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SHORT COMMUNICATION

# Evidence for CYP2D1-Mediated Primary and Secondary O-Dealkylation of Ethylmorphine and Codeine in Rat Liver Microsomes

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**ABSTRACT.** The purpose of the present study was to investigate the role of specific CYPs responsible for the O-dealkylation of ethylmorphine (EM) and codeine (CD) to morphine (M), as well as that of norethylmorphine (NEM) and norcodeine (NCD) to normorphine (NM) in rat liver microsomes. Liver microsomes metabolize EM and CD to M, and NEM and NCD to NM, in the presence of an NADPH-generating system. The metabolites of EM and CD were determined by HPLC with UV and electrochemical detection. In the present study, the role of CYP2D1 in O-dealkylation of EM/NEM and CD/NCD was investigated by use of specific antiCYP antibodies. When testing rabbit antirat CYP2D1, 2E1, 2C11, and 3A2 antibodies, only the antiCYP2D1 antibody inhibited the EM/NEM and CD/NCD O-dealkylase activities significantly. The maximum inhibition achieved was ~80% at a protein ratio (IgG to microsomes) of 10:1, $p = 0.001$. The contribution of CYP2D1 to the O-dealkylation of EM/NEM and CD/NCD was further confirmed by use of the specific CYP2D1 inhibitors quinine and propafenone. Five μM of quinine inhibited the EM/NEM and CD/NCD O-dealkylase activities by ~ 80%. The CYP3A inhibitor troleandomycin (TAO) failed to inhibit the CYP2D1 catalyzed reaction, but did inhibit the N-demethylation of EM and CD. The O-dealkylation of NEM and NCD was also impaired in Dark Agouti rat (DA) liver microsomes. Taken together, the immunoinhibition and chemical-inhibitor studies of rat liver microsomes provided convincing evidence for the involvement of CYP2D1, the rat counterpart of human CYP2D6, in the metabolism of EM/NEM and CD/NCD to the corresponding O-dealkylated metabolites. BIOCHEM PHARMACOL 53;4:603–609, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** CYP2D1; codeine, ethylmorphine; norcodeine; norethylmorphine; O-dealkylation

It has been demonstrated [1] that the O-dealkylation of EM† to M in humans cosegregates with the debrisoquine/sparteine type genetic metabolic polymorphism in which CYP2D6 is involved. The genetic polymorphism of CYP2D6 is of clinical importance in human drug metabolism because several commonly used drugs are substrates for this enzyme. Among the known substrates is the opioid CD, which is structurally similar to EM, the only difference being the length of the alkyl group in the C-3 position of the molecule. The metabolism of EM as compared to CD is, however, less well characterized.

The O-dealkylation of NEM and NCD, the N-demethylated metabolites of EM and CD to NM, could possibly also be mediated by the CYP2D6 enzyme. However, this possibility has, to our knowledge, not been investigated in any detail.

The use of specific antibodies can be a useful tool in *in vitro* drug metabolism studies [2–4]. By the use of specific antibodies in hepatic microsomal incubations, we now provide further evidence for the role of CYP2D1, the rat counterpart of the human CYP2D6 [5, 6], in the O-dealkylation of EM/NEM and CD/NCD. Furthermore, we have used chemical inhibitors of the CYP2D1 and CYP3A isozymes in incubations with Wistar liver microsomes and microsomes from female DA rats to elucidate the metabolic pathways of CD and EM.

## MATERIALS AND METHODS

### *Chemicals and Reagents*

EM was obtained from Weiders Farmasøytiske A/S (Oslo, Norway). CD and M were purchased from Norsk Medisinaldepot (Oslo, Norway). NEM was synthesized from EM [7]. Isocitrate, isocitrate dehydrogenase, NADP, NCD, NM, propafenone, quinine, and TAO were all purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal rabbit antirat CYP2D1 antibody was a generous gift from Prof. J. P. Hardwick (Northeastern Ohio Universities, College of Medicine, Rootstown, OH). Polyclonal rabbit antirat

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† *Abbreviations:* CD, codeine; DA, Dark Agouti rat; EM, ethylmorphine; M, morphine; NCD, norcodeine; NEM, norethylmorphine; NM, normorphine; TAO, troleandomycin.

CYP2C11, 2E1, and 3A2 antibodies were a generous gift from Prof. Y. Funae (Osaka City University Medical School, Osaka, Japan).

### *Preparation of Liver Microsomes*

Male Wistar rats (250–270 g), female Wistar rats (160–180 g), and female DA rats (160–180 g) (Møllegaard Hansens Avlslaboratorier A/S Ejby, Denmark) were killed by decapitation. The liver microsomes were prepared according to previously described methods [8, 9]. The protein concentration was determined according to Lowry *et al.* [10] and BSA was used as standard.

### *Microsomal Incubation*

The microsomal incubations were performed by the method of Mikus *et al.* [11]. Unless otherwise specified, all EM and CD incubations were performed with male Wistar rat liver microsomes, and all NEM and NCD incubations were done with female Wistar rat liver microsomes. EM, CD, NEM, and NCD was prepared in 0.1 M $NaH_2PO_4 \cdot H_2O$ buffer (pH 7.4), and the incubation mixture was then adjusted with incubation buffer to a final total volume of 0.5 mL. The reaction was terminated by the addition of 0.5 mL of 0.2 M $Na_2HPO_4$ buffer (pH 10) on ice. The dependency of O-dealkylations on protein concentration was assessed by incubating 50 and 100 μM of EM/NEM and CD/NCD for 20 min with protein concentrations ranging from 0.125 to 0.75 mg/mL. Time dependency was determined by incubating 0.25 mg microsomal protein/mL with 50 or 100 μM of the substrates for 10–30 min. For the experiments, substrate concentrations of 100 μM were chosen. The protein concentration was 0.25 mg/mL and the incubation time 20 min.

### *Immunoinhibition Studies*

Liver microsomes from Wistar rats (0.25 mg microsomal protein/mL) were preincubated for 25 min at 23° with an equal to 10-fold higher concentration of either rabbit antirat CYP2D1, 2E1, 2C11, or 3A2 antibodies or preimmune IgG (as control), followed by incubation for 20 min with EM, NEM CD, or NCD in a small volume (100 μL, as described above.

### *Chemical Inhibition Studies*

The inhibition of M and NM formation from EM/CD and NEM/NCD, respectively, was studied by use of 0.5 to 10 μM of propafenone/quinine, or 5 to 100 μM of TAO in rat liver microsomal incubations (100 μL, 0.25 mg microsomal protein/mL) for 20 min as described above. Propafenone and quinine were dissolved in water, whereas TAO was dissolved in methanol.

### *Sample Purification and Analysis*

The earlier extraction method used [12] was slightly modified. The sample was adjusted to pH 9.2, transferred to the extraction cartridge, and then washed with 2 × 1 mL of 5 mM $Na_2NPO_4$ buffer (pH 9.2). The analytes were further eluted with 0.5 mL of 65% (v/v) aqueous 10 mM $Na_2HPO_4$ containing 3 mM SDS (pH 2.1) and 35% (v/v) acetonitrile. The extraction recoveries of the respective compounds were (in %, n = 6): 85 ± 8 for NM; 86 ± 5 for M; 90 ± 6 for EM, 88 ± 6 for CD, and 91 ± 5 for NEM; 87 ± 7 for NCD. The HPLC system used has been described previously [12].

### *Data Analysis and Statistics*

All the O-dealkylase activities reported in this paper were based on 20-min incubations. Twenty-min incubations are within the linear time-period of these reactions, when the experimental setup is as reported here. For EM, NEM, CD, and NCD, the control O-dealkylase activities were 2.33, 1.18, 2.85, and 1.68 nmol/mL/min/mg protein, respectively. The percent inhibition of EM/NEM and CD/NCD O-dealkylase activities in the presence of antiCYP antibodies or chemical inhibitors compared to the corresponding control values (in the absence of inhibition) was calculated.

Comparisons between results obtained from controls and antiCYP antibodies and chemical inhibitor samples were performed by the Mann-Whitney test and a p value $< 0.05$ was considered significant.

## RESULTS

### *Metabolism of EM and CD in Microsomes of Wistar Rats*

EM and CD were metabolized to M, NEM/NCD, and NM in liver microsomes of Wistar rats. This metabolic pattern is similar to that observed earlier in incubations with hepatocytes obtained from Wistar rats, except for the lack of conjugation reactions in microsomal incubations [12]. As control experiments, we also performed incubations in the absence of the NADPH-generating system and without liver microsomal protein. Under both these conditions, no metabolism of EM and CD occurred (data not shown).

### *Metabolism of NEM and NCD*

NEM and NCD were metabolized to NM in liver microsomes of female Wistar rats.

### *Immunoinhibition Studies of O-dealkylase Activity*

The effects of antiCYP antibodies raised against CYP2D1, 2E1, 2C11, and 3A2 on the O-dealkylation of EM/NEM and CD/NCD were examined (Fig. 1). We observed a dose-dependent inhibition of the O-dealkylase activity of EM and CD by the antiCYP2D1 antibody (Fig. 1, top panels). The maximum inhibition achieved was approximately 80%

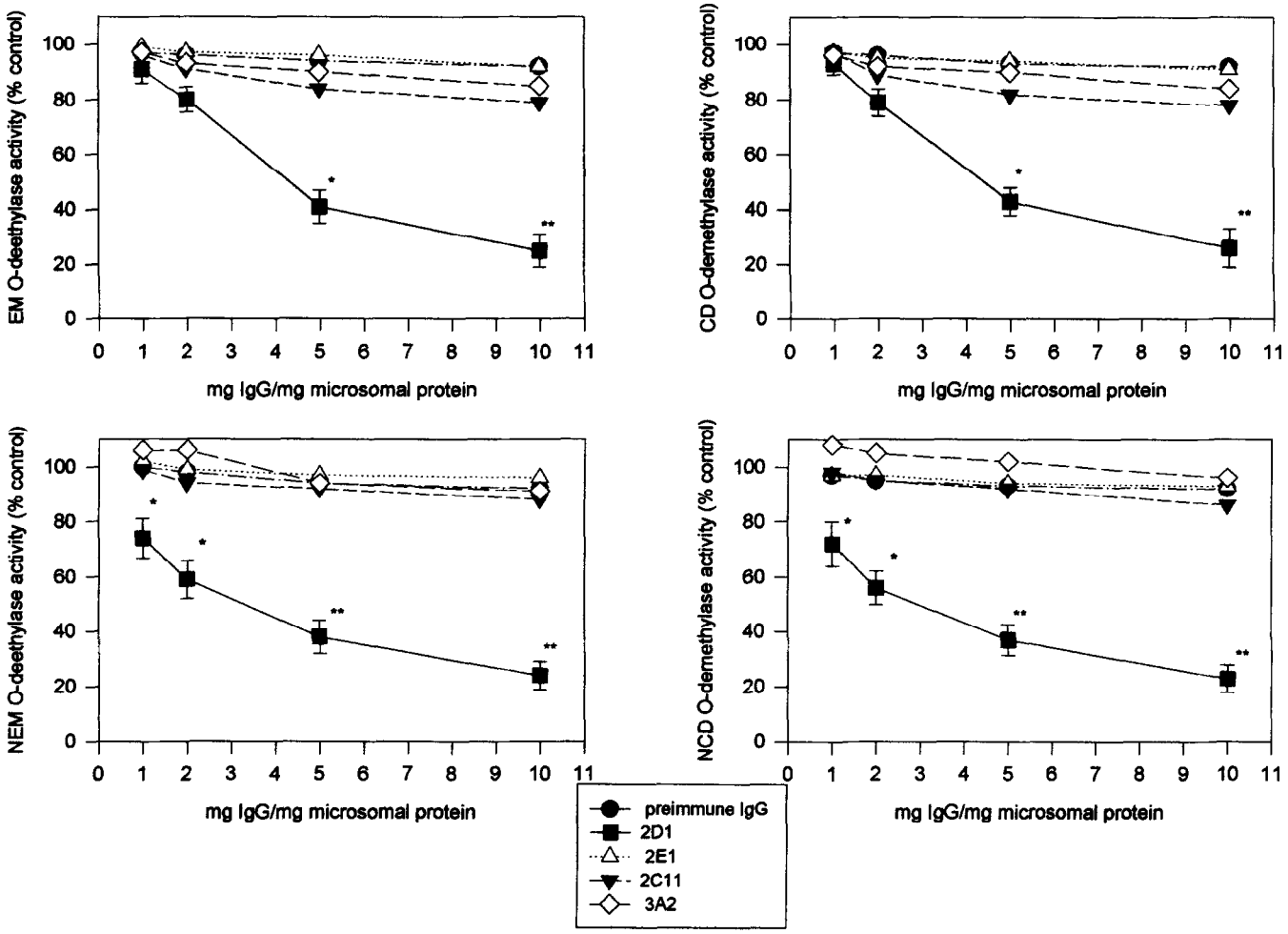


**FIG. 1. Inhibition of EM, NEM, CD, and NCD O-dealkylase activity by polyclonal rabbit antirat antibodies or rabbit preimmune IgG is shown. Results are expressed as percentage of the activity observed in the absence of the antirat antibodies. Data are mean values of 4 incubations (SD only indicated for the CYP2D1 incubations). EM and CD were incubated with the liver microsomes from male Wistar rats, and NEM and NCD were incubated with the liver microsomes from female Wistar rats. Significant changes as compared to controls are indicated, *p = 0.03, **p = 0.001.**

at a protein ratio (IgG to microsomes) of 10:1, p = 0.001. Furthermore, at the same relative antibody concentration, the antiCYP2C11 and antiCYP3A2 showed ca. 20% and 10% inhibition of the O-dealkylation activity, respectively. The antiCYP2E1 and preimmune IgG had almost no effect on the O-dealkylase activity of EM and CD (Fig. 1, top panels). Using a protein ratio (IgG to microsomal protein) of 10:1, the rabbit antiCYP2D1 antibody also resulted in an approximately 80% decrease in NM formation from NEM and NCD, p = 0.001 (Fig. 1, lower panels).

### *Immunoinhibition Studies of N-demethylase Activity*

In contrast, the antiCYP2D1 antibodies had almost no effect on the N-demethylation of EM and CD. Incubating EM and CD with either antiCYP2C11 or 3A2 antibodies, however, resulted in a significant inhibition of NEM and NCD formation (Fig. 2). With a protein ratio (IgG to microsomal protein) of 10:1, the antiCYP2C11 and 3A2 antibodies inhibited the N-demethylase pathway by approximately 60–70%.

### *Inhibition of CYPs by Chemical Inhibitors*

Propafenone and quinine are known inhibitors of CYP2D1 [11, 13]. As shown in Fig. 3, these two CYP2D1 inhibitors effectively inhibited the O-dealkylation of EM and CD in our microsomal preparations from Wistar rats (Fig. 3, top panels). Even at a low concentration of quinine (0.5 μM), approximately 60% of the O-dealkylase activity was inhibited. At higher concentrations of the inhibitors, the O-dealkylase activities were decreased by 70% and 80% in the presence of propafenone and quinine, respectively. On the other hand, the potent CYP3A inhibitor TAO [14] failed to inhibit the CYP2D1 catalyzed reactions even at concentrations as high as 100 μM (data not shown). However, TAO (100 μM) inhibited the N-demethylation of EM and CD by 40%.

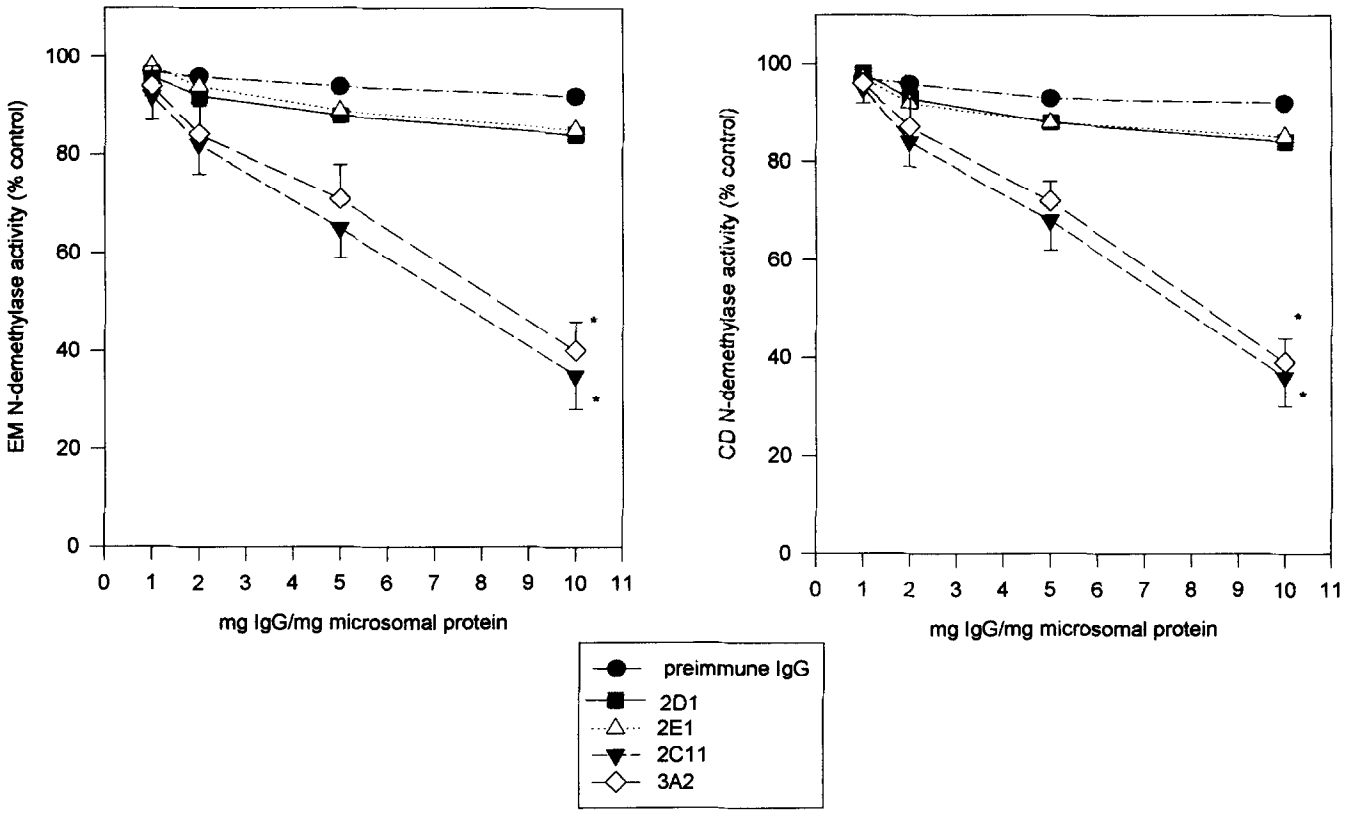


**FIG. 2. Inhibition of EM and CD N-demethylase activity by polyclonal rabbit antirat antibodies are shown in the left and right panels, respectively. Results are expressed as percentage of the activity in the absence of the antirat antibodies. Mean values of 4 incubations are shown (SD indicated for CYP2C11 and 3A2), * p= 0.03.**

### *O-dealkylation of EM and CD in Female DA Rat Liver Microsomes*

The EM and CD O-dealkylase activities in DA rat liver microsomes were 23 ± 5% (n = 4) and 25 ± 4%, respectively, of that of Wistar rats, As expected, the NEM and NCD O-dealkylase activities of DA rat liver microsomes were also deficient, being only 21 ± 6% and 22 ± 6%, respectively, of that observed in Wistar rats.

## DISCUSSION

Specific antibodies against different CYP isozymes have been used to characterize reactions that are mediated by the different CYP isozymes [2–4]. In the present study, we found that approximately 80% of the EM and CD O-dealkylase activity was inhibited by using a high concentration of the antiCYP2D1 antibody in the incubations, indicating that this isozyme is the form mainly responsible for EM and CD O-dealkylation to M in rat liver microsomes. However, other isozymes may also play a minor role in the O-dealkylation process, because anti-CYP2D1 did not block this reaction completely. To investigate the putative role of other CYPs, the antirat CYP2E1, 2C11, and 3A2 antibodies were also used to identify possible additional mediators of EM and CD O-dealkylation. EM and CD O-dealkylase activities were not inhibited by antiCYP2E1, but were inhibited to a minor extent by the antiCYP2C11 and 3A2 antibodies. This finding indicates that CYP2C11 and 3A2 may be of some importance in this O-dealkylation process, but the possible contribution of these isozymes under physiological *in vivo* conditions has to be verified.

Chemical inhibitors against specific CYP isozymes have often been used to characterize metabolic pathways. Quinine, an effective inhibitor of CYP2D1 [13], was added to Wistar rat liver microsomal samples to examine its effect on the O-dealkylation of EM and CD. EM and CD O-dealkylation activities in the microsomes from Wistar rat livers were inhibited by 60% and 80%, respectively, at concentrations of 0.5 μM and 5 μM of quinine. Propafenone, a well-characterized CYP2D6 substrate [15], showed a dose-dependent inhibition of EM and CD O-dealkylation activity. These observations give additional evidence for the major role of the CYP2D1 isozyme in the O-dealkylation process of EM and CD.

We also studied the O-dealkylation of NEM and NCD to NM, which is an important metabolite both in rats [16, 17] and man [18, 19]. AntiCYP2D1 antibodies inhibited this O-dealkylation reaction significantly and to approximately the same extent as the EM and CD O-dealkylation. This

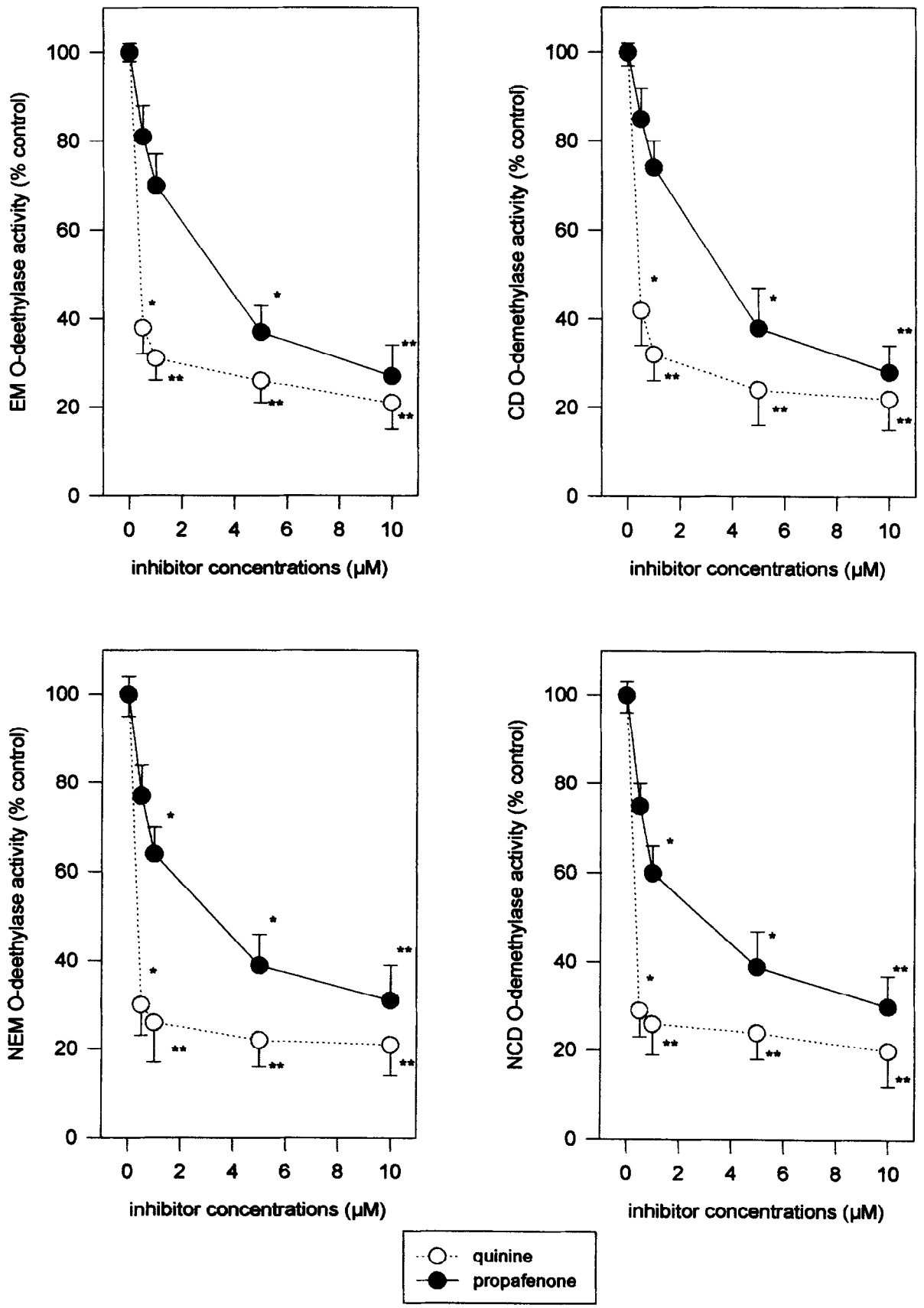


FIG. 3. Inhibition of EM, NEM, CD, and NCD O-dealkylase activity by quinine and propafenone. Results are expressed as percentage of activity in the absence of the inhibitors. EM and CD were incubated with the liver microsomes from male Wistar rats, and NEM and NCD were incubated with the liver microsomes from female Wistar rats. Mean values ± SD of 4 incubations are shown. *p = 0.03; **p = 0.001.

finding strongly supports the possibility that NEM and NCD O-dealkylation to NM is catalyzed by the CYP2D1 isozyme. The chemical inhibitors of CYP2D1 slowed the O-dealkylation of NEM and NCD markedly, further supporting this possibility.

Female DA rats are deficient in the CYP2D1 isozyme. Microsomes from these rats have only ca. 25% of the O-dealkylating capacity of Wistar rats. This metabolic capacity was comparable to what we observed after treating microsomes from Wistar rats with high concentrations of antiCYP2D1 antibodies. These observations may indicate that other isozymes than CYP2D1 may be active, even under physiological conditions.

Taken together, our findings in the incubation studies with antibodies and chemical inhibitors, and different rat strains, may indicate that the O-dealkylation of NEM and NCD is under the same pharmacogenetic control as EM and CD.

As compared to the O-dealkylation of EM and CD, the N-demethylation of these drugs was only inhibited to a minor extent by the antiCYP2D1 antibody. However, both the antiCYP2C11 and 3A2 antibodies inhibited this reaction (ca. 60%). The finding that antiCYP2C11 may inhibit the N-demethylation of EM and CD is consistent with the results of a study by Rane and Ask [20], which reported 66–73% inhibition of the N-demethylation of EM and CD by the anti-rat CYP2C11 antibody.

When TAO, a potent CYP3A inhibitor [15], was used, the N-demethylation of EM and CD was decreased by approximately 40%. This finding is consistent with our results from the antibody incubations, even though CYP3A2 had a more profound inhibition in this N-demethylation reaction. TAO had no measurable effect on EM and CD O-dealkylation. This indicates that TAO does not inhibit microsomal incubations unspecifically. The data from the TAO experiments give further support to the hypothesis that CYP3A2 is of major importance for the N-demethylation process of EM and CD.

In the present study, the extent of inhibition was investigated by the use of different concentrations of the inhibitor [21]. This procedure was chosen instead of studying the inhibition kinetics by varying the concentration of the substrates at several fixed concentrations of inhibitor because, in our hands, the kinetic values were generally quite variable, depending on the microsomal preparations and substrates used as previously described [22–25]. In addition, for such kinetics measurements larger quantities of antibodies and microsomes are needed.

The O-deethylation of EM correlated significantly with CD O-demethylation, both in a previous study of isolated rat hepatocytes [12] and in the present study with rat liver microsomes, indicating that these two opioid analogues are metabolized by the same CYP isozymes. Although the metabolism of CD has been extensively studied in human and animals, to our knowledge, this is the first study to examine the inhibition of CD O-demethylation by use of antirat CYP2D1 antibodies in rat liver microsomes.

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