J. Pharm. Pharmacol. 1998, 50: 997–1004
Received February 2, 1998
Accepted May 19, 1998

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Multiple Human Cytochromes Contribute to Biotransformation of Dextromethorphan In-vitro: Role of CYP2C9, CYP2C19, CYP2D6, and CYP3A

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Abstract

Cytochromes mediating the biotransformation of dextromethorphan to dextrorphan and 3-methoxymorphinan, its principal metabolites in man, have been studied by use of liver microsomes and microsomes containing individual cytochromes expressed by cDNA-transfected human lymphoblastoid cells.

In-vitro formation of dextrorphan from dextromethorphan by liver microsomes was mediated principally by a high-affinity enzyme (K_m (substrate concentration producing maximum reaction velocity) 3–13 μM). Formation of dextrorphan from 25 μM dextromethorphan was strongly inhibited by quinidine (IC50 (concentration resulting in 50% inhibition) = 0·37 μM); inhibition by sulphaphenazole was approximately 18% and omeprazole and ketoconazole had minimal effect. Dextrorphan was formed from dextromethorphan by microsomes from cDNA-transfected lymphoblastoid cells expressing CYP2C9, -2C19, and -2D6 but not by those expressing CYP1A2, -2E1 or -3A4. Despite the low in-vivo abundance of CYP2D6, this cytochrome was identified as the dominant enzyme mediating dextrorphan formation at substrate concentrations below 10 μM. Formation of 3-methoxy-morphinan from dextromethorphan in liver microsomes proceeded with a mean K_m of 259 μM. For formation of 3-methoxymorphinan from 25 μM dextromethorphan the IC50 for ketoconazole was 1·15 μM; sulphaphenazole, omeprazole and quinidine had little effect. 3-Methoxymorphinan was formed by microsomes from cDNA-transfected lymphoblastoid cells expressing CYP2C9, -2C19, -2D6, and -3A4, but not by those expressing CYP1A2 or -2E1. CYP2C19 had the highest affinity (K_m = 49 μM) whereas CYP3A4 had the lowest (K_m = 1155 μM). Relative abundances of the four cytochromes were determined in liver microsomes by use of the relative activity factor approach. After adjustment for relative abundance, CYP3A4 was identified as the dominant enzyme mediating 3-methoxymorphinan formation from dextromethorphan, although CYP2C9 and -2C19 were estimated to contribute to 3-methoxymorphinan formation, particularly at low substrate concentrations.

Although formation of dextrorphan from dextromethorphan appears to be sufficiently specific to be used as an in-vitro or in-vivo index reaction for profiling of CYP2D6 activity, the findings raise questions about the specificity of 3-methoxymorphinan formation as an index of CYP3A activity.

Biotransformation of dextromethorphan to dextrorphan is commonly used as an index reaction for profiling the activity of CYP2D6 both in-vivo and in various in-vitro systems (Dayer et al 1989; Jacqz-Aigrain et al 1993; Kerry et al 1994; Kroemer & Eichelbaum 1995; Schadel et al 1995; Ducharme et al 1996; Engel et al 1996; Jones et al 1996b; Rodrigues 1996). Parallel transformation of dextromethorphan to a second metabolite, 3-methoxymorphinan, has received recent attention as another index reaction that might concurrently reflect CYP3A activity both in-vivo and in-vitro

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(Ducharme et al 1996; Jones et al 1996a,b). The dextromethorphan/3-methoxymorphinan urinary metabolic ratio in-vivo is sensitive to the 3A-inducing effects of rifampin, and the inhibiting effects of erythromycin (Jones et al 1996a). In-vitro a major contribution of 3A isoforms to formation of 3-methoxymorphinan is indicated by findings such as inhibition by relatively specific inhibitory probes such as ketoconazole and gestodene and by anti-3A antibodies, as well as cosegregation with other 3A index reactions (Gorski et al 1994; Schmider et al 1996, 1997). However, the specificity of 3-methoxymorphinan formation as an index reaction for P450-3A activity is not completely established, since the inhibitory K_i of ketoconazole for this reaction is one or more orders of magnitude higher than for other pure 3A reactions, and inhibition by anti-3A antibodies is incomplete (Gorski et al 1994; Schmider et al 1997).

This study has identified the specific cytochromes contributing to the formation of dextrorphan and 3-methoxymorphinan from dextromethorphan using human liver microsomes and pure human cytochromes contained in microsomes from cDNA-transfected human lymphoblastoid cells.

Materials and Methods

Materials

Liver samples from donors with no known liver disease were provided by the International Institute for the Advancement of Medicine, Exton, PA, or the Liver Tissue Procurement and Distribution System, University of Minnesota, Minneapolis, MN. Microsomes were prepared by ultracentrifugation; microsomal pellets were suspended in 0·1 M potassium phosphate buffer (pH 7·4) containing 20% glycerol and stored at −80°C until use (von Moltke et al 1994, 1995a,b, 1996a,b,c; Schmider et al 1996, 1997). Microsomes containing individual cytochromes expressed by cDNA-transfected human lymphoblastoid cells (Crespi 1995; Gentest, Woburn, MA) were similarly stored at −80°C until used. Chemical reagents and drugs were obtained from commercial sources, or kindly provided by their pharmaceutical manufacturers.

Incubations

Samples were incubated in a water bath with gentle oscillation of the tubes. Incubation mixtures contained 50 mM phosphate buffer (pH 7·4), 5 mM Mg^{2+}, 0·5 mM $NADP^+$, and an isocitrate–isocitric dehydrogenase regenerating system (375 μM isocitric acid, 1 unit mL^{-1} isocitrate dehydrogenase). Different quantities of dextromethorphan in methanol solution, to yield final incubate concentrations ranging from 5·0 to 1250 μM, were added to a series of incubation tubes. The solvent was evaporated to dryness at 40°C under mild vacuum. Reactions were initiated by addition of microsomal protein (up to 0·5 mg mL^{-1}). After 20 min at 37°C, reactions were stopped by cooling on ice and addition of acetonitrile (50 μL). Pronethalol (4 μg mL^{-1}) was added as an internal standard, the incubation mixture was centrifuged (Eppendorf model 5415C) at 14 000 rev min^{-1}, and the supernatant was transferred to an autosampling vial for HPLC analysis of dextrorphan and 3-methoxymorphinan as described elsewhere (Schmider et al 1996, 1997). The rates of formation of both metabolites were linearly dependent on time (to at least 60 min) and protein concentration (to at least 1 mg mL^{-1}). Reaction velocities were calculated in units of nmol min^{-1} (mg protein)$^{-1}$. All incubations were performed in duplicate.

Further studies were performed using a fixed concentration of dextromethorphan (25 μM). Different concentrations of a number of possible inhibitors (ketoconazole, quinidine, sulphaphenazole or omeprazole) were co-added, reactions were initiated by addition of the microsomal protein, and mixtures were processed as described above.

To evaluate formation of dextrorphan and 3-methoxmorphinan from dextromethorphan by specific human cytochromes, two concentrations (25 μM and 250 μM) of dextromethorphan were incubated with microsomes containing individual CYP1A2, -2C9, -2C19, -2D6, -2E1, and -3A4 at a microsomal protein concentration of 1 mg mL^{-1}. These analyses revealed no detectable metabolite formation by CYP1A2, or -2E1. Accordingly for each of the pure CYP2C9, -2C19, -2D6 and -3A4 the relationship between dextromethorphan concentration (5–1000 μM) and rate of metabolite formation was determined as described above, and expressed in units of pmol min^{-1} (pmol CYP)$^{-1}$. Because of the limited quantities of pure cytochromes available, only single incubations were performed at each substrate concentration.

Data analysis

For studies of formation of 3-methoxymorphinan by liver microsomes, Eadie–Hofstee plots were consistent with a single-enzyme Michaelis–Menten kinetic profile. The equation consistent with this model was fitted to data points by non-linear least-squares regression (SAS PROC NLIN; SAS Institute, Cary, NC), yielding values of maximum reaction velocity (V_{max}), and sub-strate concentration producing a reaction velocity of 0·5 V_{max} (K_m). Eadie–Hofstee plots for dextrorphan formation

were equivocal. Accordingly both one-enzyme and two-enzyme models were used to analyse the data. Goodness-of-fit was based on visual assessment and on evaluation of r^2 values.

For studies of inhibitor effects at fixed concentrations of dextromethorphan, reaction velocities in the presence of inhibitor were expressed as a percentage ratio (R_v) of the control velocity with no inhibitor present. When applicable, 50% inhibitory concentrations (IC50) were determined by use of non-linear regression as described previously (Venkatakrishnan et al 1998).

For studies using individual cytochromes with varying concentrations of dextromethorphan, the Michaelis–Menten equation was fitted to data points to determine V_{max} and K_m values for each cytochrome. For dextrorphan formation by P450-2D6, the model was modified to incorporate uncompetitive substrate inhibition (von Moltke et al 1996c; Venkatakrishnan et al 1998).

Results

Liver microsomes

In two cases dextrorphan formation by liver microsomes was best described by a one-enzyme Michaelis–Menten model (Figure 1) with K_m values of 8·1 and 13·0 μM (Table 1); in the other two cases a two-enzyme model best described the data (Figure 1); the high-affinity component, accounting for most of the intrinsic clearance (V_{max}/K_m ratio) via this pathway, had K_m values of

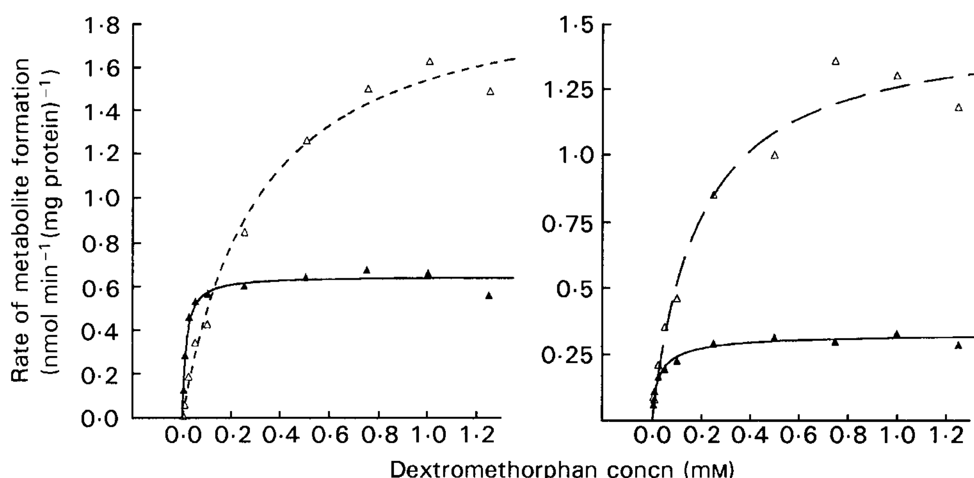

Figure 1. Rates of formation of dextrorphan (▲) and 3-methoxymorphinan (△) from dextromethorphan by microsomal preparations from sample A (left), for which dextrorphan formation was consistent with a one-enzyme model, and sample C (right), for which dextrorphan formation was consistent with a two-enzyme model. Each point represents the mean of duplicate determinations. Lines represent functions consistent with the Michaelis–Menten model for dextrorphan (—) and 3-methoxymorphinan (– –). See Table 1 for enzyme kinetic analysis.

Table 1. Formation of dextrorphan and 3-methoxymorphinan from dextromethorphan by human liver microsomes.

	Liver sample			
	A	B	C	D
Dextrorphan formation				
High-affinity:				
V_{max} (nmol min^{-1} (mg protein)$^{-1}$)	0·646	0·046	0·15	0·202
K_m (μM)	13·0	3·6	8·5	8·1
V_{max}/K_m × 1000	49·88	12·78	17·65	24·94
Low-affinity:				
V_{max} (nmol min^{-1} (mg protein)$^{-1}$)	–	0·258	0·166	–
K_m (μM)	–	82·3	69·8	–
V_{max}/K_m × 1000	–	3·13	2·38	–
3-Methoxymorphinan formation				
V_{max} (nmol min^{-1} (mg protein)$^{-1}$)	2·01	0·79	1·49	0·96
K_m (μM)	304	335	188	209
V_{max}/K_m × 1000	6·61	2·36	7·93	4·59

V_{max}, maximum reaction velocity; K_m, substrate concentration producing 50% of maximum reaction velocity.

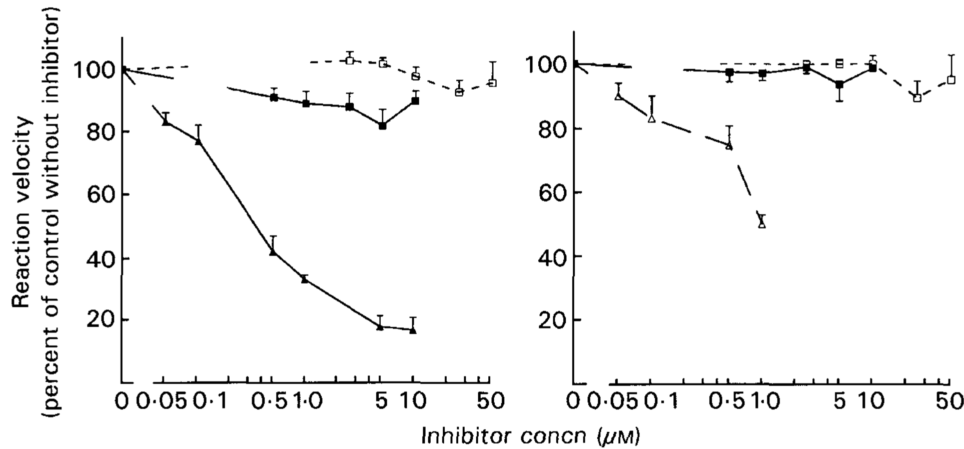

Figure 2. Effects of different concentrations of inhibitors on the rates of formation of dextrorphan (left) or 3-methoxymorphinan (right) from dextromethorphan (25 μM). Reaction rates are expressed as a percent of the control velocity without inhibitor (mean ± s.e., n = 4, for each point). Symbols are: quinidine, ▲, ———; ketoconazole, △, — —; sulphaphenazole, ■, ———; omeprazole, □, — -.

3·6 and 8·5 μM. Formation of 3-methoxymorphinan by liver microsomes was consistent with a single-enzyme Michaelis–Menten model with a mean K_m of 259 μM (Table 1). Formation of dextrorphan accounted for the majority of net intrinsic clearance.

Quinidine was a highly potent inhibitor of dextrorphan formation, with a mean (± s.e.) IC50 of 0·37 (± 0·07) μM and a previously described K_i value of 0·1 μM (Schmider et al 1996). The rate of formation of dextrorphan was reduced to 82% of control by sulphaphenazole at 10 μM (Figure 2). Neither omeprazole at concentrations up to 50 μM nor ketoconazole had a significant effect on the rate of formation of dextrorphan.

The rate of formation of 3-methoxymorphinan was reduced to 50% of control by 1 μM ketoconazole (Figure 2). The IC50 for ketoconazole was 1·15 (± 0·22) μM, and the previously reported K_i was 0·37 μM (Schmider et al 1996). The rate of formation of 3-methoxymorphinan was not affected by more than 10% by sulphaphenazole, omeprazole or quinidine.

Human cytochromes

Dextrorphan was formed by cytochromes CYP2C9, -2C19 and -2D6. CYP1A2, -2E1 and -3A4 produced no detectable activity (Figure 3, Table 2). The highest affinity of the three enzymes was attributable to CYP2D6, the K_m of which (5·2 μM)

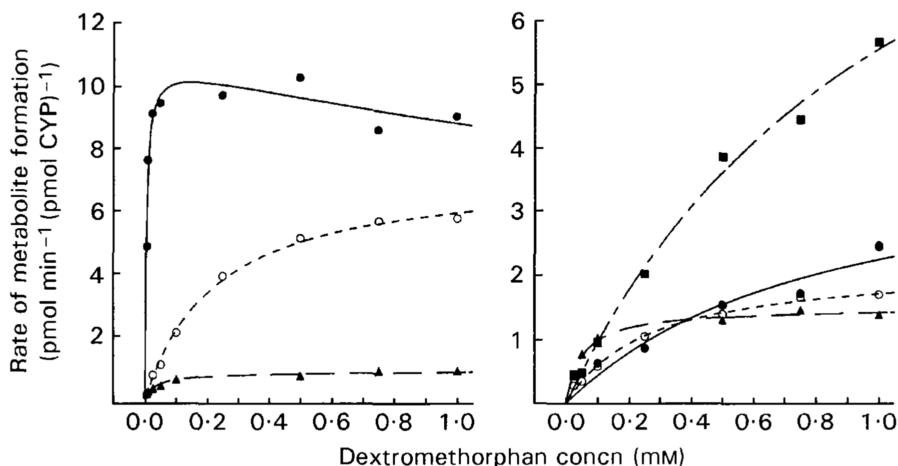

Figure 3. Rates of formation of dextrorphan (left) or 3-methoxymorphinan (right) from dextromethorphan by cytochromes contained in microsomes expressed by cDNA-transfected human lymphoblastoid cells. Lines represent Michaelis–Menten functions (Table 2). Data have not been adjusted for in-vivo abundance of the cytochromes. (Very low values for CYP2C19 at dextromethorphan = 250 μM, apparently due to excessive addition of internal standard, and a high value for dextrorphan formation by CYP2D6 at dextromethorphan = 100 μM, which is unexplained, were excluded from the data analysis.) Symbols are: CYP2D6, ●, ———; CYP2C9, ○, - - -; CYP2C19, ▲, — — —; CYP3A4, ■, — -.

Table 2. Formation of dextrorphan and 3-methoxymorphinan from dextromethorphan by individual human cytochromes.

	CYP3A4	CYP2C9	CYP2C19	CYP2D6
Dextrorphan formation				
V_{max} (pmol min^{-1} (pmol CYP)$^{-1}$)	–	7·16	0·84	10·8*
K_m (μM)	–	229	32·9	5·2*
$V_{max}/K_m \times 1000$	–	31·3	25·7	2087
Abundance-normalized values:				
V_{max} (%)	–	84·7	3·3	12·0
V_{max}/K_m (%)	–	13·3	3·6	83·1
3-Methoxymorphinan formation				
V_{max} (pmol min^{-1} (pmol CYP)$^{-1}$)	11·90	2·10	1·45	4·07
K_m (μM)	1155	254	49·2	838
$V_{max}/K_m \times 1000$	10·3	8·3	29·4	4·9
Abundance-normalized values:				
V_{max} (%)	87·9	8·6	1·9	1·6
V_{max}/K_m (%)	50·5	22·3	26·0	1·2

V_{max}, maximum reaction velocity; K_m, substrate concentration producing 50% of maximum reaction velocity. * Uncompetitive substrate inhibition constant 4225 μM.

was similar to the high-K_m component of liver microsomes. Formation of dextrorphan by CYP2C19 and -2C9 proceeded with higher K_m and lower intrinsic clearance compared with -2D6.

3-Methoxymorphinan was formed by CYP2C9, -2C19, -2D6, and -3A4, but not by CYP1A2 or -2E1. The highest affinity and highest intrinsic clearance were attributable to CYP2C19 (Figure 3, Table 2). CYP3A4 formed 3-methoxymorphinan with low affinity ($K_m = 1155\ \mu$M). The mean K_m for 3-methoxymorphinan formation by human liver microsomes was similar only to that of P450-2C9 ($K_m = 254\ \mu$M).

Estimated contribution of individual cytochromes

The relative contribution of a specific cytochrome to net reaction velocity in liver microsomes in-vitro, or in-vivo, will depend on the V_{max}, K_m and intrinsic clearance for that specific cytochrome, as well as on the abundance of that cytochrome (Shimada et al 1994; von Moltke et al 1995b). We determined the average relative abundances of CYP3A, -2C9, -2C19 and -2D6 in a series of microsomal preparations from 10–12 liver samples, using the relative activity factor (RAF) approach as described elsewhere (Crespi 1995; Kobayashi et al 1997). RAF for each of the four isoforms was calculated as the ratio of the V_{max} value for a relatively isoform-specific reaction in liver microsomes divided by the V_{max} for the same reaction by the cDNA-expressed isoform as described above (Venkatakrishnan K et al, unpublished data). The isoform-specific reactions were: diazepam 3-hydroxylation for CYP3A, tolbutamide methylhydroxylation for -2C9, *S*-mephenytoin 4′-hydroxylation for -2C19, and the high-affinity component of nortriptyline 10-hydroxylation for -2D6. Mean RAF values (expressed as a percentage of the sum of the four absolute RAF values) were: 56% for CYP3A, 30·9% for -2C9, 10·1% for -2C19, and 2·9% for -2D6.

V_{max} and intrinsic clearance values for the individual cytochromes were multiplied by these RAFs, and the values were normalized to 100% (Table 2). The resulting values were used to estimate the contribution of each cytochrome to net reaction velocity in relation to the concentration of substrate (dextromethorphan), focusing on the low range of substrate concentrations.

Formation of dextrorphan is clearly dominated by CYP2D6 in the low-substrate concentration range, despite the low abundance of this enzyme (Figure 4). Because of the high relative V_{max} of CYP2C9 after adjustment for abundance, this enzyme will assume increasing importance at high substrate concentrations.

Formation of 3-methoxymorphinan is dominated by CYP3A4, but at low substrate concentrations -2C9 and -2C19 are estimated to make significant contributions (Figure 4). CYP2D6 makes only a minor contribution to 3-methoxymorphinan formation.

Discussion

The findings from this study with human liver microsomes are consistent with our previous results (Schmider et al 1996, 1997). Formation of dextrorphan is the principal metabolic pathway for biotransformation of dextromethorphan. More than one enzyme seems to contribute to this pathway, but the dominant component is a high-affinity reaction with a K_m value in the range 3–13 μM. The reaction was strongly inhibited by quinidine, but

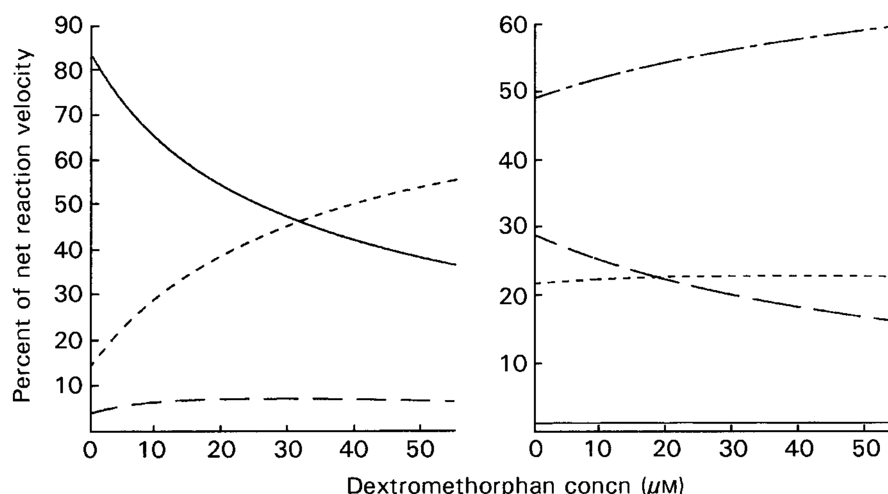

Figure 4. Predicted relative contributions of CYP2C9, -2C19, -2D6, and -3A4 to the net rate of formation of dextrorphan (left) and of 3-methoxymorphinan (right), from dextromethorphan. Contributions have been adjusted for abundances of the individual cytochromes, by use of the relative activity factor method as described in the text. Lines are: CYP2D6, ———; CYP2C9, - - - - -; CYP2C19, — —; CYP3A4, — - —.

other relatively isoform-specific inhibitors (ketoconazole, sulphaphenazole, omeprazole) had minimal effect on dextrorphan formation at a substrate concentration of 25 μM. Studies using individual cytochromes contained in microsomes expressed by cDNA-transfected human lymphoblastoid cells indicated that CYP2C9, -2C19, and -2D6 were all capable of biotransforming dextromethorphan to dextrorphan. Consideration of the intrinsic clearance for the three enzymes, along with their average relative abundances in the liver microsomes, indicated that CYP2D6 is the quantitatively dominant enzyme at substrate concentrations below 10 μM. Thus the data support application of the conversion of dextromethorphan to dextrorphan as an index reaction for profiling CYP2D6 activity in man both in-vitro and in-vivo.

Formation of 3-methoxymorphinan from dextromethorphan in liver microsomes proceeded as an apparent single-enzyme reaction, with a mean K_m of 259 μM, similar to that reported previously (Schmider et al 1997). The reaction was strongly inhibited by ketoconazole, but the susceptibility to ketoconazole inhibition (based on IC50 or K_i values) was less than that of other reactions established as being pure index reactions. For example, the mean ketoconazole K_i for in-vitro hydroxylation of alprazolam (von Moltke et al 1994), triazolam (von Moltke et al 1996b), and midazolam (von Moltke et al 1996c) consistently falls below 0·1 μM, compared with the ketoconazole K_i of 0·37 μM for formation of 3-methoxymorphinan. The reaction is, furthermore, incompletely inhibited by troleandomycin, gestodene and by anti-3A antibodies (Gorski et al 1994; Schmider et al 1997). The important but not exclusive participation of CYP3A isoforms in 3-methoxymorphinan formation is supported by pure cytochrome data, indicating that CYP2C9, -2C19, and -2D6 can mediate this reaction in addition to -3A4. On the basis of chemical inhibition data we previously suggested that CYP2E1 might contribute to 3-methoxymorphinan formation (Schmider et al 1997); however neither -1A2 nor -2E1 produced detectable amounts of 3-methoxymorphinan. The observed K_m for the liver microsomes is apparently a hybrid value, since it was not similar to the K_m for either pure CYP3A4, or for CYP2C19, the enzyme with the highest affinity and highest intrinsic clearance. After normalization for estimated average relative abundance, CYP3A emerged as the dominant enzyme, particularly in the higher range of substrate concentrations typically used for in-vitro studies. This is consistent with the minimal inhibition of this reaction in-vitro by sulphaphenazole or omeprazole at substrate concentrations of 25 μM. However at low substrate concentrations, as might be encountered in-vivo, CYP2C9 and -2C19 are estimated to make potentially important contributions to 3-methoxymorphinan formation along with CYP3A4. It should be emphasized that these estimates are based on average relative abundance values. Considerable variation can be expected both in-vivo in man and among liver samples in-vitro.

Limitations inherent in extrapolation of in-vitro data to drug metabolism in-vivo are well recognized (Bertz & Granneman 1997; Greenblatt & von

Moltke 1997; von Moltke et al 1998). For example, the role of CYP3A isoforms in 3-methoxymorphinan formation after oral administration of dextromethorphan in-vivo might be greater than estimated by this model, since CYP3A isoforms are dominant in the gastrointestinal tract mucosa. Furthermore our estimate of P450-2C19 abundance based on relative activity factors is higher than estimates based on immunoquantitation (Inoue et al 1997). Despite this the findings raise concerns about the specificity of 3-methoxymorphinan formation as an in-vivo index of CYP3A activity in man. Although the in-vivo dextromethorphan/3-methoxymorphinan urinary metabolic ratio is clearly sensitive to factors that cause large changes in CYP3A activity (Jones et al 1996a), it is not clear whether this index is specific enough to reflect more subtle within- and between-subject differences in CYP3A activity, or whether the index is in fact insensitive, for example, to factors influencing CYP2C9 or -2C19 activity. The question requires further study of the effect of isoform-specific inhibitors (such as ketoconazole or omeprazole) on 3-methoxymorphinan formation from dextromethorphan in-vivo, or the influence of genetic variations in 2C19 activity.

Acknowledgements

This work was supported by Grants MH-34223, DA-05258, MH-19924 and RR-00054 from the Department of Health and Human Services. Dr von Moltke is the recipient of a Scientist Development Award (K21-MH-01237) from the National Institutes of Mental Health. Dr Schmider was the recipient of a Merck Sharp and Dohme International Fellowship in Clinical Pharmacology.

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