquine also inhibited morphine formation. Both quinine and quinidine inhibited codeine O-demethylation with quinine having a stronger effect; this stereoselectivity in inhibition has been described previously [28]. The inhibition constant for quinine was the smallest of all substances tested and quinine was 23-times more potent in inhibiting morphine formation from codeine compared to inhibiting 4-hydroxydebrisoquine formation from debrisoquine [28]. It was recently found, in the rat tail-flick model, that quinine attenuated codeine-induced analgesia presumably by inhibiting morphine formation [29]. Our results using liver microsomes confirm this above mechanism.

Racemic methadone was a potent competitive inhibitor of morphine formation in the SD rat. This is in contrast to human liver microsomes in which methadone and also codeine did not inhibit the 2-hydroxylation of desmethylimipramine, another substrate of cytochrome P450IID6 [30]. However, our findings support the data of Maany *et al.* [31] who reported that methadone increased desmethylimipramine plasma concentrations in patients by 70–170%. It is not known whether methadone is only a potent selective inhibitor or is also a substrate of cytochrome P450IID6 in man or P450IID1 in the rat.

Inhibition was observed with 3-methoxymorphinan (the N-demethylated metabolite of dextromethorphan), norcodeine, dihydrocodeine, hydrocodone and oxycodone. These substances could be O-demethylated at the 3-position, the same position as for codeine O-demethylation. Their metabolism in man has been poorly described, if at all, and it is not known whether their disposition is under pharmacogenetic control. Current studies in our laboratory are aimed to investigate this hypothesis.

Three substances naturally occurring in the opium poppy—thebaine, papaverine and noscapine [32]—were also tested for inhibition of codeine O-demethylation. Papaverine was reported to inhibit both sparteine oxidation and mephenytoin hydroxylation in human liver microsomes, two reactions with different genetic polymorphisms [33]. All three substances were inhibitors of morphine formation and since their metabolism also involves O-demethylation, it is possible that their disposition co-segregates with codeine or dextromethorphan O-demethylation in man.

In conclusion, we have shown that the female Sprague–Dawley and the female Dark–Agouti rat are good animal counterparts of the human EM and PM phenotype with respect to codeine O-demethylation. These strains could be used to study the pharmacodynamic consequences of the genetic polymorphism in codeine O-demethylation. The outcome of these studies could impact on the therapy of pain control. The value of this animal model in predicting drug–drug interactions in humans remains to be established.

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