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Determination of Cytochrome P450 Metabolic Activity Using Selective Markers

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1. Introduction

Cytochrome P450 enzymes (CYP) play a pivot role in phase I of xenobiotic biotransformation. Many of CYP enzymes are known to be polymorphic, with many allelic variants. As a consequence of huge number of possible allele combinations, a wide range of metabolic activity can be observed among patients in population. Individual CYP activity may be also affected by various xenobiotics – either induction or inhibition of distinct CYP isoenzyme may cause failure or toxicity of pharmacotherapy. Due to this, some prediction of metabolic activity is worthwhile in clinical practice. This chapter is going to briefly summarize the possibilities of *in vivo* and *in vitro* CYP metabolic activity assessments of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 enzymes.

2. *In vivo* enzyme activity assessment

2.1 Clinical need for CYP phenotyping

It is useful to assess CYP metabolic activity prior to or during the pharmacotherapy and to adjust the individual dosage according to the patient's phenotype at least in some cases.

The best studied CYP enzyme concerning polymorphisms, enzyme inhibition and dosage individualization according to phenotype is probably CYP2D6, followed by CYP2C9 and CYP2C19. These enzymes are highly polymorphic, contrary to CYP3A4, where the metabolic activity may vary due to differences in CYP3A4 gene expression and pharmacokinetic interactions [Ingelman-Sundberg 2004]. Nevertheless, any shift in metabolic capacity of individual CYP enzyme may result either in decreased or increased therapeutic response or intensity of adverse effects. For example, after paroxetine administration to the patients on tamoxifen, a decrease in plasma levels of active metabolite of tamoxifen was detected [Stearns et al. 2003]. This can be crucially important in breast cancer treatment. On the other hand, CYP2D6 ultrarapid metabolizer phenotype may cause failure of pharmacotherapy due to the very low and thus ineffective drug plasma levels [Corruble 2008]. In antipsychotic treatment, an association has been observed between extrapyramidal adverse effects and CYP2D6 genotype [Fleeman et al. 2011]. Significant clinical consequences of CYP2D6 genotype or enzyme inhibition were described also in beta-blockers, antianginal, antiarrhythmic drugs, antihistamines and antiemetics. The clinical

impact of enzyme polymorphism or changes due to inhibition or induction usually depends on the contribution of other CYP forms to the total drug's elimination. By this, the relative therapeutic potency of the parent drug or any of its metabolites may be altered [Zhou 2009].

Methodological approaches such as assessment of metabolic ratio of specific substrate to metabolite(s) in saliva, plasma / serum or urine are most widely used to assess metabolic activity *in vivo*. Besides determination of the concentrations of probe and metabolite in biological fluids, specific substrates are used in various breath tests. Rarely some other approaches are used, e.g. the pupillometry after opioid administration.

It is essentially important to differentiate between *in vivo* and *in vitro* metabolic activity assessments, as they show very often discrepant results.

One of the greatest advantages of genotype assessment is that it does not need to be repeated because it does not change with time or under the simultaneous influences of drugs and other factors. On the other side the disadvantage of the pharmacogenetic testing is, that genotype does not always correlate with observed metabolic activity recorded using probe drug(s). This discrepancy may be caused by various epigenetic factors as well as by inhibition or induction of enzyme metabolic activity caused by other xenobiotics coadministered.

2.2 Conventional probe substrates for *in vivo* metabolic activity assessment

Metabolic activity of various CYP enzymes is most often assessed using selective substrate of distinct CYP enzyme ("marker of metabolic activity"), i.e. a drug (or substance) which is ideally metabolized by the single CYP enzyme [Pelkonen et al. 1998, Pelkonen et al. 2008]. Ideal marker should be a non-toxic substance, with regard to its possible usage *in vivo*. Moreover, such ideal marker should be easily available substance (i.e. registered as a therapeutic drug), which is assessable in biological fluids together with its main metabolite(s). Pharmacokinetics of ideal marker should be determined by metabolism and not by intensity of liver perfusion, protein binding or elimination of unchanged drug [Frank et al. 2007, Kivisto & Kroemer 1997, Pelkonen et al. 1998]. Phenotype classification *in vivo* is based on the drug to metabolite concentration ratio in biological fluids (metabolic ratio): $MR = [C_{drug}/C_{metabolite}]$. With regard to the phenotype (or MR), metabolizers can be classified into 4 different categories – poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM) and ultrarapid metabolizers (UM) [Zanger et al. 2004]. Bimodal or trimodal distribution of log-transformed MR is observed in some probe substrates. Histograms of log-transformed MR may refer to cut-off values of MR which distinguish EM from UM, PM or IM.. Doses of medication may be adjusted according to the current phenotype, following the principles of personalized medicine. Probe drugs with unimodal distribution of their MRs cannot reflect genetically encoded differences in the metabolism. Despite this, such probe substrates have also been used for CYP metabolic activity prognosis, but with varying degree of predictive success [Benet 2005]. Some of the probe drugs are metabolized by multiple CYPs, as observed in warfarin or caffeine [Kaminsky & Zhang 1997, Tassaneeyakul et al. 1994]. Moreover, metabolic fate of some substrates may be enantiomer specific: *R*-warfarin is primarily metabolized by CYP1A2 and CYP3A4, *S*-warfarin by CYP2C9 [Kaminsky & Zhang 1997]. Despite this, the major metabolic pathway may serve as a tool for distinct isoenzyme metabolic activity assessment (*S*-warfarin for CYP2C9 phenotype assessment). Phenotype expressed as MR is basically determined by the

genotype, but may be also influenced by age [Kamali et al. 2004], sex [Nafziger et al. 1998], habits [Bozikas et al. 2004], co-medication and/or liver disease [Rost et al. 1995].

The above mentioned principles are also utilized in drug development, where drugs are evaluated concerning their CYP-mediated interactions prior to the registration and launch on the basis of the enzyme-specific reaction [Zlokarnik et al. 2005].

2.2.1 CYP1A2

Many substrates have been tried for CYP1A2 metabolic activity assessment, but caffeine is the most widely used one, although other enzymes are involved in biotransformation of caffeine and its metabolites (xanthinoxidase, N-acetyl transferase and with lesser extent also CYP2E1, CYP2A6, CYP3A4, CYP3A5) [Dorne et al. 2001, Tassaneeyakul et al. 1994]. Many metabolic ratios were used in phenotyping studies, with "caffeine metabolic ratio" (CMR) as the well determined marker of CYP1A2 metabolic activity [Hakooz 2009]. On the other hand, simple paraxanthine to caffeine molar concentration ratio assessed in serum/plasma or saliva is also used [van Troostwijk et al. 2003]. Other substrates are used much rarely in phenotyping studies. Probes for CYP1A2 metabolic activity assessment are summarized in Table 1. Moreover, various concentration ratios of caffeine and its metabolites are also used for determination of NAT2 (arylamine N-acetyltransferase), CYP2A6 and xanthinoxidase metabolic activities [Begas et al. 2007, Hakooz 2009, Nyeki et al. 2001].

Probe	CYP1A2-specific reaction
caffeine	N-demethylation
phenacetine	O-deethylation
theophylline	N-demethylation

Table 1. Probes for CYP1A2 metabolic activity assessment [Ou-Yang et al. 2000, Takata et al. 2006]

2.2.2 CYP2C9

CYP2C9 is known to be polymorphic, with more than 40 alleles identified [Ingelman-Sundberg et al. 2011]. Ten years ago it was thought that with CYP2C9 genotyping it will be possible to avoid adverse reactions in patients receiving warfarin [Ma et al. 2002]. These expectations were mostly calmed by reality, that CYP2C9 genotype is not the single major factor influencing warfarin toxicity.

Most often used CYP2C9-specific reactions are summarized in the Table 2. Diclofenac 4'-hydroxylation and tolbutamide methylhydroxylation [Zhou et al. 2009] seem to be the most frequently used, however, tolbutamide methylhydroxylation is also catalyzed by CYP2C19 [Wester et al. 2000]. Phenotyping with the use of substrates with low therapeutic index (phenytoin, oral anticoagulants) would be unsafe since the enzyme activity in CYP2C9*3 homozygotes is nearly absent, and these subjects could suffer from drug toxicities or adverse drug reactions [Zhou et al. 2009]. *In vivo* biotransformation of celecoxib, a cyclooxygenase (COX)-2 inhibitor, is affected by CYP2C9 polymorphisms [Guengerich 2005] and thus is object of interest as a potential probe substrate. CYP2C9 protein levels correlate with hexobarbital C3-hydroxylation activity [Kato et al. 1992] and Rendic mentions hexobarbital among CYP2C9 probe substrates [Rendic 2002].

Probe	CYP2C9-specific reaction
diclofenac	4'-hydroxylation
losartan	oxidation
phenytoin	4'-hydroxylation
S-flurbiprofen	4'-hydroxylation
S-warfarin	7-hydroxylation
tolbutamide	methylhydroxylation

Table 2. Probes for CYP2C9 metabolic activity assessment [Zhou et al. 2009]

2.2.3 CYP2C19

Following CYP2D6 and CYP2C9, CYP2C19 is the third well investigated polymorphic drug-metabolizing enzyme. CYP2C19 metabolizes many psychoactive drugs. Many of them are able to inhibit CYP2C19 metabolic activity. Probes for CYP2C19 metabolic activity assessment are summarized in Table 3.

Probe	CYP2C19-specific reaction
chloroguanine (proguanil)	conversion to cycloguanine
omeprazole	5-hydroxylation
S-mephenytoin	4'-hydroxylation

Table 3. Probes for CYP2C19 metabolic activity assessment [FDA 2006, Nolin & Frye 2003, Tenneze et al. 1999]

2.2.4 CYP2D6

Suitable substrates for *in vivo* CYP2D6 phenotyping are listed in the Table 4. Among them, bufuralol and dextromethorphan are the most preferred substrates for *in vitro* preclinical studies [Zhou 2009]. Tramadol is metabolized to *O*-demethyltramadol (M1) by CYP2D6, but in studies *in vitro* was shown that M1 formation is mediated also by CYP2B6 in high extent. Moreover, correlation of tramadol/M1 metabolic ratio with MR of dextromethorphan/dextrorphan is modest and therefore tramadol use as a probe substrate is limited [Frank et al. 2007]. Since debrisoquine and sparteine are currently not available as registered drugs, dextromethorphan remains the most widely used probe drug for CYP2D6 metabolic activity assessment *in vivo* [Zhou 2009].

Probe	CYP2D6-specific reaction
codeine	<i>O</i> -demethylation
debrisoquine	4-hydroxylation
dextromethorphan	<i>O</i> -demethylation
metoprolol	α -hydroxylation
sparteine	dehydrogenation
tramadol	<i>O</i> -demethylation

Table 4. Probes for CYP2D6 metabolic activity assessment [Rasmussen et al. 1998, Rendic 2002, Zanger et al. 2004, Zhou 2009].

2.2.5 CYP3A4

CYP3A4 is the predominant enzyme of CYP3A subfamily and plays the pivot role in drug metabolism [Guengerich 2005]. Hepatic CYP3A4 metabolizes about 50 % of clinically used drugs [Guengerich 1999] and CYP3A4 is the most abundant intestinal CYP enzyme [Guengerich 2005]. CYP3A4 is not supposed to be polymorphic as e.g. CYP2D6, but CYP3A4 activity may vary among individuals from 5 up to 50 fold [Ma et al. 2002]. Metabolic activity of CYP3A4 (expressed as log MR) has unimodal distribution; variation is probably a consequence of both genetic and non-genetic factors [Guengerich 1999, Ozdemir et al. 2000, Shimada et al. 1994, Wilkinson 1996]. Common probes for CYP3A4 metabolic activity assessment are listed in the Table 5.

Probe	CYP3A4-specific reaction
alfentanil	demethylation
alprazolam	4-hydroxylation
codeine	O-demethylation
cortisol	6- β hydroxylation
dapsone	N-hydroxylation
dextromethorphan	N-demethylation
erythromycin	N-demethylation
lidocaine	N-deethylation
midazolam	1-hydroxylation
nifedipine	oxidation
quinidine	3-hydroxylation /N-oxidation
testosterone	6- β hydroxylation
triazolam	1-hydroxylation

Table 5. Probes for CYP3A4 metabolic activity assessment [Liu et al. 2007, Rasmussen et al. 1998, Rendic 2002, Wennerholm et al. 2005]

Since none of suggested probes for CYP3A4 phenotyping is metabolized uniquely by this enzyme, and the active site of CYP3A4 is thought to be large and able to bind multiple substrates simultaneously, it is recommended to use at least 2 structurally unrelated probe substrates for precise enzyme activity evaluation [Ekroos & Sjogren 2006, Foti et al. 2010, Liu et al. 2007]. The apparent metabolic activity of CYP3A4 (assessed using probe substrates) may be affected by P-glycoprotein mediated decrease of availability of probe substrates [Guengerich 2005].

By this, some authors suggest that there are no useful CYP3A4 substrates for accurate prediction of its metabolic activity [Benet 2005].

2.3 Differentiation between poor and extensive metabolizers: MR cut-off values

2.3.1 CYP1A2

Caffeine is the most frequently used probe drug and several metabolic ratios are used for CYP1A2 phenotyping. Urinary MR of (AFMU + 1U + 1X)/17U (also named as caffeine metabolic ratio, CMR) is probably mostly utilized [Campbell et al. 1987], followed by urinary MR of (17X + 17U)/137X [Muscat et al. 2008, Schrenk et al. 1998], salivary and

plasmatic MR of 17X /137X [Fuhr et al. 1996, Simon et al. 2001] or MR of (AFMU + 1U + 1X + 17U + 17X)/137X in serum [Aklillu et al. 2003]. Due to several MRs used, there is not the only one, clear, and widely accepted MR cut-off value distinguishing poor and extensive metabolizers. Furthermore, bimodal or trimodal distribution of MR may be observed depending on the kind of ratio used [Muscat et al. 2008]. Using MR of (AFMU + 1U + 1X + 17U + 17X)/137X in serum, log MR of 0.96 (corresponding MR = 9.12) was found to distinguish PM from EM [Aklillu et al. 2003]. Other authors assessed CYP1A2 phenotype using molar ratio of 17X/137X in the 4 h urine samples, and observed a bimodal distribution with a cut point of 1.85 separating poore and extensive phenotypes [Muscat et al. 2008]. Histograms of serum 17X/137X ratio indicated the antimode of 0.16 [Han et al. 2001].

Metabolic ratio of AFMU/(AFMU +1U +1X) with an apparent antimode at 0.25 may serve for NAT2 phenotyping - subjects with metabolic ratios < 0.25 were then classified as slow acetylators and those with metabolic ratios > 0.25 as fast acetylators [Begas et al. 2007, Bendriss et al. 2000]. Some other authors suggest and use antimode of 0.34 of the same ratio [Nyeki et al. 2001, Tang et al. 1991].

Total salivary caffeine assessment (TOSCA) as a measure of general liver function was used for identification of patients with liver cirrhosis, with a cut-off value of 4.2 µg/ml, but TOSCA was not used for CYP1A2 phenotyping [Tarantino et al. 2006].

*abbreviations

137X	= 1,3,7 trimethylxanthine or caffeine
17X	= 1,7-dimethylxanthine or paraxanthine
AFMU	= 5-acetylamino-6-formylamino-3-methyluracil
1U	= 1-methyluric acid
1X	= 1-methylxanthine
17U	= 1,7-dimethyluric acid

2.3.2 CYP2C9

Phenytoin: Phenytoin hydroxylation index (amount of phenytoin administered/0-32 hr urinary output of hydroxyphenytoin) seems to be bimodally distributed in population [Horsmans et al. 1997]. MR of 4-hydroxyphenytoin/phenytoin reveals a bimodal distribution although substantial overlap of this MR was seen between genetic variants of CYP2C9 [Aynacioglu et al. 1999]. Nevertheless, clear cut-off MR values are not suggested.

Tolbutamide: Tolbutamide metabolism appears to be depend on CYP2C9 genotype [Lee et al. 2005], but there was not suggested a clear MR (tolbutamide/4-hydroxytolbutamide) cut-off value to distinguish poor and extensive metabolizers.

2.3.3 CYP2C19

Omeprazole: Omeprazole is hydroxylated in position 5 to form 5-hydroxyomeprazole. A bimodal distribution of omeprazole to 5-hydroxyomeprazole metabolic ratio histograms and probit plots is observed. Using above mentioned MR, there was found antimode of 5.6 to distinguish between poor and extensive metabolizers in Chinese population [Wang et al. 2007]. Different antimode of 12.0 distinguishing poor and extensive metabolizers was found when used MR of omeprazole + omeprazole sulfone /5'-hydroxyomeprazole [Rost et al. 1995].

Mephenytoin: Both urinary 4-hydroxymephenytoin and the *S/R* enantiomer ratio of mephenytoin are able to discriminate between extensive and poor metabolizers [Wedlund et al. 1984]. In Chinese population sample, when *S/R* mephenytoin ratio was analyzed, probit plot suggested antimode of 0.8 distinguishing poor and extensive metabolizers [Demorais et al. 1995]. Antimode of 2 % of dose excreted as 4-hydroxymephenytoin has been used to distinguish poor and extensive metabolizers [Demorais et al. 1995]. An antimode of 1.0 in the log-mephenytoin hydroxylation index [\log_{10} (μmol dose *S*-mephenytoin/ μmol 4'-hydroxymephenytoin excreted in 8-h urine)], was used to classify the extensive and poor metabolizers [Yin et al. 2004].

Proguanil: Bimodal distribution of proguanil/cycloguanil MR was observed in Polynesian population with antimode of 10.0 [Wanwimolruk et al. 1998]. The same antimode was reported by Australian study [Coller et al. 1997].

2.3.4 CYP2D6

Dextromethorphan: Eight-hour urinary MR of dextromethorphan to dextrorphan (conjugated + unconjugated) with 0.3 as a cut-off value is well established and used to differentiate between extensive and poor metabolizers [Chladek et al. 2000, Ito et al. 2010, Lotsch et al. 2009, O'Mathuna et al. 2008]. Urinary metabolic ratios based on free compounds (with an antimode of 4.0) also correlated with the conventional MR [Yeh et al. 2003]. Collection of urine during 8 hour (sometimes also 24 h) interval could be demanding process. Therefore alternative procedures have been developed for easier phenotyping. In addition to urine, saliva or plasma samples can also be used for the determination of MR [Chladek et al. 1997]. The serum $\text{MR}_{\text{DEM/DOR}}$ may serve as alternative tool for CYP2D6 phenotyping as also our results indicate tight correlation ($r^2 = 0.87$) exists between $\text{MR}_{\text{DEM/DOR}}$ measured in serum (3h postdose) and $\text{MR}_{\text{DEM/DOR}}$ measured in urine (0-8 h postdose). Another approach has been tried for CYP2D6 phenotyping [Hu et al. 1998], in which a single or multiple-dose controlled-release dextromethorphan tablets were administered. It has been demonstrated that there is a good correlation between MR after single-dose and multiple-dose dextromethorphan as well as between MRs assessed in various kinds of samples [Hu et al. 1998, Yeh et al. 2003]. Plasmatic (or serum) 3, 4 or 6 hour MRs and salivary 2, 3, 4, 5, 6 hour MRs were investigated regarding possibility to discriminate EM from PM and alternatively also from IM [Frank et al. 2007]. However, only 3 studies have determined the cut-off values for plasma or serum MR, namely those of Shiran et al. who determined the value of 0.1 for differentiating between EM and PM (using 3-hour postdose $\text{MR}_{\text{DEM/DOR}}$) [Shiran et al. 2003]. The others, observed antimode of 2.0 to delineate EM from IM (using any time point in steady state) [Yeh et al. 2003], and Kohler et al. found the intercept separating EM from PM to be 0.126 (1-hour postdose $\text{MR}_{\text{DEM/DOR}}$) [Kohler et al. 1997]. These results are in accordance with our results showing a cut-off found for serum $\text{MR}_{\text{DEM/DOR}}$ to discriminate between poor metabolizers from either extensive or extensive+intermediate metabolizers, but not for serum $\text{MR}_{\text{DEM/DOR}}$ to discriminate extensive metabolizers from intermediate metabolizers. On the other hand, it has been suggested, that it is not possible to delineate EM from IM using plasma MR [Hu et al. 1998]. In conclusion, there is a lack of evidence for clear plasma/serum or saliva dextromethorphan MR cut-off values. As the most used and best proved remains the urinary 0-8 h dextromethorphan to dextrorphan MR [Frank et al. 2007], but alternative procedures for phenotyping may be useful in some groups of patients.

Debrisoquine: MR of debrisoquine/4-OH debrisoquine in urine collected for 8 hours serves as a well proved determinant of metabolic status: a bimodal distribution was observed in Caucasian population with antimode of 12.6 separating EM (MR < 12.6) from PM (MR > 12.6) [Eiermann et al. 1998, Sachse et al. 1997]. For debrisoquine, there is not recommended any other sampling interval or other way of phenotypization except of urine 0 - 8h MR.

Sparteine: MR of sparteine/sum of dehydrosparteines in urine collected 0 - 6 h post-dose is used for phenotype assessment, with 20.0 as the conventional cut-off point between PM and EM subjects [Basci et al. 1994, Paar et al. 1997]. This value was suggested to be slightly modified to 14.0 in another study [Halling et al. 2005].

2.3.5 CYP3A4

There is a lack of evidence for existence of a bimodal distribution of CYP3A4 activity and therefore there is no consensus for antimode or cut-off values in any of used probe substrates.

2.4 Breath tests and other approaches for CYP enzyme activity assessment

Erythromycine breath test (EBT) is probably one of the best proved models for CYP3A4 activity assessment. The hepatic CYP3A4 catalyzes *N*-demethylation of [¹⁴C]-erythromycin with subsequent formation of CO₂, and therefore the metabolic activity of this enzyme may be expressed as the amount of the expired ¹⁴CO₂ [Liu et al. 2007, Watkins 1994].

Similar breath tests detecting radiolabeled metabolites (or ratio of ¹⁴CO₂ or ¹³CO₂ to ¹²CO₂) were designed for phynotyping CYP2C19 with use of [¹³C] pantoprazole [Furuta et al. 2009], and CYP1A2 with the use of [¹⁴C] caffeine [Kalow & Tang 1993]. Multiple CYP metabolic activities (CYP2C19, 1A2, 3A4, and 2C9) were evaluated with [¹³C]-aminophenazone ("aminopyrine breath test") [Kodaira et al. 2011]. CYP2E1 activity in rats was measured using [¹⁴C] nitrosodimethylamine as a probe substrate [Bastien & Villeneuve 1998].

2.5 Miotic response to opioids

Significant correlations were observed between alfentanil pharmacokinetic parameters and its induced miotic kinetic parameters either under ambient or dark conditions. This means that pupillary response after alfentanil administration may be used as noninvasive measure of CYP3A4 metabolic activity [Baririan et al. 2005, Kharasch et al. 2004, Klees et al. 2005].

O-demethylation of tramadol has been tried as a probe reaction for CYP2D6 phenotyping [Frank et al. 2007]. Pharmacokinetic parameters of its metabolite, *O*-demethyltramadol (M1) correlates well with pupillary constriction after tramadol administration. Since intrinsic efficacy of M1 is higher than of tramadol, higher pupillary constriction was observed in EM subjects [Slanar et al. 2007]. Interestingly, it was observed not only different patterns of miotic response between PM and EM groups, but also difference in the kinetic parameters (median time to reach max. miosis) between heterozygous and homozygous EM [Slanar et al. 2007]. It seems possible to discriminate EM from PM on the basis of the miotic response after tramadol administration [Slanar et al. 2007].

2.6 Cocktail approaches

In early phase of drug development, there are utilized so called "high-throughput" methods increasing the efficiency and effectiveness of assay to assess metabolic activity of many CYP enzymes in short time [Smith et al. 2007, Testino & Patonay 2003, Zlokarnik et al. 2005]. Investigative methodologies for CYP enzymes often utilize so-called "cocktail" of markers to assess the metabolic activity of multiple CYP forms in one session [Asimus et al. 2007, Frye et al. 1997, Kumar et al. 2007, Smith et al. 2007, Tanaka et al. 2003, Yao, M. et al. 2007, Yao, Y.M. et al. 2007]. This approach is used either for *in vivo* or *in vitro* metabolic assessment. The markers of metabolic activity and their enzyme-specific metabolites can be assessed either simultaneously or separately [Blakey et al. 2004, Sharma et al. 2004]. A well known advantage of the cocktail approach is obtaining information of multiple CYP enzymes in a single experimental session [Tanaka et al. 2003]. Several different cocktails of markers have been used for this purpose (Table 6) [Asimus et al. 2007, Dierks et al. 2001, Sharma et al. 2004, Zhou et al. 2004]; as a "cocktail" is sometimes called any combination of at least 2 probe drugs, but generally from three up to six probes have been tried for *in vivo* CYP phenotyping [Zhou et al. 2004]. Among the most frequently used is e.g. "Pittsburgh cocktail": caffeine, chlorzoxazone, dapsone, debrisoquine, and mephenytoin for the combined assessment of CYP1A2, CYP2E1, CYP2D6, CYP2C19, CYP3A and N-acetyltransferase activities [Zhou et al. 2004]. Unfortunately, the disadvantages of cocktail approach are also well defined: the frequent occurrence of adverse effects due to various pharmacodynamic-based interactions, more sample consumption (collection) for analysis and more complicated analytical methods [Tanaka et al. 2003]. After developing precise analytical methods, the cocktail approach became widely used and is now one of the basic analytical tools in early drug development (*in vitro* cocktails) [Zlokarnik et al. 2005]. Methods utilizing reaction with a marker and subsequent chromatographic analysis are recommended by the FDA in initial drug evaluations for investigating pharmacokinetic interactions with CYP enzymes [FDA 2006] (in contrast to some other high throughput methods). On the other hand, a "cocktail" approach can open a further question - can one marker influence the biotransformation rate of the other one? In some of these "cocktails", it was proved that the rates of biotransformation of single substrates are not affected by other drugs coadministered, but there are some examples that one probe substrate may affect the biotransformation rate of another one. Namely, there were reported an inhibition of midazolam metabolism by chlorzoxazone in humans [Palmer et al. 2001], inhibition of nifedipine aromatization and sparteine dehydrogenation by quinidine in humans [Schellens et al. 1991], increase in phenacetin deethylation by tolbutamide in rats [Jurica et al. 2009]. Therefore, such assay may provide invalid (or at least inaccurate) results [Palmer et al. 2001]. Review of cocktails used *in vivo* is given in the Table 6. Except of these, only few other combinations of probe substrates have been used, e.g. assessment of CYP2A6 metabolic activity (coumarin) with CYP2D6 (dextromethorphan) and CYP2C19 (mephenytoin) [Endres et al. 1996].

3. *In vitro* CYP activity assessment

The specific probe substrates for CYP activity assessment and inhibition studies are used in the preclinical drug development as well as for clinical purpose. Research in this field is carried out on various models (human liver microsomes, recombinant CYP enzymes, hepatocytes, precision-cut human liver slices, organ perfusions etc.) which enables use of wide range of techniques and model substrates (e.g. luminometric, fluorometric,

Note	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4	Ref.
GW cocktail	caffeine	diclofenac	mephenytoin	debrisoquine	chlorzoxazone	midazolam	[Scott et al. 1999]
Karolinska cocktail	caffeine	diclofenac losartan	mephenytoin omeprazole	debrisoquine		midazolam quinine	[Palmer et al. 2001] [Christensen et al. 2003]
Coperstown 5+1 cocktail	caffeine	warfarin + vit. K tolbutamide	mephenytoin omeprazole	debrisoquine dextromethorphan	chlorzoxazone	dapsone midazolam	[Frye et al. 1997] [Shelepova et al. 2003]
Coperstown cocktail	caffeine	tolbutamide	mephenytoin	dextromethorphan		midazolam	[Bruce et al. 2001, Gorski et al. 2000, Wang et al. 2001]
	caffeine	tolbutamide	omeprazole	dextromethorphan		midazolam	[Tomalik-Scharte et al. 2005]
	caffeine	tolbutamide	omeprazole	dextromethorphan		midazolam	[Streetman et al. 2000]
	caffeine	tolbutamide	omeprazole	dextromethorphan		dapsone midazolam midazolam	[Gupta et al. 2011] [Zhang et al. 2010] [Ardjomand- Woelkart et al. 2011]
used in rats	theophylline	tolbutamide tolbutamide tolbutamide	mephenytoin	dextromethorphan dextromethorphan dextromethorphan		lidocaine	[Tanaka et al. 1995]
used in rats	caffeine	phenytoin	mephenytoin	debrisoquine	trimethadione	dapsone lidocaine	[Black et al. 1992] [Tanaka et al. 1994]
	caffeine		mephenytoin	dextromethorphan		dapsone	[Brockmoller & Roots 1994]
	caffeine	flurbiprofen	mephenytoin	metoprolol		dapsone	[Setiabudy et al. 1995]
	caffeine		omeprazole				[Jenkins et al. 2010]

Table 6. Probe substrate combinations for *in vivo* CYP activity assessment (adapted from [Tanaka et al. 2003, Zhou et al. 2004])

radiolabeled etc.). *In vitro* methods are mostly used in preclinical drug development to predict possible CYP-mediated pharmacokinetic interactions. Unfortunately, there is a consensus that a prediction of *in vivo* interactions from *in vitro* data is not reliable. Moreover, as some authors hypothesize, in case of CYP3A4, any inductive or inhibitory effect observed in study with one probe CYP3A4 substrate can not accurately predict the extent of the *in vivo* interaction for another CYP3A4 substrate [Benet 2005]. FDA recommends suitable probe substrates for *in vitro* experiments in the drug development [FDA 2006]. These substrates are also mentioned in the appropriate tables below.

3.1 Conventional probe substrates used *in vitro*

3.1.1 CYP1A2

Simple *in vitro* spectrophotometric methods like ethoxyresorufin *O*-deethylation (EROD) and methoxyresorufin *O*-demethylation (MROD) are frequently utilized in CYP1A metabolic activity assessment. These probe substrates were used in various models with liver microsomes from human, monkey, rat and mouse [FDA 2006, Hanioka et al. 2000, Chun et al. 1999]. Besides these, conventional probe substrates (registered drugs) are used *in vitro* as well (Table 7).

Probe	CYP1A2-specific reaction
acetanilide	C ₄ -hydroxylation
7-ethoxyresorufin	<i>O</i> -deethylation
7-methoxyresorufin	<i>O</i> -demethylation
caffeine	<i>N</i> -demethylation
phenacetine	<i>O</i> -deethylation
tacrine	1-hydroxylation
theophylline	<i>N</i> -demethylation

Table 7. *In vitro* probes for CYP1A2 metabolic activity assessment [Henderson et al. 2000, Rendic 2002, Yuan et al. 2002]

3.1.2 CYP2C9

Despite that probe substrates are in general supposed to be highly specific and selective towards individual CYP forms, it was reported that with use of (*S*)-warfarin as a CYP2C9 probe, significantly lower *K_i* values were obtained when compared to diclofenac, flurbiprofen, phenytoin or tolbutamide [Foti et al. 2010, Kumar et al. 2006]. Selective probe substrates for CYP2C9 *in vitro* activity assessment are summarized in the Table 8.

3.1.3 CYP2C19

Probe substrates for CYP2C19 activity assessment are summarized in the Table 9. As in the case of CYP2C9, also in CYP2C19 there were reported different sensitivities of probe substrates to inhibitors. (*S*)-mephenytoin was the most sensitive to a set of inhibitors when compared to (*S*)- or (*R*)-omeprazole or (*S*)-fluoxetine [Foti & Wahlstrom 2008, Foti et al. 2010].

Probe	CYP2C9-specific reaction
celecoxib	methyl hydroxylation
diclofenac	4'-hydroxylation
losartan	oxidation
naproxen	O-demethylation
Ppenytoin	4'-hydroxylation
S-flurbiprofen	4'-hydroxylation
S-warfarin	7-hydroxylation
tolbutamide	methylhydroxylation

Table 8. *In vitro* probes for CYP2C9 metabolic activity assessment [Hummel et al. 2004, Rendic 2002, Tang et al. 2000, Yuan et al. 2002]

Probe	CYP2C19-specific reaction
R-omeprazole	5-hydroxylation
S-fluoxetine	O-dealkylation
S-mephenytoin	4'-hydroxylation
S-omeprazole	5-hydroxylation

Table 9. *In vitro* probes for CYP2C19 metabolic activity assessment [FDA 2006, Yuan et al. 2002]

3.1.4 CYP2D6

In vitro CYP2D6 metabolic activity may be evaluated using the same substrates as *in vivo*. Except of these, bufuralol, and some fluorogenic probes were used to evaluate CYP2D6 activity *in vitro* [Wang et al. 2009] (Table 10). Bufuralol is also metabolized via CYP2C19 [Mankowski 1999] and CYP1A2 [Yamazaki et al. 1994], therefore its specificity for CYP2D6 may be decreased. Nevertheless, bufuralol is the most preferred *in vitro* probe substrate (60 % of *in vitro* studies) followed by dextromethorphan (30 % of *in vitro* studies) [Zhou 2009]. FDA recommends to use bufuralol, dextromethorphan or debrisoquine for *in vitro* experiments [FDA 2006].

Probe	CYP2D6-specific reaction
bufuralol	1-hydroxylation
debrisoquine	4-hydroxylation
carteolol	C ₈ -hydroxylation
dextromethorphan	O-demethylation
metoprolol	α -hydroxylation
sparteine	dehydrogenation
tramadol	O-demethylation

Table 10. *In vitro* probes for CYP2D6 metabolic activity assessment [FDA 2006, Frank et al. 2007, Kudo & Odomi 1998, Rendic 2002, Zhou 2009]

3.1.5 CYP3A4

Besides probe drugs used *in vivo*, some other conventional substrates are also used *in vitro* (listed in Table 11). As it was already mentioned above, it is strongly recommended to use at least 2 structurally unrelated probe substrates for CYP3A4 metabolic activity evaluation.

Standard inhibitors are used in preclinical drug development for evaluation of the inhibitory potency of new chemical entity or to identify individual CYP enzymes responsible for a drug's metabolism, and to determine the relative contribution of an individual CYP enzyme to biotransformation of evaluated chemical entity (Table 12).

Probe	CYP3A4-specific reaction
alfentanil	demethylation
alprazolam	4-hydroxylation
benzyloxyresorufin	O-dealkylation
cortisol	6- β hydroxylation
cyclosporine	oxidation
dextromethorphan	N-demethylation
diazepam	N-demethylation
erythromycin	N-demethylation
ethylmorphine	N-demethylation
midazolam	1-hydroxylation
nifedipine	oxidation
quinidine	3-hydroxylation / N-oxidation
terfenadine	C-hydroxylation
testosterone	6- β hydroxylation
triazolam	1-hydroxylation

Table 11. *In vitro* probes for CYP3A4 metabolic activity assessment [FDA 2006, Meyer et al. 2010, Yuan et al. 2002]

CYP enzyme	Inhibitor	Ki (μ M)
CYP1A2	α -naphthoflavone	0.01
	furafylline	0.6 - 0.73
CYP2C9	fluconazole	7
	fluvoxamine	6.4 - 19
	fluoxetine	18 - 41
	sulfaphenazole	0.3
CYP2C19	nootkatone	0.5
	ticlopidine	1.2
CYP2D6	quinidine	0.027 - 0.4
CYP3A4	itraconazole	0.27; 2.3
	ketoconazole	0.0037 - 0.18

Table 12. Inhibitors of CYP enzymes recommended by FDA for *in vitro* use (adapted from [FDA 2006])

3.2 High throughput methods for CYP metabolic activity assays

In past two decades, the role of *in vitro* drug metabolism and toxicity studies is slightly changing. Absorption, distribution, metabolism, excretion and toxicity studies (ADMET) have formerly been performed with a few compounds in the late stages of drug development. The number of compounds entering the drug discovery is increasing what requires to perform ADMET studies at earlier stages in the drug development [Atterwill & Wing 2002, Trubetskoy et al. 2005].

Therefore, there is a need for new generation of assays with higher throughput capability, sensitivity and reproducibility. These assays are often automated and miniaturized. Plate-scanning readers and handling robots, multi-channel analyzers, high-density assay plates together with automated LC-MS systems are mostly mentioned as the major support for such high throughput methods [Zlokarnik et al. 2005]. Thanks to these techniques, these approaches are also cheaper than standard procedures, maintaining sufficient selectivity, sensitivity and precision, and enable testing of large sets of compounds.

3.2.1 Radiolabeled substrates

Detection of $^{13/14}\text{C}$ or ^3H metabolites ($\text{H}^{13/14}\text{CHO}$ and/or $^{13/14}\text{CO}_2$) of radiolabeled substrates (erythromycin, caffeine, cyclosporine, aminophenazone, diazepam etc.) is used in some *in vitro* studies to assess metabolic activity of CYP3A4 [Grand et al. 2002, Kenworthy et al. 1999]. Metabolites may be detected radiochemically, ^{14}C -formaldehyde may be extracted and detected by radioluminescence in a microplate scintillation counter [Zlokarnik et al. 2005]. In general, detection of radiolabeled substrates and metabolites seems to provide better limits of detection, accuracy and sensitivity than other conventional methods. Other advantage (over fluorogenic and luminogenic probe substrates) of this approach is ability to use common probe substrates with well known specificity, kinetic parameters and solubility [Zlokarnik et al. 2005].

3.2.2 Fluorogenic substrates

Fluorogenic substrates are used *in vitro* very often. In general, these substrates are structurally modified fluorogenic compounds which are metabolized via various CYP forms to generate a fluorescent dye. These substrates are commercially available and widely used in preclinical drug development. Due to insufficient selectivity towards single CYP enzyme, these substrates are mostly used in recombinant CYP enzymes [Foti et al. 2010]. On the other hand, some of the fluorogenic substrates seem to be isoform-specific in lower concentrations, such as 3-*O*-methylfluorescein, which was evaluated for selectivity towards CYP2C19 [Sudsakorn et al. 2007].

These difficulties are pronounced in many probe substrates - the most widely used fluorogenic substrate for assessment of CYP2C9 activity *in vitro* is 7-methoxy-4-trifluoromethylcoumarin (MFC) [Crespi & Stresser 2000]. Despite this, some controversy about the selectivity of MFC towards CYP2C9 (the participation of several CYP enzymes in MFC *O*-demethylation) was also reported [Porrogi et al. 2008]. Nevertheless, MFC-based fluorometric CYP2C9 assays are described as "rapid with a high-throughput screening capacity, easy to perform, and amenable to automation" [Zhou et al. 2009]. Overview of fluorogenic substrates for individual CYP enzymes is given in the Table 13.

CYP enzyme	Probe substrate	Product / commercial name
CYP1A2	3-cyano-7-ethoxycoumarin (CEC)	3-cyano-7-hydroxycoumarin (CHC)
	7-ethoxy-methyloxy-3-cyanocoumarin (EOMCC)	Vivid Blue
CYP2C9	7-methoxy-4-(trifluoromethyl)-coumarin (7-MFC)	7-hydroxy-4-(trifluoromethyl)-coumarin (HFC)
	dibenzylfluorescein (DBF)	fluorescein
	7-benzyloxy-methyloxy-3-cyanocoumarin (BOMCC)	Vivid Blue
	N-octyloxymethyl-resorufin (OOMR)	Vivid Red
CYP2C19	benzyloxy-methyl-fluorescein (BOMF)	Vivid Green
	3-cyano-7-ethoxycoumarin (CEC)	3-cyano-7-hydroxycoumarin (CHC)
	dibenzylfluorescein (DBF)	fluorescein
	3-O-methylfluorescein (OMF)	fluorescein
	7-ethoxy-methyloxy-3-cyanocoumarin (EOMCC)	Vivid Blue
CYP2D6	7-methoxy-4-(aminomethyl)-coumarin (MAMC)	7-hydroxy-4-(aminomethyl)-coumarin (HAMC)
	3-[2-(N,N diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC)	3-[2-(diethylamino)-ethyl]-7-hydroxy-4-methylcoumarin (AHMC)
	7- p-methoxy-benzyloxy-4-trifluorocoumarin (MOBFC)	Vivid Cyan
	7-ethoxy-methyloxy-3-cyanocoumarin (EOMCC)	Vivid Blue
CYP3A4	7-benzyloxy-trifluoromethylcoumarin (BFC)	7-hydroxy-4-(trifluoromethyl)-coumarin (HFC)
	7-benzyloxyquinoline (7-BQ)	quinolinol
	benzylresorufin	resorufin
	di(benzyloxymethoxy)fluorescein (DBF)	fluorescein
	benzyloxy-methyl-resorufin (BOMR)	Vivid Red
	dibenzylmethylfluorescein (DBOMF)	Vivid Green
	7-benzyloxy-methyloxy-3-cyanocoumarin (BOMCC)	Vivid Blue
	7-benzyloxy-methyloxy-4-(trifluoromethyl)-coumarin (BOMFC)	Vivid Cyan

Table 13. Fluorogenic substrates for *in vitro* CYP enzyme activity assessment (adapted from Foti et. al. [FDA 2006, Foti et al. 2010])

Some of disadvantages (background fluorescence of unmetabolized substrate, low aqueous solubility, and low signal-to-noise ratio) which may limit the use of fluorescent substrates [Trubetskoy et al. 2005] were resolved by structurally related derivatives (e.g.

oxyphenylmethyl-, oxymethyl-, octyloxymethyl-) of commonly available substrates such as resorufin (red), coumarin (blue and cyan) and fluorescein (green). Currently (2011), there are available 8 modified fluorescent substrates for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 metabolic activities assessment [Makings & Zlokarnik 2011]. Similarly to other common fluorogenic substrates, these derivatives were synthesized as "blocked" fluorophores with negligible background fluorescence (lower than in common fluorogenic probes). Fluorescence signal is triggered after isoenzyme specific biotransformation (Fig. 1) [Marks et al. 2003]. Moreover, these modified (Vivid®) substrates exhibit higher aqueous solubility and their molecule contains 2 potential cleavage sites [Marks et al. 2003]. Fluorogenic assays may be performed in miniaturized form in microplates.

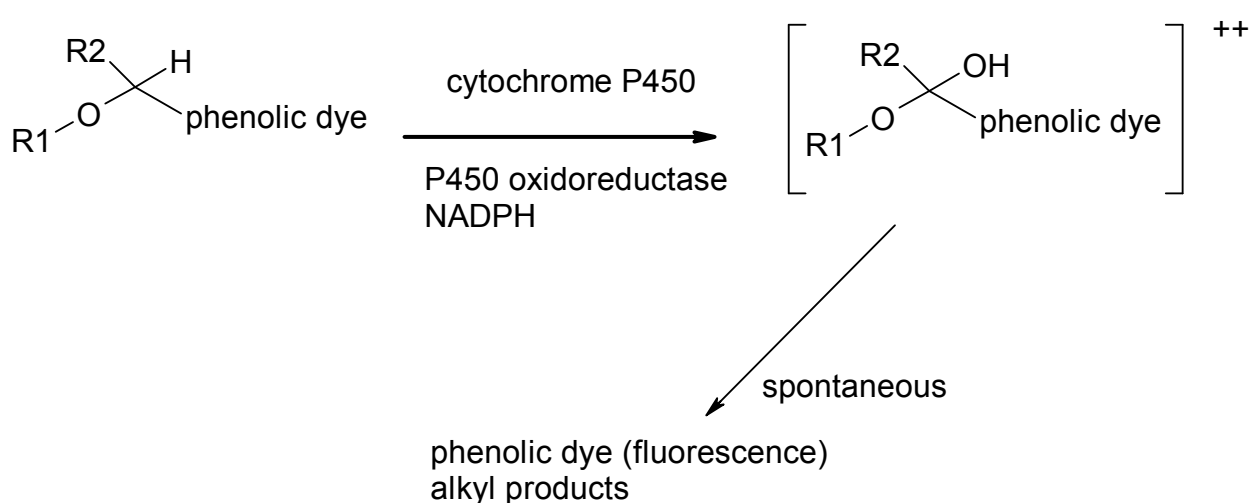


Fig. 1. Illustrative reaction of fluorescent CYP probe substrate (adapted from [Makings & Zlokarnik 2011]).

3.2.3 Luminometric substrates

Luminometric substrate probes are used for CYP activity assessment since 2003. These probes are derivatives of luciferin, substrate for the firefly luciferase which generates light. The derivatives have to be metabolized by CYP enzymes to form luciferin prior to the reaction with luciferase and light emission [Cali et al. 2003]. Then, recorded luminiscence is proportional to the amount of metabolite what is dependent on activity of the CYP enzyme. Illustrative example of reaction is given in Figure 2.

Various bioluminometric probe substrates (derivatives of luciferin) to test CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, MAO-A and MAO-B activity are available. Luciferin 6' chloroethyl ether (Luciferin -CEE) is a substrate for CYP1A1 and CYP1B1, luciferin 6' methyl ether (Luciferin-ME) for CYP1A2 and CYP2C8, 6' deoxyluciferin (luciferin-H) for CYP2C9, ethylene glycol ester of luciferin 6'-methyl ether (Luciferin-ME EGE) for CYP2D6, 6-deoxyluciferin ethyleneglycol ester (Luciferin-H EGE) for CYP2C19, luciferin 6' benzyl ether (Luciferin -BE) and luciferin 6' pentafluorobenzyl ether (Luciferin-PFBE) for CYP3A4 and CYP3A7, luciferin phenylpiperazine xylene ether (Luciferin-PPXE) and the latest one developed, luciferin isopropylacetal (Luciferin-IPA) for CYP3A4.

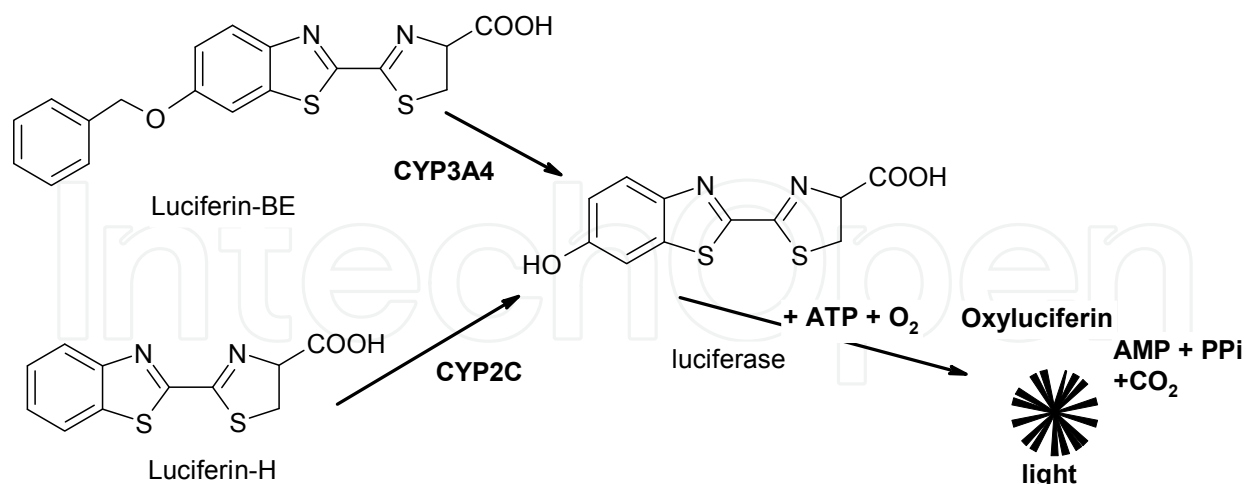


Fig. 2. Examples of CYP - mediated activation of luciferin (adapted from [Cali et al. 2011])

These methods may be also automated, miniaturized and used in "high-throughput" mode, in 3 or 6 μ l 1536-well plates and low volume 384- well plates. The signal half-life of over 2 hours enables batch processing of plates [Cali et al. 2008].

4. Conclusion

The selection of probe drug for either assessment of CYP metabolic activity *in vitro* or *in vivo* phenotyping is crucial for results of CYP metabolic activity assessment. Despite undeniable selectivity of probe substrates it seems that selection of distinct probe substrate may influence results obtained either *in vitro* or *in vivo* [Kumar et al. 2006, Stein et al. 1996]. The selection of appropriate probe drugs also depends on the system used - for *in vivo* phenotyping are used only conventional substrates (the registered drugs or endogenous substrate). A wide scale of substrates may be chosen for various *in vitro* systems such as human liver microsomes, recombinant CYP enzymes, hepatocytes, tissue slices and isolated organ perfusions. Conventional probe substrates mimics well the properties of other drugs, but the metabolites have to be analyzed by HPLC, mostly with MS, UV, fluorescence or electrochemical detection, or occasionally also with capillary electrophoretic methods [Konecny et al. 2007].

In addition, in CYP3A4 it is recommended to use at least 2 structurally unrelated probe substrates because of presence of multiple substrate binding domains within CYP3A4 protein [Khan et al. 2002, Korzekwa et al. 1998, Schrag & Wienkers 2001, Tucker et al. 2001]. Published crystal structures of CYP enzymes confirmed the ability of individual CYP enzyme to metabolize a wide range of substrates or to bind multiple substrate/inhibitor molecules simultaneously [Ekroos & Sjogren 2006, Foti et al. 2010].

In some CYP forms, it seems that polymorphisms may have variable consequences in different substrates, as was shown in the case of CYP2D6 and CYP2C19. This phenomenon is also described as allele-dependent differences of substrates [Benet 2005]. In detail, this means that in the case of polymorphism CYP2D6*17, the rate of metabolism depends on the substrate used [Bogni et al. 2005, Zhou 2009].

Fluorogenic and bioluminometric probe substrates proved sufficiently their usability in early stages of drug development; these methods of CYP metabolic activity assessment are reproducible, robust and sensitive. On the other hand, since there is occasionally reported lack of correlation in specificity and sensitivity between fluorogenic and conventional probes (mostly in CYP3A4), follow-up studies with conventional probe substrates are strictly recommended to be performed in the clinical phase of new drug development [Cohen et al. 2003].

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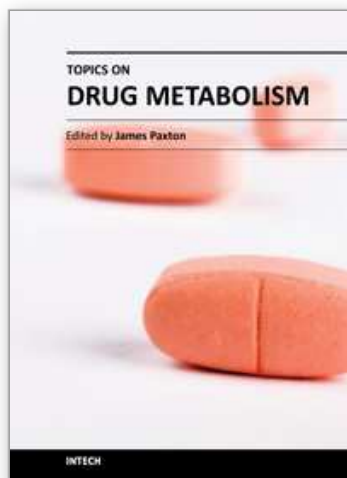
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In order to avoid late-stage drug failure due to factors such as undesirable metabolic instability, toxic metabolites, drug-drug interactions, and polymorphic metabolism, an enormous amount of effort has been expended by both the pharmaceutical industry and academia towards developing more powerful techniques and screening assays to identify the metabolic profiles and enzymes involved in drug metabolism. This book presents some in-depth reviews of selected topics in drug metabolism. Among the key topics covered are: the interplay between drug transport and metabolism in oral bioavailability; the influence of genetic and epigenetic factors on drug metabolism; impact of disease on transport and metabolism; and the use of novel microdosing techniques and novel LC/MS and genomic technologies to predict the metabolic parameters and profiles of potential new drug candidates.

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