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Nefiracetam metabolism by human liver microsomes: role of cytochrome P450 3A4 and cytochrome P450 1A2 in 5-hydroxynefiracetam formation

Yuko Fujimaki, Nobuko Arai, Tsunenori Nakazawa and Masayoshi Fujimaki

Abstract

An in-vitro study was conducted to investigate the metabolism of nefiracetam in human liver microsomes and to identify the enzymes responsible for the metabolism. Nefiracetam was hydroxylated by human liver microsomes to 5-hydroxynefiracetam (5-OHN). Eadie-Hofstee plots for the formation of 5-OHN suggested substrate activation. The kinetic parameters, apparent K_m, V_{max}, and Hill coefficient, for the formation of 5-OHN by pooled human liver microsomes were 4012 μM, 2.66 nmol min^{-1} (mg protein)$^{-1}$, and 1.65, respectively. The formation of 5-OHN was significantly correlated with cytochrome P450 (CYP)3A4-mediated testosterone 6β-hydroxylase activity and dextromethorphan *N*-demethylase activity. The 5-OHN formation was inhibited (94%) by antibody to human CYP3A4/5. The 5-OHN formation was also inhibited by the CYP3A4 inhibitors ketoconazole and troleandomycin, but not significantly inhibited by several other P450 inhibitors. The microsomes containing cDNA-expressed CYP3A4 formed 5-OHN with sigmoidal kinetics. CYP3A5-containing microsomes did not form 5-OHN. These results indicated that CYP3A, most likely CYP3A4, was the major isozyme responsible for the formation of 5-OHN in human liver microsomes. CYP1A2 and CYP2C19 microsomes were also capable of forming 5-OHN. However, the contribution of CYP1A2 was considered to be relatively minor compared with that of CYP3A4, and the contribution of CYP2C19 was assumed to be negligible, based on the result of the immunoinhibition study and taking into account both the turnover rate by each isozyme and the relative abundance of each isozyme in human liver. We conclude that on average the formation of 5-OHN, the major metabolite of nefiracetam, is principally mediated by CYP3A4 with a relatively minor contribution by CYP1A2.

Drug Metabolism and Analytical Chemistry Research Laboratory, New Product Research Laboratories III, Daiichi Pharmaceutical Co., Ltd, Tokyo, Japan

Yuko Fujimaki, Nobuko Arai, Tsunenori Nakazawa, Masayoshi Fujimaki

Correspondence: Y. Fujimaki, Drug Metabolism and Analytical Chemistry Research Laboratory, Daiichi Pharmaceutical Co., Ltd, 1-16-13 Kitakasai, Edogawa-ku, Tokyo 134-8630, Japan.

Introduction

Nefiracetam (*N*-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl)acetamide), a cyclic derivative of γ-aminobutyric acid (Figure 1), is a potent facilitator of neuronal calcium-channel activity and shows anti-amnesic effects by interacting with GABAergic and AChergic neuronal systems (Kameyama et al 1990; Kojima et al 1990; Nabeshima et al 1990a, b, 1991; Watabe et al 1993; Kawajiri et al 1994; Yoshii & Watabe 1994; Yoshii et al 1997). This compound is now undergoing clinical trials as a novel neurotransmission-enhancer for the treatment of sequelae of cerebrovascular disorders and Alzheimer's disease.

Previous studies have shown that nefiracetam elimination is mainly dependent on its hepatic clearance in man, because less than 10% of the drug is excreted

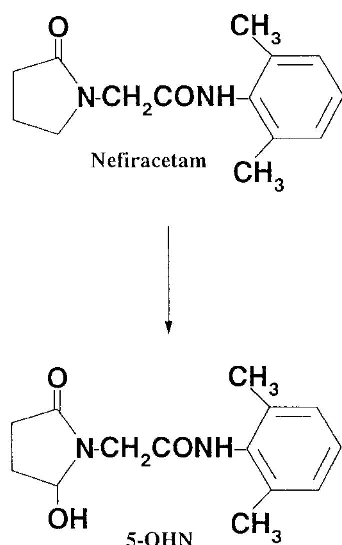

Figure 1 Hydroxylation of nefiracetam to 5-hydroxynefiracetam (5-OHN).

unchanged in the urine and faeces (Fujimaki et al 1992). The metabolic pathways of nefiracetam are identified as: hydroxylation of the pyrrolidine ring and hydroxylation of the dimethylphenyl moiety followed by sulfate conjugation; the major metabolite is 5-hydroxynefiracetam (5-OHN; Figure 1) (Fujimaki et al 1990, 1993).

The purpose of the current study was to investigate the first step metabolism of nefiracetam in human liver microsomes and to identify the cytochrome P450 isoforms responsible for this metabolism. This information is important for predicting possible drug interactions.

Materials and Methods

Chemicals and reagents

Nefiracetam (Lot no. 9501) was synthesized in the Technology Research Laboratories of Daiichi Pharmaceutical Co., Ltd (Tokyo, Japan), and the authentic samples of the nefiracetam metabolite (5-OHN) and an internal standard (2-oxo-1-pyrrolidinyl-*N*-phenylacetamide) used for the determination of 5-OHN were synthesized in the Drug Metabolism and Analytical Chemistry Research Laboratory, Daiichi Pharmaceutical Co., Ltd (Tokyo, Japan). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADPH, diethyldithiocarbamic acid (DDC), tolbutamide, and troleandomycin (TAO) were purchased from Sigma Chemical Co. (St Louis, MO). Quinidine was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan), sulfaphenazole was from Research Biochemical International (Natick, USA), ketoconazole was from ICN Biochemicals Inc. (OH), and furafylline and (*S*)-mephenytoin were from Ultrafine Chemicals (Manchester, UK). Coumarin and α-naphthoflavone (ANF) were obtained from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). Pooled human liver microsomes from seven human livers and individual human liver microsomes from 16 donors (Reaction Phenotyping Kit) were obtained from Xenotech LLC (KS). Liver samples from two donors (sample K14 and K24) were generously provided by Dr T. Inaba (University of Toronto), and microsomal fractions were prepared by differential centrifugation. The relative cytochrome P450 (CYP)3A activity of these liver samples was K14 (++++) and K24 (+), and the relative CYP1A2 activity was K14 (++) and K24 (++). Antibodies to human CYP1A1/2, CYP2C, and CYP2D6 were obtained from Daiichi Pure Chemical Co., Ltd (Tokyo, Japan) and antibody to human CYP3A4/5 was from Amersham Life Science (Tokyo, Japan). The cross-reactivity of each antibody was investigated for human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 activity using microsomes containing each recombinant human CYP isozyme. The cross-reactivity of antibody CYP2C for human CYP2C19 activity and the cross-reactivity of antibody to CYP3A4/5 for CYP3A4 and CYP3A5 activity was also investigated. The immunoinhibitory profiles for human CYPs are as follows: anti-CYP1A1/2 antibody cross-reacts with CYP1A1 and CYP1A2; anti-CYP2C antibody cross-reacts with CYP2C9 and CYP2C19; anti-CYP2D6 antibody cross-reacts with CYP2D6; and anti-CYP3A4/5 antibody cross-reacts with CYP3A4 and CYP3A5. Microsomes prepared from baculovirus-infected insect cells (BTI-TN-5B1-4) expressing human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP4A11 and control microsomes were purchased from Gentest Corporation (MA). Except for the control microsomes, all microsome preparations contained cDNA-expressed NADPH-CYP reductase, and CYP2E1 and CYP3A4 microsomes also contained cDNA-expressed CYPB5. The reagents used for HPLC analyses were of HPLC grade, and all other chemicals were of the highest quality available.

Incubation procedure for nefiracetam metabolism

Metabolism by human liver microsomes

The typical incubation mixture (final vol. 0.8 mL) con-

sisted of 50 mM Tris-HCl buffer (pH 7.5), an NADPH-generating system (10 mM glucose-6-phosphate, 0.33 unit mL^{-1} glucose-6-phosphate dehydrogenase, 4 mM $MgCl_2$, 0.5 mM NADPH), human liver microsomes (1 (mg protein) mL^{-1}) and nefiracetam. After 3 min pre-incubation at 37°C, the reaction was initiated by the addition of the NADPH-generating system, and stopped by adding ice-cold ethanol after 30 min incubation. The resultant mixture was vigorously mixed after the addition of the internal standard and centrifuged at 3000 rev min^{-1} for 15 min. The supernatant was evaporated to dryness, and the residue was redissolved in 25% acetonitrile in 25 mM phosphate buffer (pH 7) for HPLC analysis. The kinetics of metabolite formation by pooled human liver microsomes was studied in triplicate with the substrate concentration range of 8 μM to 8 mM. The kinetics of metabolite formation by microsomes from two individual liver samples (K14 and K24) was also investigated. The formation rate of 5-OHN in human liver microsomes was fitted to the Hill equation ($v = V_{max} \times [S]^N/(K_m + [S]^N)$, and the apparent K_m, V_{max}, and Hill coefficient (N) values were obtained using the non-linear least-square regression program (Kaleida Graph, Synergy Software).

Correlation study

Correlation of the formation of 5-OHN with several CYP isoform-specific activities in the microsomes prepared from 16 different human livers (Reaction Phenotyping Kit) was studied in duplicate with the nefiracetam concentration of 30 μM. The metabolic activities of the microsomes for 7-ethoxyresorufin *O*-deethylation, coumarin 7-hydroxylation, 7-ethoxy-4-trifluoromethylcoumarin deethylation, taxol 6-hydroxylation, tolbutamide methylhydroxylation, *S*-mephenytoin 4′-hydroxylation, dextromethorphan *O*-demethylation, chlorzoxazone 6-hydroxylation, *p*-nitrophenol hydroxylation, testosterone 6β-hydroxylation, dextromethorphan *N*-demethylation, and lauric acid 12-hydroxylation, were taken from the data sheet of the Kit. Statistical analyses were performed to calculate Pearson correlation coefficients between the formation rate of 5-OHN and several CYP isoform-specific activities using the Macintosh StatView program (Abacus Concepts).

Immunoinhibition study

Immunoinhibition of the metabolism was examined in duplicate with antibodies to human CYP1A1, CYP1A1/1A2, CYP2C, CYP2D6, or CYP3A4/5. Pooled human liver microsomes (0.2 mg protein) were pre-incubated with antibodies to human CYP for 30 min at room temperature before the start of the 3-min pre-incubation with nefiracetam (30 μM) at 37°C, and thereafter the incubation was performed for 30 min with the NADPH-generating system.

Chemical inhibition study

In chemical inhibition experiments, the chemicals used as CYP isoform-specific substrates/inhibitors were: furafylline (5 μM, 50 μM) for CYP1A2; coumarin (10 μM) for CYP2A6, sulfaphenazole (5 μM, 50 μM) for CYP2C9/10, tolbutamide (400 μM) for CYP2C8/9/10, *S*-mephenytoin (500 μM) for CYP2C19, quinidine (1 μM, 5 μM) for CYP2D6, DDC (100 μM) for CYP2E1, TAO (10 μM) and ketoconazole (5 μM) for CYP3A (Yun et al 1991; Gonzalez 1997; Jang & Benet 1997; Kumar et al 1997; Lee & Slattery 1997; Rodrigues et al 1997). Incubations were performed in duplicate. The chemicals were added just before the addition of nefiracetam (30 μM), and incubated with pooled human liver microsomes (1 (mg protein)$^{-1}$ mL^{-1}) as described before. For the mechanism-based inhibitors (i.e. furafylline, DDC, and TAO), the reaction mixture was pre-incubated for 10 min at 37°C in the presence of inhibitor, human liver microsomes, and the NADPH-generating system before the addition of substrate (30 μM). The same approach was used with ANF (5 μM, 10 μM, 20 μM), which is an inhibitor of CYP1A1/1A2 and a stimulator of CYP3A4 (Buening et al 1981). The effect of nifedipine, a substrate of CYP3A4, on nefiracetam metabolism was also examined. Nifedipine (100 μM) was added to the incubation mixture containing human liver microsomes, the NADPH-generating system, and nefiracetam (30 μM) and incubated for 30 min at 37°C after the 3-min pre-incubation.

Metabolism by recombinant human liver CYP

The ability of recombinant human liver CYP isozymes (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP4A11) to produce 5-OHN from nefiracetam was assessed in duplicate in an incubation system (final vol. 500 μL) consisting of the NADPH-generating system, nefiracetam (30 μM), and microsomal protein specifically expressing human CYP (50 pmol mL^{-1}). The reaction was started by adding the NADPH-generating system, and incubations were performed for 30 min as described for human liver micro-

somes. A control study was also conducted using control vesicle microsomes. Kinetic experiments with microsomes expressing either CYP2C19 or CYP3A4 were also conducted with the substrate concentration range from 4 to 500 μM, and the profiles were compared with those of human liver microsomes.

Analysis of microsomal incubations

The metabolic profile of nefiracetam in human liver microsomes was analysed with the HPLC system consisting of the pump L-6000 (Hitachi, Japan), the column TSKgel ODS-80T_M (4.6 mm i.d. × 150 mm; Tosoh, Japan), and the guard column (3.2 mm i.d. × 15 mm; Tosoh, Japan). The mobile phase was 13% acetonitrile in 25 mM phosphate buffer (pH 7). The flow rate was 1.0 mL min^{-1}. The eluent was monitored by an L-4000 UV detector (Hitachi, Japan) set at 210 nm. ESI/LC/MS was performed to identify metabolites formed in microsomal incubations of nefiracetam using a TSQ700 mass spectrometer. Samples were injected onto an Inertsil ODS-3 column (GL Science, Japan) with 2:3 (v/v) methanol–10 mM ammonium formate (pH 4.7) at a flow rate of 0.2 mL min^{-1}. The formation of 5-OHN was determined using the semi-micro HPLC system consisting of the pumps PU610, an autosampler AS640 and a column oven CO630 (all from GL Sciences, Japan). An ODS column (Capcell pak C18 UG120, 2.0 mm i.d. × 150 mm; Shiseido, Japan) was used at 40°C preceded by a pretreatment column (TSK guardgel ODS-80T_M, 3.2 mm i.d. × 15 mm; Tosoh, Japan). The mobile phase for pretreatment was 25 mM phosphate buffer (pH 7.4). For analysis, the mixture of acetonitrile and 25 mM phosphate buffer (pH 7.4) was used as the mobile phase with 6% acetonitrile for 0–5 min and 6–50% acetonitrile for 5–20 min (linear gradient). The flow rate was 0.5 mL min^{-1} for pretreatment and 0.2 mL min^{-1} for analysis. The eluate was monitored by UV620 (GL Sciences, Japan) at 210 nm. The quantification limit of 5-OHN was 20 pmol with an inter-assay coefficient of variation of 14%.

Results

Nefiracetam metabolism

A typical HPLC chromatogram of the supernatant after incubation of nefiracetam with human liver microsomes in the presence of an NADPH-generating system is presented in Figure 2. The structure of the metabolite produced after incubation was identified as 5-OHN by

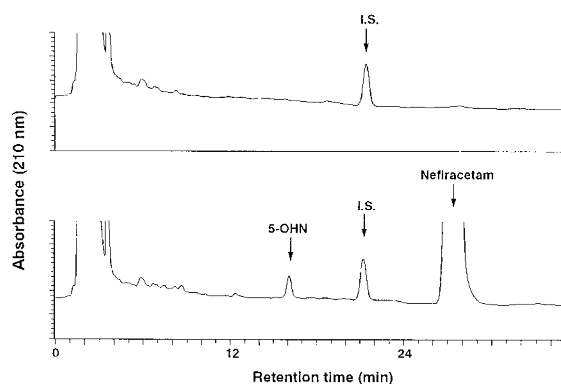

Figure 2 Typical HPLC profile of nefiracetam incubated with human liver microsomes. Nefiracetam (60 μM) was incubated for 20 min at 37°C in the presence of an NADPH-generating system with human liver microsomes.

ESI/LC/MS analysis, as described previously (Fujimaki et al 1995). The formation of 5-OHN by human liver microsomes (1 (mg protein) mL^{-1}) was linear with respect to incubation times of up to 30 min (data not shown).

Kinetics of 5-OHN formation

In-vitro kinetic parameters of 5-OHN formation in pooled microsomes from seven human livers were determined with substrate concentrations ranging from 8 μM to 8 mM. The kinetics of 5-OHN formation in individual liver microsomes from two donors (K14 and K24) were also examined. Figure 3 shows the substrate–velocity plots and the corresponding Eadie-Hofstee plots for 5-OHN formation. The 5-OHN formation activity of K14 microsomes with relatively high CYP3A4 activity and medium CYP1A2 activity was higher than that of K24 microsomes with relatively low CYP3A4 activity and medium CYP1A2 activity. Since the kinetic profiles of 5-OHN formation were sigmoidal and atypical Eadie-Hofstee plots were obtained for all human liver microsomes, the data were fitted to a sigmoidal V_{max} model equivalent to the Hill equation. The obtained V_{max}, K_m, and Hill coefficient values are presented in Table 1. The Hill coefficient values indicating the degree of sigmoidicity were 1.50–1.65.

Correlation between various CYP isoform-specific activities and the formation of 5-OHN

The rates of 5-OHN formation with the nefiracetam concentration of 30 μM ranged from 0.83 to 7.43 pmol min^{-1} (mg protein)$^{-1}$ in liver microsomes prepared from

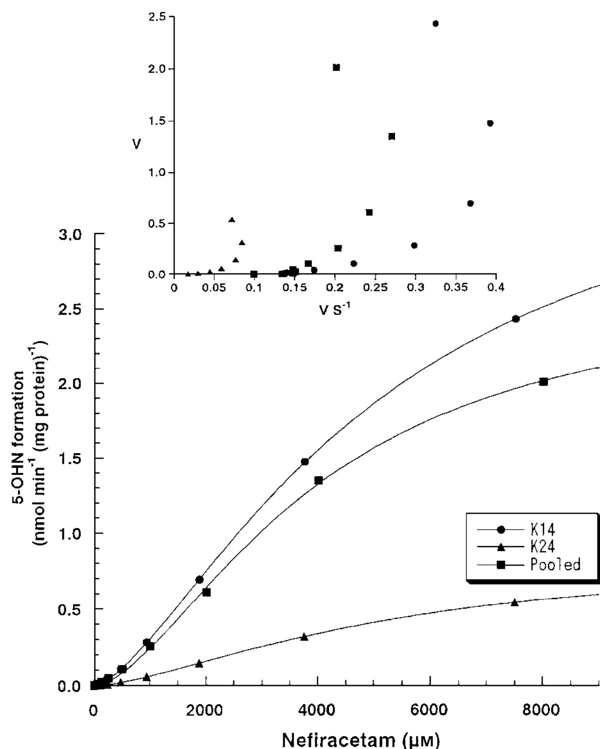

Figure 3 Formation of 5-hydroxynefiracetam (5-OHN) from nefiracetam by human liver microsomes. Inset shows Eadie-Hofstee plots for 5-OHN formation.

Table 1 Kinetic parameters for nefiracetam 5-hydroxylation by human liver microsomes.

Parameter	Pooled	K14	K24
V_{max} (nmol min^{-1} (mg protein)$^{-1}$)	2.66	3.78	0.85
K_m (μM)	4012	5071	5274
V_{max}/K_m (μL min^{-1} (mg protein)$^{-1}$)	0.66	0.75	0.16
Hill number	1.65	1.50	1.54

Nefiracetam was added to the microsomal incubation mixture at a concentration of 8 μM–8 mM and incubated for 30 min at 37°C.

16 different human liver samples, showing 9-fold interindividual variability (Figure 4). The correlation between several metabolic activities selective for CYP isozymes and the formation of 5-OHN was assessed (Table 2). The formation of 5-OHN was significantly correlated with CYP3A-mediated testosterone 6β-hydroxylase activity (r = 0.849; $P < 0.0001$; Figure 4) and dextromethorphan *N*-demethylase activity (r = 0.936; $P < 0.0001$). It was also well correlated with CYP2B6-mediated 7-ethoxy-4-trifluoromethyl coumarin deethylase activity (r = 0.766; $P = 0.0003$). No good correlation was found between the formation of 5-OHN and other CYP isozyme selective activities.

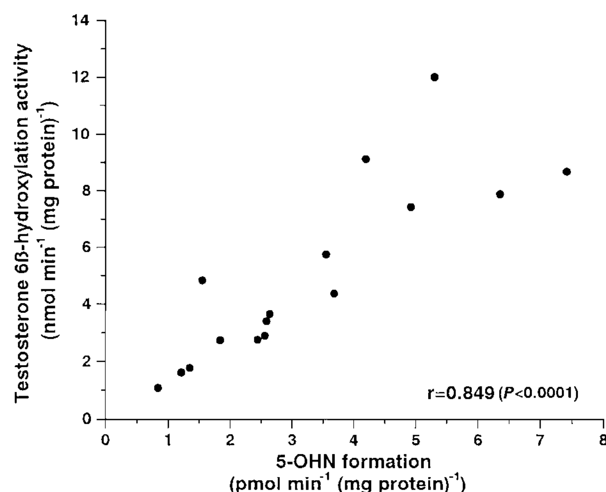

Figure 4 Correlation between 5-hydroxynefiracetam (5-OHN) formation and testosterone 6β-hydroxylation activity in microsomes from 16 human livers.

Immunoinhibition studies

Immunoinhibition studies with antibodies to CYP1A1, CYP1A1/2, CYP2C, CYP2D6 and CYP3A4/5 were conducted (Figure 5). Antibodies to CYP3A4/5 and CYP1A1/1A2 inhibited the 5-OHN formation in human liver microsomes by 83–94% and 39–46%, respectively. The inhibition of 5-OHN formation by other antibodies was < 12%.

Effects of CYP isoform-selective substrates or inhibitors on the formation of 5-OHN

The effects of CYP isoform-selective substrates or inhibitors on the formation of 5-OHN were also examined (Figure 6). The CYP3A inhibitors ketoconazole and TAO inhibited the 5-OHN formation by 77 and 65%, respectively. In contrast, furafylline (the CYP1A2 inhibitor), coumarin (the CYP2A6 substrate), sulfaphenazole (the CYP2C9/10 inhibitor), tolbutamide (the CYP2C8/9/10 substrate), *S*-mephenytoin (the CYP2C19 substrate), quinidine (the CYP2D6 inhibitor), and DDC (the CYP2E1 inhibitor) had no significant effects. Nifedipine (100 μM), a substrate of CYP3A4, inhibited the formation of 5-OHN by 31%.

Table 2 Correlation between 5-hydroxynefiracetam formation and isoform-specific cytochrome activity in microsomes from 16 different human livers.

Catalytic activity	P450	r	*P*
7-Ethoxyresorufin *O*-dealkylation	CYP1A2	0.326	0.2227
Coumarin 7-hydroxylation	CYP2A6	0.463	0.0708
7-Ethoxy-4-trifluoromethylcoumarin deethylation	CYP2B6	0.766	0.0003
Taxol 6-hydroxylation	CYP2C8	0.587	0.0153
Tolbutamide methylhydroxylation	CYP2C9	0.284	0.2917
S-Mephenytoin 4′-hydroxylation	CYP2C19	0.400	0.1267
Dextromethorphan *O*-demethylation	CYP2D6	0.199	0.4663
Chlorzoxazone 6-hydroxylation	CYP2E1	0.042	0.8809
p-Nitrophenol hydroxylation	CYP2E1	0.248	0.3616
Testosterone 6β-hydroxylation	CYP3A4/5	0.849	< 0.0001
Dextromethorphan *N*-demethylation	CYP3A4	0.936	< 0.0001
Lauric acid 12-hydroxylation	CYP4A9	0.130	0.6380

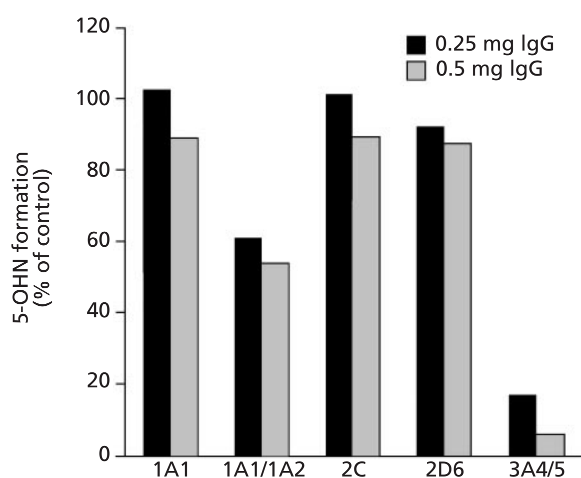

Figure 5 Effects of anti-CYP antibodies on the formation of 5-hydroxynefiracetam (5-OHN) in human liver microsomes. After pre-incubation of pooled human liver microsomes (0.2 mg protein) with antibodies to human CYPs for 30 min at room temperature, nefiracetam (30 μM) was added and incubated for 30 min with the NADPH-generating system. For control reactions, normal serum was added instead of the antiserum. Each bar represents the mean of duplicate incubations.

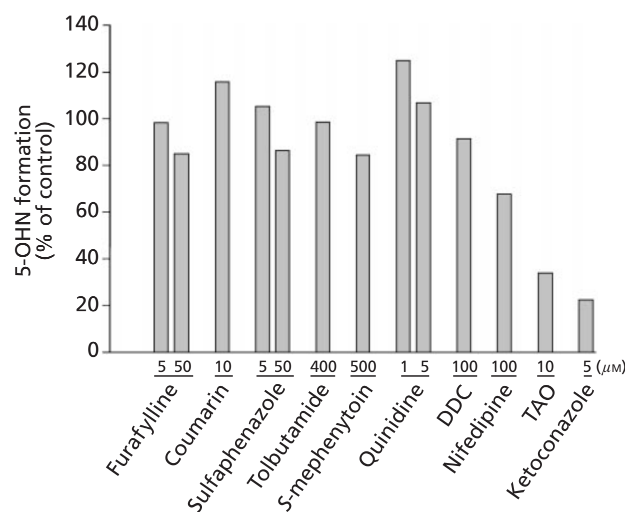

Figure 6 Effects of various compounds on 5-hydroxynefiracetam (5-OHN) formation in human liver microsomes. Compounds were added just before the addition of nefiracetam (30 μM) and incubated with pooled human liver microsomes (1 mg protein mL^{-1}). The compounds were dissolved in buffer or methanol. An equivalent quantity of buffer or methanol was added for control incubations. For the mechanism-based inhibitors (furafylline, diethyldithiocarbamic acid (DDC), and troleandomycin (TAO)), the reaction mixture was pre-incubated for 10 min at 37°C with the inhibitor, human liver microsomes and the NADPH-generating system before the addition of nefiracetam. Each bar represents the mean of duplicate incubations.

Formation of 5-OHN in microsomes containing recombinant human CYP isozymes

The CYP isoforms responsible for 5-OHN formation were also elucidated using microsomes containing recombinant human CYP isozymes (Figure 7). The formation of 5-OHN was observed in microsomes from baculovirus-infected insect cells containing cDNA-expressed human CYP2C19, CYP3A4, and CYP1A2 with the turnover numbers of 0.90, 0.49 and 0.22 pmol min^{-1} (pmol CYP)$^{-1}$, respectively. No activity was detected with control microsomes or microsomes containing cDNA-expressed CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2D6, CYP2E1, CYP3A5, and CYP4A11. The formation of 5-OHN by microsomes containing cDNA-expressed CYP3A4 and CYP2C19 was examined further with nefiracetam over the concentration range 4–500 μM to compare the kinetic

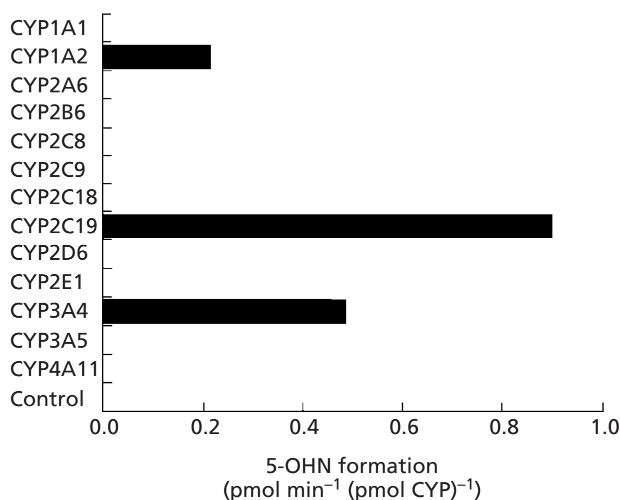

Figure 7 Formation of 5-hydroxynefiracetam (5-OHN) by recombinant human CYPs. Nefiracetam (30 μM) was incubated with microsomal protein specifically expressing human CYP (50 pmol mL^{-1}) in the presence of an NADPH-generating system. A control study was also conducted using control vesicle microsomes. Each bar represents the mean of duplicate incubations.

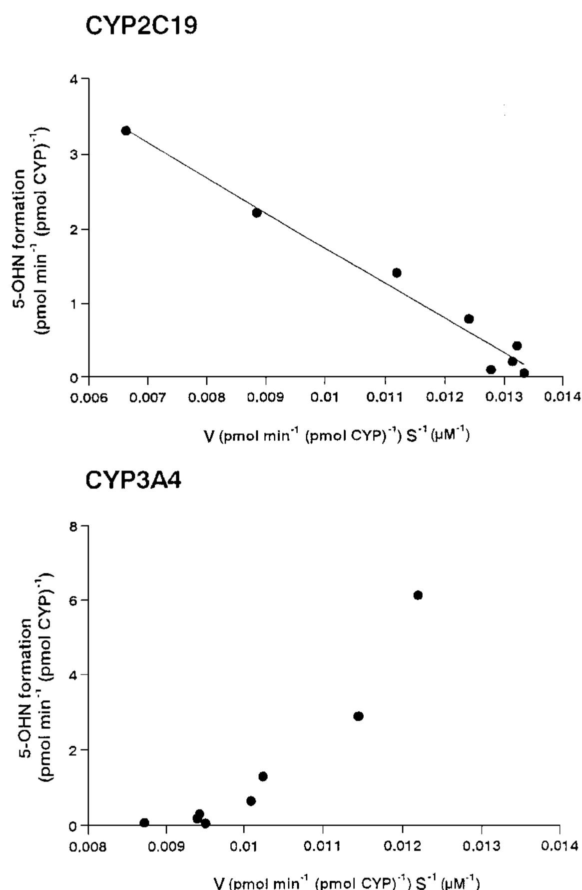

Figure 8 Eadie-Hofstee plots for 5-hydroxynefiracetam (5-OHN) formation by recombinant human CYPs. Nefiracetam (4–500 μM) was incubated with recombinant human CYP2C19 and CYP3A4. Each data point represents the mean value of duplicate incubations.

profiles with those of human liver microsomes. As shown in Figure 8, the Eadie-Hofstee plots for 5-OHN formation by CYP2C19 were monophasic (estimated V_{max} and K_m values were 6.22 pmol min^{-1} (pmol CYP)$^{-1}$ and 442 μM, respectively), whereas the Eadie-Hofstee plots by CYP3A4 showed an atypical profile as observed in the human liver microsomes (estimated V_{max}, K_m, and Hill coefficient values were 51.0 pmol min^{-1} (pmol CYP)$^{-1}$, 5618 μM, and 1.14, respectively).

Effect of ANF on the formation of 5-OHN

ANF was added to the incubation mixture containing nefiracetam (30 μM) and human liver microsomes. Increasing concentrations of ANF stimulated 5-OHN formation (Figure 9). An approximate 3-fold increase was observed in the presence of 20 μM ANF.

Discussion

In the present study, the metabolite of nefiracetam formed in human liver microsomes was identified as 5-OHN, which has also been reported as the main metabolite in man in-vivo (Fujimaki et al 1993).

The kinetics of 5-OHN formation by human liver microsomes was studied over the substrate concentration range 8 μM–8 mM, which included the range of serum concentrations (20 μg mL^{-1}, 80 μM) observed in humans receiving 450 mg (150 mg t.i.d.) nefiracetam (Hasegawa et al 1994). The 5-OHN formation rate in human liver microsomes accelerated disproportionately with increasing nefiracetam concentrations, and displayed sigmoidal kinetic characteristics (Figure 3), suggesting substrate activation with Hill coefficient values of < 2.

Several reports are available on the in-vitro activation of CYPs (Schwab et al 1988; Schmider et al 1995; Ueng et al 1997; Venkatakrishnan et al 1998; Witherow & Houston 1999), and activation by the substrate increasing its own rate of metabolism (autoactivation), as well as activation by one compound affecting the metabolism of another compound are both reported for many CYP-catalysed reactions including CYP3A4. Some mechanisms have been proposed to explain CYP3A4 activation. Ueng et al (1997) proposed an allosteric model with two

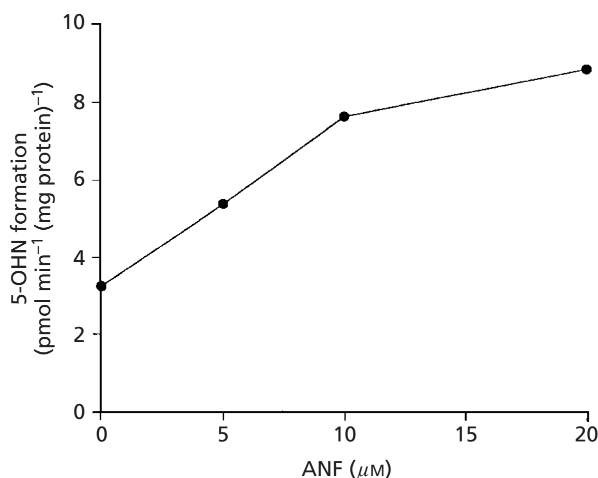

Figure 9 Effects of α-naphthoflavone (ANF) on 5-hydroxynefiracetam (5-OHN) formation in human liver microsomes. Incubations were conducted with nefiracetam (30 μM), ANF (5, 10, 20 μM) and human liver microsomes in the presence of an NADPH-generation system. Each data point represents the mean value of duplicate incubations.

distinct binding sites; the affinity of the substrate binding site was increased for the substrate. Shou et al (1994) described simultaneous binding of substrate and activator in the active site, Korzekwa et al (1998) reported the cooperative binding of multiple substrates (an effector is also a substrate), and Ludwig et al (1999) suggested that CYP3A4 possesses at least two different substrate-binding sites. The mechanism of the autoactivation in the formation of 5-OHN is unknown and requires further study.

The present study indicated that on average 5-OHN formation was mainly catalysed by CYP3A4 with a relatively minor contribution by CYP1A2. To obtain these results, we used four in-vitro approaches: correlation of the metabolite formation rate with predetermined marker CYP activity in different human liver microsomes; immunoinhibition by the antibodies to human CYPs; chemical inhibition by specific CYP substrates/inhibitors; and screening for the formation of the metabolite with heterologously expressed human CYPs.

The formation of 5-OHN was significantly correlated with CYP3A4-mediated testosterone 6β-hydroxylase activity and dextromethorphan *N*-demethylase activity (Waxman et al 1988; Jacqz-Aigrain et al 1993). The formation was inhibited by antibody to CYP3A4/5, and also inhibited by the CYP3A4 inhibitors ketoconazole and TAO (Maurice et al 1992; Newton et al 1994). Furthermore, ANF, which is known to activate CYP3A activity (Buening et al 1981), caused three-times the formation rate of 5-OHN (Figure 9). These results strongly suggest that the CYP3A subfamily plays a major role in the formation of 5-OHN. The CYP isoforms responsible for 5-OHN formation were further elucidated using the microsomes containing cDNA-expressed human CYPs. Nefiracetam was metabolized to 5-OHN by not only CYP3A4 microsomes but also CYP2C19 and CYP1A2 microsomes, and CYP2C19 microsomes formed 5-OHN at a higher rate than did the CYP3A4 and CYP1A2 microsomes (Figure 7). However, taking into account both the turnover rate by each isozyme and the relative level of each isozyme in human liver (Shimada et al 1994), the contribution of CYP2C19, CYP3A4, and CYP1A2 to the formation of 5-OHN was estimated as 2.1, 85.3, and 12.6%, respectively. Moreover, substrate activation was observed in the kinetics of 5-OHN formation by recombinant CYP3A4 as observed by human liver microsomes, but not observed by CYP2C19 (Figure 8). In addition, CYP3A5 microsomes did not form 5-OHN (Figure 7). Together, these data indicate that CYP3A, most likely CYP3A4, was the major isozyme responsible for the formation of 5-OHN.

Recombinant CYP1A2 exhibited 5-OHN formation activity, and CYP1A2 antibody partially inhibited the 5-OHN formation (46%). Thus, CYP1A2 also seems to play a role in the formation of 5-OHN. However, the 5-OHN formation in 16 human liver microsomes did not correlate well with the 7-ethoxyresorufin *O*-dealkylation activity, furafylline did not sufficiently inhibit the 5-OHN formation, and the contribution of CYP1A2 estimated from the turnover rate and the relative level of the isozyme in human liver was 12.6% of the total 5-OHN formation. So, it is considered that on average the contribution of CYP1A2 to 5-OHN formation is relatively minor compared with that of CYP3A4, and the formation of 5-OHN in man is expected to depend mainly on the activity of CYP3A4. The 5-OHN formation in liver microsomes from K14 donor with relatively higher CYP3A4 activity and medium CYP1A2 activity was higher than that in liver microsomes from K24 donor with relatively lower CYP3A4 activity and medium CYP1A2 activity (Figure 3), and the 5-OHN formation in human liver microsomes used in the correlation study (obtained from Xenotech LLC) was also higher in microsomes with high CYP3A4 and low CYP1A2 activity than those in microsomes with low CYP3A4 and high CYP1A2 activity (data not shown). It is possible, however, to consider that the contribution of CYP1A2 to the formation of 5-OHN will be much more predominant in human liver microsomes with extensively low CYP3A4 and high CYP1A2 activity,

because the activity of CYPs differ in individuals, so that the percentage contributions of CYPs differ in individuals.

Even though recombinant CYP2C19 was capable of forming 5-OHN with the highest activity in all the isozymes examined, its contribution was estimated as 2.1 %, and CYP2C antibody, which cross-reacts with CYP2C19, did not inhibit 5-OHN formation. Thus, the role of CYP2C19 might be negligible for the formation of 5-OHN in human liver microsomes.

The correlation between 5-OHN formation and CYP2B6-mediated 7-ethoxy-4-trifluoromethylcoumarin deethylation ($r = 0.766$; $P < 0.0003$) was considered fortuitous, because CYP2B6-mediated 7-ethoxy-4-trifluoromethylcoumarin deethylation and CYP3A4-mediated testosterone 6β-hydroxylation activities were highly correlated ($r = 0.852$; $P < 0.0001$) in 16 human liver microsomes used for these correlation studies (data not shown).

Nifedipine is a calcium antagonist used extensively to treat angina and hypertension and is known to be a substrate of CYP3A4 (Guengerich et al 1986). In this study, the effect of nifedipine on the metabolism of nefiracetam was also investigated. Nifedipine (100 μM) inhibited the formation of 5-OHN by approximately 30 %. It was assumed that the inhibition of 5-OHN formation by nifedipine could be underestimated due to the autoactivation of the formation of 5-OHN; however, it was unlikely that nifedipine affected the metabolism of nefiracetam, since the C_{max} value of nifedipine in clinical use was reported to be approximately 0.33 μM (Kleinbloesem et al 1994).

In conclusion, it was demonstrated that the main metabolic pathway of nefiracetam by human liver microsomes was the 5-hydroxylation of the pyrrolidine ring (the formation of 5-OHN), and that on average CYP3A4 predominantly mediated this pathway by exhibiting substrate activation in human liver microsomes, with a relatively minor contribution by CYP1A2. Thus, the clinical pharmacokinetics of nefiracetam would be expected to depend mainly on the activity of CYP3A4, which is predominantly responsible for the formation of the major metabolite 5-OHN. However, the percentage contributions of CYPs differ in individuals, so that the contribution of CYP1A2 to the formation of 5-OHN will be much more predominant in human liver microsomes with extensively low CYP3A4 and high CYP1A2 activity. Although the formation of 5-OHN was not significantly affected by nifedipine in-vitro, the likelihood of in-vivo interactions between nefiracetam and other CYP3A-metabolized drugs cannot totally be negated, and further investigation of drug interactions of nefiracetam via CYP3A4 and CYP1A2 both in-vitro and in-vivo is required.

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