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Limited applicability of 7-methoxy-4-trifluoromethylcoumarin as a CYP2C9-selective substrate

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Abstract:
Fluorometric substrates selective for various cytochrome P450 isoforms (P450s) have great advantages in *in vitro* enzyme inhibition and induction studies because they are highly sensitive and suitable for rapid screening. 7-Methoxy-4-trifluoromethylcoumarin (MFC) has been reported as a CYP2C9-selective substrate. The present study investigated the relative catalytic selectivity of several human P450s in the *O*-demethylation of MFC and the applicability of MFC as a probe substrate for CYP2C9. The CYP2C9-selectivity in liver microsomes was not supported by the correlation analysis within a series of microsomes from individual donors or by studies using chemical inhibitors. MFC *O*-demethylation of microsomes did not correlate with tolbutamide 4-hydroxylation, the classical CYP2C9-marker activity, suggesting the possible participation of some of the other P450s. Results of inhibition studies using model P450 inhibitors also brought the CYP2C9-selectivity of MFC *O*-demethylation into question. In microsomes containing cDNA-expressed individual P450s, CYP2B6 and CYP2E1 seemed to be the most active in the *O*-demethylation of MFC. Our results support the participation of several P450 enzymes (CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4) in MFC *O*-dealkylation. Therefore, MFC cannot be considered a suitable probe substrate in models that express several P450s, such as liver microsomes or primary hepatocytes. Moreover, MFC is a more potent fluorogenic substrate for CYP2B6 and CYP2E1 than for CYP2C9 in microsomes containing cDNA-expressed P450s.

Key words:
7-methoxy-4-trifluoromethylcoumarin *O*-demethylation, CYP2C9-selectivity, fluorogenic substrate, high-throughput screening, drug-drug interaction

Abbreviations: MFC – 7-methyl-4-trifluoromethylcoumarin, P450 – cytochrome P450

Introduction

Cytochrome P450 (P450) enzymes are major participants in the oxidative metabolism of a wide range of structurally diverse xenobiotics, including drugs, pesticides and food additives. Members of the P450 superfamily, which are enzymes of CYP1-3, are responsible for the metabolism and disposition of more than 90% of the commercially available therapeutics [20]. The different P450s vary in terms of their catalytic specificity, regulation of expression and sensitivity to inhibitors [16, 21]. The inhibition or the induction of P450 enzymes by a xenobiotic can alter the patient's

response to foreign compounds that may result in changes in the pharmacological or toxicological action. Therefore, it is of great clinical interest to study and better understand the metabolic drug interactions as a side effect of drug therapy. Increased blood levels of a drug in patients as a consequence of drug interactions may cause unexpected toxic side effects, whereas the increased elimination of a drug leads to the loss of the pharmacological effect [34]. Early prediction of the clinically significant drug interactions of a drug candidate may contribute to the reduction of adverse effects.

Investigations of P450 catalytic activity usually involve the conversion of the isoform selective substrates to the respective metabolites. Selective enzyme assays provide a useful tool in the *in vitro* measurements of individual P450 activities. There are several molecules available as selective probes for individual P450s; however, most of the metabolite detection methods involve laborious HPLC separations [6, 10]. Fluorescence-based methods offer highly sensitive and rapid assays for *in vitro* drug interaction studies that do not require metabolite separation, which allows for the parallel monitoring of large reaction arrays on plate readers, thus enhancing the sample throughput. These methods use non-fluorescent substrates that are metabolized by P450s to fluorescent metabolites [7]. The catalytic selectivity of P450s toward several fluorogenic substrates has been proven and their utility has been confirmed using both cDNA-expressed P450s and liver microsomes.

CYP2C enzymes play an important role in the oxidative metabolism of several drugs, such as warfarin, diclofenac, proguanil, (*S*)-mephenytoin, and proton pump inhibitors (e.g., omeprazole, pantoprazole) [21]. Human CYP2Cs account for about 20% of the hepatic P450 enzymes and contribute to the metabolism of more than 20% of the drugs on the market. As with all major drug metabolizing enzymes, co-administration of known substrates or inhibitors is a major source of adverse drug interaction events among drugs principally eliminated by the CYP2C subfamily. CYP2C9 is one of the most abundant CYP2Cs and is involved in a number of clinically-significant drug interactions [23]. A number of reactions have been suggested as a selective probe for the CYP2C9 enzyme, such as tolbutamide 4-hydroxylation [24], diclofenac 4'-hydroxylation [19], and (*S*)-warfarin 7-hydroxylation [28]. These CYP2C9-marker assays require time and labor-intensive analytical procedures, which limit the sample throughput.

Donato et al. [11] and Crespi et al. [9] have described several fluorescence assays for rapid measurements of P450 activities in intact cells or in microsomes containing individual P450 isoforms. The assays are based on the direct incubation of monolayers of cells or microsomes that contain cDNA-expressed P450s with a fluorogenic substrate followed by fluorometric quantification of the product formed. 7-Methoxy-4-trifluoromethylcoumarin (MFC) has been reported to be a marker substrate for CYP2C9 [9]. In the present work, we aimed to determine both the relative catalytic selectivity of several human P450s during *O*-demethylation of MFC (Fig. 1) and the applicability of MFC as a probe substrate for CYP2C9.

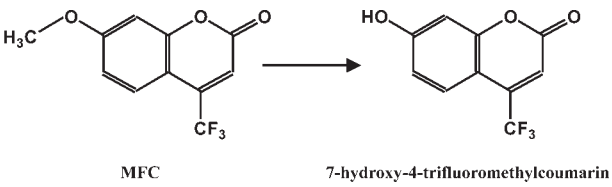

Fig. 1. 7-Methoxy-4-trifluoromethylcoumarin (MFC) *O*-demethylation

Materials and Methods

Chemicals

α-Naphthoflavone, 8-methoxypsoralen, sulfaphenazole, tranylcypromine, quinidine, diethyl-dithiocarbamate, ketoconazole, pentoxyresorufin, mephenytoin, chlorzoxazone, midazolam, nifedipine, MFC and its metabolite, 7-hydroxy-4-trifluoromethylcoumarin were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Coumarin, D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase, methanol, dichloromethane, and acetonitrile of HPLC grade were obtained from Merck (Darmstadt, Germany). Tolbutamide was from Research Biochemicals International (Natick, MA, USA). All other chemicals used in this study were from Reanal Finechemical Co. (Budapest, Hungary).

Preparation of human liver microsomes

Human liver tissues from kidney transplant donors were from the Transplantation and Surgical Clinic, Semmelweis University (Budapest, Hungary). Per-

mission of the Hungarian Regional Committee of Science and Research Ethics was obtained to use human tissues for scientific purposes. Human liver tissues were homogenized in a 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 154 mM KCl. The hepatic microsomal fraction was prepared by differential centrifugation [35]. All procedures of preparation were performed at 0–4°C. Protein content of microsomes was determined by the method of Lowry et al. [22], using bovine serum albumin as the standard.

Microsomal P450 enzyme assays

Published methods were followed to determine the following selective enzyme activities: coumarin 7-hydroxylation for CYP2A6 [27], mephenytoin *N*-demethylation for CYP2B6 [15], tolbutamide 4-hydroxylation for CYP2C9 [24], mephenytoin 4'-hydroxylation for CYP2C19 [31], chlorzoxazone 6-hydroxylation for CYP2E1 [26], nifedipine oxidation [13], midazolam 4- and 1'-hydroxylation [18] for CYP3A4/5. The incubation mixture contained the NADPH-generating system (1 mM NADPH, 10 mM glucose 6-phosphate, 5 mM $MgCl_2$ and 2 units/ml glucose 6-phosphate-dehydrogenase), human liver microsomes and the various selective substrates for P450 forms (coumarin, mephenytoin, tolbutamide, chlorzoxazone, midazolam or nifedipine). P450 enzyme assays were performed in triplicate and the rates of enzyme activities were determined under linear conditions for the microsomal protein concentration and for the incubation time. Reactions were terminated by the addition of ice-cold methanol. HPLC and fluorometric analyses were performed according to published methods [13, 15, 18, 24, 26, 27, 31].

Determination of MFC *O*-demethylation activity

MFC *O*-demethylation activity was determined in human liver microsomes from thirty-two donors and in microsomes containing cDNA-expressed P450 enzymes (Supersomes CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2E1, CYP3A4 obtained from BD Bioscience, Woburn, MA, USA). The incubation mixture contained the NADPH-generating system (1 mM NADPH, 10 mM glucose 6-phosphate, 5 mM $MgCl_2$ and 2 units/ml glucose 6-phosphate-dehydrogenase), human liver microsomes or cDNA-expressed P450s and MFC at various concentrations (3.25–100 μM). After incubating for 10 to 30 min, the reactions were terminated by the addition of ice-cold methanol. The amount of 7-hydroxy-4-trifluoromethylcoumarin produced was determined by fluorometric analysis at an excitation wavelength of 405 nm and an emission wavelength of 535 nm using an RF-5301PC fluorometer (Shimadzu, Kyoto, Japan). Kinetic data were calculated according to the procedures by the graphical analysis of Hanes' plots and the results are expressed as the mean ± SD of three independent experiments.

Inhibition studies

Inhibition of MFC *O*-demethylation was carried out in the absence or presence of various P450 inhibitors (α-naphthoflavone for CYP1A2, 8-methoxypsoralen for CYP2A6, pentoxyresorufin for CYP2B6, sulfaphenazole for CYP2C9, tranylcypromine for CYP2C19, quinidine for CYP2D6, diethyl-dithiocarbamate for CYP2E1, ketoconazole for CYP3A4/5) [3, 25, 29]. For those P450 inhibitors that were able to significantly decrease MFC *O*-demethylation, the K_i values (inhibition constants) were determined by using different concentrations of MFC and inhibitors (0.1–200 μM). The K_i values were calculated from Dixon plots of velocity^{-1} versus inhibitor concentration at the three MFC concentrations. The apparent K_i values were estimated from the intercept of the three lines of Dixon plots and expressed as the mean ± SD of the intercepts.

Statistical analysis

Correlation analyses between hepatic microsomal P450 activities and MFC *O*-demethylation were performed by GraphPad InStat software, version 3.05 (GraphPad Software, San Diego, CA). Statistically significant contribution of respective P450 activity to MFC *O*-demethylation was considered if the p value of multiple correlation analysis was lower than 0.01.

Results

The main goals of the present study were to investigate both the catalytic selectivity of CYP2C9 and the relative contribution of human P450s to MFC *O*-demethylation. Our further aim was to estimate the applicability of MFC as a fluorogenic probe substrate for CYP2C9, using human liver microsomes and recombinant enzymes.

Michaelis-Menten kinetics of MFC *O*-demethylation

Michaelis-Menten kinetic parameters for the fluorescence assay of MFC *O*-demethylation were determined using human liver microsomes. MFC was intensively demethylated with an apparent K_m value of 7.76 ± 0.73 μM in human liver microsomes. The Michaelis-Menten constant for hepatic microsomes was much lower than for microsomes containing cDNA-expressed CYP2C9 (55 μM [33]) or in cells expressing CYP2C9 (50.7 μM [11]). The maximal rate of MFC *O*-demethylation, determined in the hepatic microsomes of thirty-two human donors, was found to be 778.5 ± 471.4 pmol/mg protein/min (range: 164.6–2173.1 pmol/mg protein/min).

Involvement of various P450 enzymes in *O*-demethylation of MFC

To estimate the involvement of P450 enzymes in MFC *O*-demethylation, we followed the following three approaches: i) a series of P450-selective inhibitors was examined with MFC *O*-dealkylation, ii) correlation analyses between MFC *O*-demethylation and classical P450 marker reactions were performed in human liver microsomes from thirty-two donors, and iii) MFC biotransformation was investigated in microsomes containing cDNA-expressed individual P450s (Supersomes).

The participation of P450 enzymes in MFC *O*-demethylation was determined using well known chemical inhibitors selective for P450 enzymes. A significant decrease in MFC *O*-demethylation was observed in the presence of sulfaphenazol, which is selective for CYP2C9. This is fairly consistent with the preceding studies that reported MFC to be a probe substrate for CYP2C9 [9]. However, 8-methoxypsoralen (for CYP2A6), pentoxyresorufin (for CYP2B6), tranylcypromine (for CYP2C19), diethyl-dithiocarbamate (for CYP2E1), and ketoconazole (for CYP3A4/5) were also able to substantially reduce the microsomal MFC *O*-demethylation activity. The inhibition constants (apparent K_i values) of these compounds were found to be in a micromolar range similar to that of sulfaphenazole (Tab. 1). The inhibition of CYP1A2 and CYP2D6 (by α-naphthoflavone and quinidine, respectively) had no effect on the *O*-demethylation step of MFC metabolism. These results indicate the involvement of several P450s (CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4/5) in microsomal MFC *O*-demethylation. It should be noted that a high concentration of diethyl-dithiocarbamate (> 100 μM) also inhibits other P450s (CYP1A2, CYP2A6, CYP2B6 and CYP3A4). However, decreasing the concentration of diethyl-dithiocarbamate to 10 μM leads to the inhibition of CYP2A6 and CYP2B6 [5]. Therefore, additional studies were required to evaluate whether the inhibition of MFC *O*-dealkylation by diethyl-dithiocarbamate indicates the participation of CYP2E1 or/and some other P450s (e.g., CYP2A6, CYP2B6).

Tab. 1. Inhibition of MFC *O*-demethylation by selective P450 inhibitors

Inhibitor	K_i value (μM)
α-Naphthoflavone (CYP1A2)	no inhibition
8-Methoxypsoralen (CYP2A6)	17.62 ± 5.935
Pentoxyresorufin (CYP2B6)	12.31 ± 6.89
Sulfaphenazole (CYP2C9)	4.14 ± 2.835
Tranylcypromine (CYP2C19)	15.31 ± 2.994
Quinidine (CYP2D6)	no inhibition
Diethyl-dithiocarbamate (CYP2E1)	11.46 ± 1.242
Ketoconazole (CYP3A4/5)	15.77 ± 6.263

Microsomal enzyme assays with substrates that are selective for the individual P450s (coumarin 7-hydroxylation of CYP2A6, mephenytoin *N*-demethylation for CYP2B6, tolbutamide 4-hydroxylation for CYP2C9, mephenytoin 4-hydroxylation for CYP2C19, chlorzoxazone 6-hydroxylation for CYP2E1, nifedipine oxidation, midazolam 1'- and 4-hydroxylation for CYP3A4/5) were used to characterize the human enzymes that are responsible for the majority of oxidative drug metabolism. MFC *O*-demethylation activity and P450 selective marker reactions were compared in a panel of liver microsomes from thirty-two individual donors. The *O*-dealkylase activity for MFC did not display a strong correlation ($r = 0.5461$) with tolbutamide 4-hydroxylation, which is considered to be the marker reaction of CYP2C9 (Fig. 2A) [24]. On the other hand, the results of simple correlation analysis predicted a significant correlation between MFC *O*-demethylation and mephenytoin *N*-demethylation activity of CYP2B6 or chlorzoxazone 6-hydroxylation of CYP2E1 ($r > 0.75$, $p < 0.0001$) (Fig. 2). Although a statistically significant correlation was also observed between MFC *O*-dealkylation and midazolam

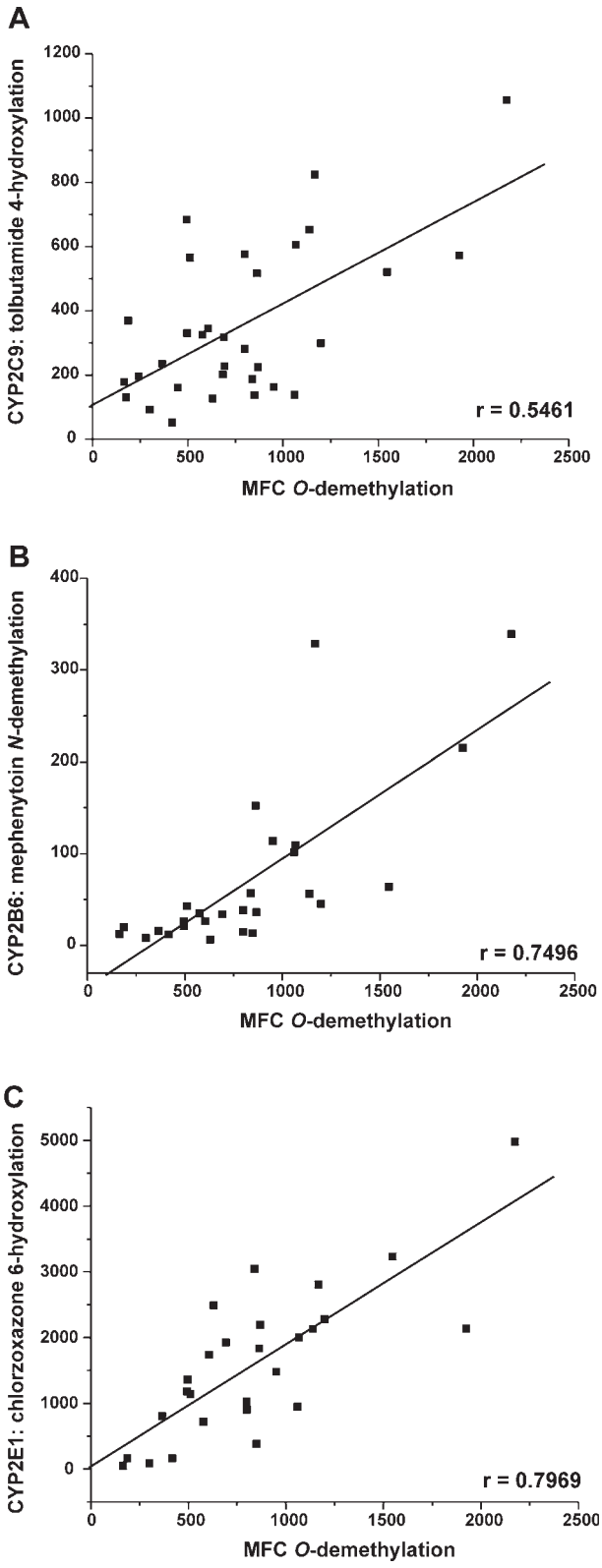

Fig. 2. Correlation between MFC *O*-demethylation activity and P450 marker activities of CYP2C9 **(A)**, CYP2B6 **(B)** and CYP2E1 **(C)**. Enzyme activities were determined using human liver microsomes from thirty-two donors. Activities are expressed as mg protein/min

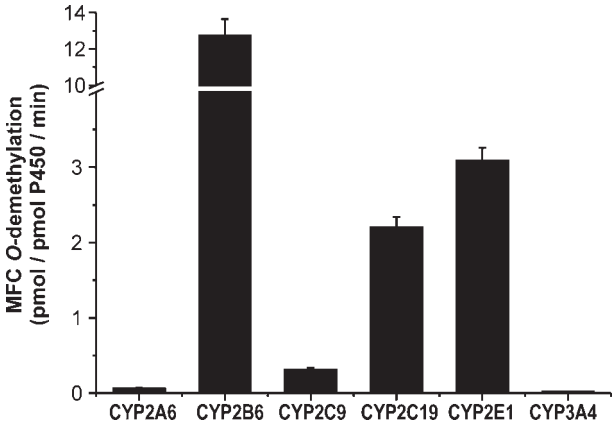

Fig. 3. MFC *O*-demethylation activity of cDNA-expressed P450s

1'-hydroxylation of CYP3A4/5, the other CYP3A4/5 activities (midazolam 4-hydroxylation and nifedipine oxidation) did not correlate with MFC *O*-demethylation. These findings were confirmed by the results of multiple correlation analyses accounting for the potential participation of all P450s investigated. Since the overall p value of the multiple correlation model was extremely low ($p < 0.0001$), the question arose which individual P450 activity was influencing MFC *O*-dealkylation. Significant contributions of CYP2B6 and CYP2E1 to MFC *O*-demethylation were indicated by the multiple model ($p < 0.01$). Although ketoconazole (selective for CYP3A4) inhibited MFC *O*-demethylation at a K_i of 15.77 μM, the results of the correlation analysis did not support the significant contribution of CYP3A4. The results of MFC *O*-dealkylation in cDNA-expressed P450 enzymes also assumed that the participation of CYP3A4 in this reaction was rather questionable.

With the use of the panel of cDNA-expressed human P450 enzymes, MFC was found to be demethylated with the highest turnover number (12.74 ± 0.92 / min) by CYP2B6 (Fig. 3). However, MFC did not seem to be selective for CYP2B6. cDNA-expressed CYP2E1 and CYP2C19 showed some preference for MFC, but these enzymes metabolized MFC at a rate about four to six times lower than observed for CYP2B6. Although CYP2C9 could also *O*-dealkylate MFC, it did not seem to be efficient in MFC metabolism (0.31 ± 0.026 / min). Furthermore, MFC was hardly considered to be a high affinity substrate for cDNA-expressed CYP2A6 and CYP3A4. The results

obtained in microsomes containing cDNA-expressed individual P450s confirmed some of the findings of *in vitro* inhibition studies and of the following correlation analyses: i) MFC was found to be a nonselective P450 substrate; ii) CYP2B6 and CYP2E1 potently contributes to MFC *O*-demethylation; iii) and our observations did not support a primary role for CYP2C9 in MFC *O*-dealkylation.

Discussion

Analysis of P450-mediated drug metabolism and drug interactions has become one of the early tasks in the drug development processes. Various *in vitro* assay methodologies have been developed to estimate metabolic drug interactions [6, 10]; however, these require time- and labor-intensive analytical tools, such as HPLC (UV) or LC-MS, which present a major limitation to their use in high-throughput P450-inhibition studies [14, 17]. Assays that make the use of P450 specific substrates that produce fluorescent metabolites, do not require metabolite separation and facilitate higher sample throughput. In connection with this, an important question may arise whether fluorescence-based assays can offer a reliable replacement of the conventional P450-selective marker reactions. Fluorometric assays for CYP1As and CYP2Bs using *O*-alkyl derivatives of resorufin [4] or coumarin for CYP2A6 [27] have been reported to be useful probes for this purpose. Attempts have been made to develop novel fluorogenic substrates for CYP2C9, CYP2D6 and CYP3A4 [8]. Some probes (e.g., 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin selective for rat CYP2D2 or human CYP2D6) can be used in heterogeneous enzyme systems, such as liver microsomes or primary cultures of hepatocytes, whereas 7-benzyloxyquinoline or 7-benzyloxy-4-trifluoromethylcoumarin are suitable for high throughput testing of CYP3A4 only in microsomes which individually express recombinant P450 enzymes [32].

Several authors [1, 2, 12, 33] applied MFC *O*-dealkylation as a marker reaction for human CYP2C9 in enzyme kinetic or high throughput inhibition studies. The applicability of MFC for high throughput screening has been proposed, although with some limitations. MFC *O*-demethylation has been reported to be catalyzed by CYP2C9 in microsomes containing cDNA-expressed P450 [12, 33] or in intact cells expressing individual P450s [11]. CYP2E1 has also been assumed to contribute to *O*-demethylation of MFC; however, the K_m value for CYP2E1 seems to be much higher (approximately 200 μM) than for CYP2C9 (55 μM) [33]. The present work describes the relative selectivity of MFC towards human P450s in liver microsomes and estimates the contribution of various P450 isoforms to MFC *O*-dealkylation.

The reliability of MFC *O*-demethylation as a fluorogenic probe for CYP2C9 was determined by several approaches, including the use of biological systems of human liver microsomes and cDNA-expressed individual P450 enzymes. The effect of chemical inhibitors that are selective for major drug-metabolizing P450s was tested in human liver microsomes [3, 25, 29]. MFC *O*-demethylation and P450 marker activities for correlation analysis were also performed using the heterogeneous enzyme system. Furthermore, MFC *O*-dealkylation was investigated in microsomes containing cDNA-expressed individual P450s. The results of P450 inhibition studies supported the potential participation of several P450 enzymes (CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4/5) in MFC *O*-dealkylation in human liver microsomes. This data suggested that MFC *O*-demethylation activity cannot be considered a selective fluorescent marker reaction for CYP2C9 in biological systems expressing several P450s. Correlation analysis between MFC *O*-demethylation and classical P450 marker activities provided further evidence for the contribution of CYP2B6 and CYP2E1, whereas some slight contribution of CYP2A6, CYP2C9 and CYP3A4/5 might be assumed.

Fairly good agreement with the results of inhibition studies and correlation analysis between MFC *O*-demethylation and classical P450 marker reactions using liver microsomes was found for MFC metabolism by cDNA-expressed CYP2B6 and CYP2E1. CYP2B6 seemed to be the most active in MFC *O*-demethylation. CYP2E1 and CYP2C19 also contributed to MFC metabolism to some extent; however, the results obtained in microsomes expressing individual P450s did not confirm the effective participation of CYP2C9 and CYP3A4. In liver microsomes, MFC *O*-demethylation by an individual isoform depends on both the catalytic activity and the relative contribution of the isoform to the total P450 content [30]. We attempted to roughly estimate the contribution of P450 isoforms to MFC *O*-dealkylation in liver microsomes on the basis of enzyme activities in cDNA-expressed Super-

Tab. 2. Estimation of P450-isoform contribution to the MFC *O*-dealkylation in human liver microsomes

P450	Relative contribution of the isoform to the total P450 content in liver[a]	Predicted velocity in liver microsomes (pmol hydroxy-FC/pmol of total P450/min)[b]	Relative contribution of the isoform to MFC *O*-demethylation in liver microsomes (%)
CYP1A2	0.127	–	–
CYP2A6	0.04	0.0024	0.701
CYP2B6	0.002	0.0255	7.438
CYP2C9	0.156	0.0484	14.135
CYP2C19	0.026	0.0573	16.721
CYP2D6	0.015	–	–
CYP2E1	0.066	0.2037	59.473
CYP3A4	0.288	0.00525	1.533

[a] Data according to Shimada et al. [30]; [b] Hydroxy-FC stands for 7-hydroxy-4-trifluoromethylcoumarin

somes (Tab. 2). Accordingly, the contribution of CYP2E1 to the *O*-demethylation in liver microsomes was relatively greater than that of CYP2B6, even if the catalytic activity of CYP2B6 was higher than that of CYP2E1. In regards to the other investigated P450 isoforms, CYP2C9 and CYP2C19 contributed to a lesser, but not negligible degree. Since the results of the inhibition studies did not indicate the participation of CYP1A2 and CYP2D6 in MFC *O*-demethylation, the relative contribution of these enzymes were not taken into account. Although the estimation of the relative contributions of various P450s could be considered rather rough, the main player(s) in MFC *O*-dealkylation could be identified.

In conclusion, our findings suggest that MFC cannot be considered to be a selective substrate for human CYP2C9. Nonselective substrates are not appropriate for *in vitro* models expressing several P450s, such as liver microsomes or primary hepatocytes. This limitation can be addressed by the use of individually expressed P450 enzymes, although MFC did not seem to be a high affinity substrate for CYP2C9. Our results verify that several P450s are involved in MFC *O*-demethylation. Moreover, CYP2E1 may be considered as the primary catalyst. In the aggregate, it is possible to use MFC as a fluorogenic substrate for CYP2B6 and CYP2E1 in systems expressing individual P450s; MFC *O*-demethylation, however, does not seem to be applicable for studies in heterogeneous systems.

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