In Vitro High Throughput Screening of Compounds for Favorable Metabolic Properties in Drug Discovery

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Abstract: Drug metabolism can have profound effects on the pharmacological and toxicological profile of therapeutic agents. In the pharmaceutical industry, many *in vitro* techniques are in place or under development to screen and optimize compounds for favorable metabolic properties in the drug discovery phase. These *in vitro* technologies are meant to address important issues such as: (1) is the compound a potent inhibitor of drug metabolising enzymes (DMEs)? (2) does the compound induce the expression of DMEs? (3) how labile is the compound to metabolic degradation? (4) which specific enzyme(s) is responsible for the compound's biotransformation? and (5) to which metabolites is the compound metabolized? Answers to these questions provide a basis for judging whether a compound is likely to have acceptable pharmacokinetic properties *in vivo*. To address these issues on the increasing number of compounds inundating the drug discovery programs, high throughput assays are essential. A combination of biochemical advances in the understanding of the function and regulation of DMEs (in particular, cytochromes P450, CYPs) and automated analytical technologies are revolutionizing drug metabolism research. Automated LC-MS based metabolic stability, fluorescence, radiometric and LC-MS based CYP inhibition assays are now in routine use. Automatible models for studying CYP induction based on enzyme activity, quantitative RT-PCR and reporter gene systems are being developed. We will review the utility and limitations of these HTS approaches and highlight on-going developments and emerging technologies to answer metabolism questions at the different stages of the drug discovery process.

1.0 INTRODUCTION

Drug metabolism and pharmacokinetic (DMPK) activities have recently changed from a documentation process to being a key player in the selection and optimization of compounds in the drug discovery process. This dramatic change has been driven by the observation that up to 40% of new chemical entities (NCE) failed to make it to the market due to pharmacokinetic problems [1]. A recent study also showed that adverse drug reactions (ADR), most of which are pharmacokinetic based, are the 4-6th leading cause of death in hospitalized patients in the USA [2]. For DMPK research to effectively participate in the drug discovery process, high-throughput screening (HTS) technologies are essential for determining metabolic, absorption and kinetic properties of compounds. *In vitro* HTS techniques to address metabolic issues are now in place or under development in major pharmaceutical companies and contract laboratories. Drug regulatory authorities like the Food and Drug Administration (FDA) have reported an increase in the inclusion of *in vitro* data in NDA (new drug applications) and acknowledged the importance of this information in evaluating the acceptability of the drug and labelling of product [3].

Drug metabolism is a major determinant of the pharmacokinetics of a compound and hence a potential source of unacceptable pharmacological and toxicological effects. Of the many human drug metabolising enzymes (DMEs), cytochromes

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P450 (CYP) have been shown to be responsible for the metabolism of most therapeutics whose elimination is facilitated by metabolism [4]. The *in vitro* HTS systems in current use are therefore biased towards answering metabolism questions like: (1) Is the compound a potent inhibitor of a drug metabolising CYP? (2) Does the drug induce the expression of a CYP? (3) Which specific CYP isoform(s) is responsible for biotransformation of the compound? (4) How labile is the compound to metabolic degradation, and (5) to what metabolites is the compound metabolized? However, the role of the other DMEs like glucuronosyltransferases, N-acetyltransferases, epoxide hydrolases, sulfotransferases, glutathione-S-transferases and carboxylesterases should be considered since the increased chemical library diversity due to combinatorial chemistry can result in the metabolism by these enzymes and not only by CYPs [5]. Knowledge of the metabolic properties of a compound provides a basis for judging whether a compound is likely to show an acceptable pharmacokinetic profile and no undesirable pharmacokinetic drug-drug interactions *in vivo*. *In vivo* predictions based on these mechanisms have been the subject of many reviews [6, 7, 8, 9]. In recognition of the importance of *in vitro* metabolism research towards the development of safer drugs, the FDA and EMA (European Agency for the Evaluation of Medicinal Products) have issued guidelines for such studies to industry [10, 11].

A number of advances in our understanding of the biochemistry of CYP function and regulation have provided a scientific platform to design HTS assays. Major break-throughs include the cloning and recombinant expression of individual CYP genes in different systems (*E. coli*, yeast, mammalian and insect cells) [12], the finding of CYP specific substrates and inhibitors, use of human liver slices and primary hepatocytes for induction studies [13], and the recent elucidation of the mechanism of induction of the major CYPs [14]. Recent success in crystallizing a mammalian CYP [15] promises to boost the role of computational approaches in defining CYP-ligand interactions through 3D QSAR pharmacophore modeling. On the technical side, design of 96 well microtiter plate based fluorescence assays [16] and adaptation of manual HPLC methods to automated LC-MS/MS [17] have contributed to the revolution in metabolism research. The DNA chip technology has already made genotyping of CYP polymorphism a rapid process and is making CYP induction studies by differential gene expression an exciting possibility [18]. Robotic systems and data handling software have also been developed to cope with or facilitate the continually miniaturized assay formats and process the enormous amount of data generated.

To some, HTS has come to mean screening thousands of compounds through a go/no go gate criterion. Ironically, almost none of the drugs on the market could have passed through such an HTS approach. However, some are beginning to realize that HTS is not so much the number of compounds one can screen-out but a rapid way of systematically defining properties of many compounds and designing of relevant follow-up experiments. It is important to clearly define where in the discovery process the studies should be done, how the information will be used, and what quality of data are needed. The goal is to make HTS a rational process. It is important to always keep in mind some of the assumptions made in the design of HTS assays, which usually affect the quality of data generated and hence the conclusions that can be drawn from them.

Fig.(**1**) depicts an overview of the drug discovery process and how various metabolism assays provide essential information at each phase of discovery and development. In this dynamic process, medicinal chemists facilitate the optimization of the pharmacokinetic and pharmacological properties of leads by using feedback from the respective scientists. In this article, we will review the utility and limitations of the *in vitro* HTS approaches and highlight ongoing developments and emerging new technologies to answer metabolism questions at the different stages of the drug discovery process.

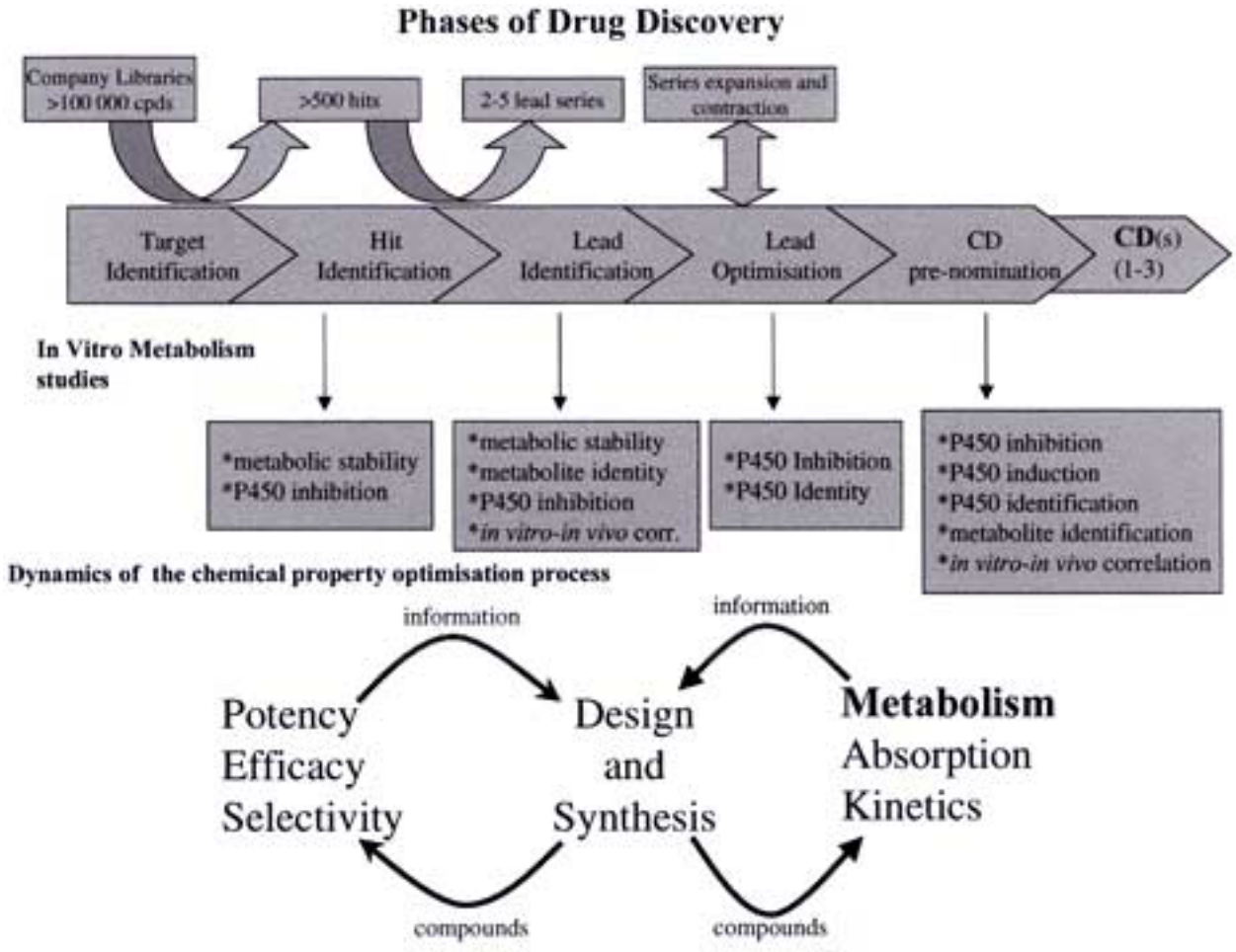

Fig. (1). A general scheme of the drug discovery process depicting the types of in vitro metabolism studies that could be performed at the various stages and the dynamics of interaction with the medicinal chemists in the optimization of lead compounds.

2.0 CYTOCHROME P450

Cytochromes P450 are a superfamily of enzymes found across all living organisms and are postulated to have evolved from a common ancestral gene. In humans, some CYPs are involved in the metabolism of endogenous molecules to maintain cellular homeostasis, whilst other CYPs have evolved to metabolize compounds foreign to the body, presumably as a defence system against chemical insults [19]. The foreign compounds metabolized by CYPs include environmental pollutants, dietary components and therapeutics. The fact that some CYPs are mainly responsible for the elimination of drugs from the body and that drugs can up- or down-regulate the expression/ activity of some of these enzymes has made them of great interest to pharmaceutical companies. Of the over 40 human CYPs (for CYP nomenclature, see http://drnelson.utmem.edu/hum.html) only approximately ten belonging to the CYP1A, 2A, 1B, 2B, 2C, 2D, 2E and 3A subfamiles are of importance in the metabolism of drugs. CYPs 1A2, 2A6, 2C9, 2C19, 2D6 and 3A4 determine the metabolism and disposition of over 90% of currently used drugs [4].

Knowledge gained in the past few years has conclusively shown that there are profound interspecies differences in CYP forms and function that make it impossible to extrapolate findings from experimental animals to humans [13, 20, 21]. Recombinant human CYPs, human liver microsomes, precision-cut liver slices and primary

hepatocytes are now recommended for the generation of *in vitro* metabolism data. Hepatic tissues are used because CYPs are at high concentrations in the liver and hence the major organ for first-pass metabolism of oral drugs. However, other tissues like the intestine have significant amounts of CYP3A4 which is of major importance for the metabolism of some orally administered drugs [8].

3.0 HTS TECHNOLOGICAL ADVANCES

While genomics has increased the number of potential targets, combinatorial chemistry has increased the number of compounds to test as potential drugs. To meet the increased screening demands, automated robotic systems are being used to run HTS assays at every stage of the drug discovery process. The pharmaceutical industry is in urgent need to cut cost and time in the preclinical phase of drug discovery from hit identification to candidate drug (CD) nomination. With respect to metabolism research, developments in assay miniaturization, mass spectrometry, microtiter plate readers, and data processing software have facilitated HTS.

Assay Miniaturization

The pros and cons of miniaturized microtiter plate formats have been exhaustively reviewed by Sills [22]. The major advantages are the cut in costs (reduced amount of test compounds and reagents used) and the increased compound throughput. Currently metabolism studies are routinely done in 96-well microtiter plates, which have been associated with a reduction in incubation volumes from 500-1000 µl in test tubes to 100-200 µl. While this is a modest level of miniaturization compared to those being applied in hit identification HTS of up to 9,600 well (0.2 µl reaction volumes) microtiter plates, the automation of the assays has increased throughput from 5-10 to hundreds of compounds per day.

As much as microtiter plate technology has changed the field of *in vitro* screening for metabolic properties, the ongoing development in microscale total analysis systems (µTAS) show even greater potential [23]. The integration of complex biochemical and analytical processes on a single chip opens the possibility of completely new assay designs and highly parallel systems. The field is developing at a rapid rate and chip based enzymatic assays [24, 25], and electrophoretic [26] and electrochromatographic [27] separations have been described in the literature. Microchip electrospray mass spectrometry [28] has gone through constant improvements and 96-nozzle sprayers are being tested today [29].

DNA Chips/DNA Microarrays

Conventional molecular biology methods for mutation detection of genetic polymorphism like polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and Southern blot are unsuitable for HTS. Also incompatible with HTS are methods for quantitative gene expression like Northern blot and reverse transcription-PCR (RT-PCR). There are now HTS techniques for quantitative gene expression, 96-well microtiter plate based quantitative real-time RT-PCR and methods for both mutation detection and quantitative gene expression based on DNA microarrays. This latter technology represents a revolutionary development in genomics and is having a great impact on metabolism research, too.

DNA gene chips or microarrays are glass surfaces (1.6-3.6 cm^2) spotted with either DNA oligodeoxynucleotide or cDNA [18]. The chips carry up to 400 000 DNA oligonucleotides representing up to 900 genes. Affymetrix has developed a chip for monitoring the polymorphic variants of cytochromes P450 2C19 and 2D6 (http://www.affymetrix.com/products/gc-cyp450.html). With this method one can analyze four samples/hr for 14 major CYP2D6 and CYP2C9 mutations. The use of this chip will contribute to the selection of population sub-groups to evaluate the clinical impact of metabolic polymorphisms. Population genotyping can also be used in retrospective analysis of results of a clinical study which could explain some observed drug responses. Gene expression chips are being used to analyse the

differential expression of genes coding for drug metabolizing enzymes. The classical effects of phenobarbital on rat CYPs were impressively demonstrated using such chips [18]. Currently many pharmaceutical companies are engaged in the development of customer-designed gene chips to detect polymorphisms of CYPs for use in clinical trials and differential expression of human CYPs upon exposure to test compounds in toxicology studies.

4.0 METABOLIC STABILITY AND METABOLITE IDENTIFICATION

The stability of a test compound to metabolic degradation is a major determinant of whether it is worth developing. This is because metabolism determines the clearance of many compounds and hence their concentrations in the body. Knowledge of whether a test compound has a low, intermediate or high clearance is important in anticipating dosage regimens. Metabolic stability studies are commonly carried out using human liver microsomes (HLM). The experimental design is fairly simple, involving measurement of either the disappearance of test compound (as is common in the early phases of discovery when the metabolites are unknown) or formation of metabolites at advanced stages of lead optimization. The incubation conditions reported in the literature vary considerably and include microsomal concentrations of 0.1 – 10 mg/ml, substrate concentrations of 0.2 –20 μM (or higher in some cases), and incubation times of 10-120 min [30, 31]. Since the *in vitro* intrinsic clearances, CL_{in}, are used to predict *in vivo* clearances, the models require that the data be generated based on a number of specific assumptions [32]. It is generally recommended that microsomal concentrations not exceed 0.5 mg/ml, test compound concentration not exceed 10 μM, and incubation times not extend beyond 60 min. LC-MS analysis is overcoming the problem of sensitivity, which previously led researchers to use high microsomal concentrations and long incubation periods to reliably quantify compound disappearance.

Single Incubation Time Point Approach

Korfmacher *et al.*, [33] have recently demonstrated the utility of an automated one incubation time point approach in 96-well microtiter plates with subsequent LC-MS analysis of parent compound disappearance. Many pharmaceutical companies are using this HTS approach to analyse 70-80 compounds per day. The use of this approach is most appropriate when dealing with many compounds as a gross metabolic stability exclusion criterion before doing other more rigorous and time consuming metabolism studies.

The $T_{1/2}$ or CL_{int} Approach

Compounds which have passed the one time point screen and those in advanced stages of the lead optimization process are subjected to more rigorous metabolic stability investigations. In this approach, the test compound disappearance is measured at several time points between 10 and 60 min. Since the $T_{1/2}$ for compound disappearance from these studies is then used to calculate the intrinsic clearance, CL_{int}, the study design is based on a number of important assumptions:

1. The test compound disappearance shows first order Michaelis-Menten kinetics (S<<Km). Since the Kms of CYP substrates are generally high, a test compound concentration of around 1 μM generally fulfils this condition.

2. The CL_{int} can then be derived from the relationship: $CL_{int} = V(ml) \times 0.693/T_{1/2}$. The $T_{1/2}$ being obtained from a plot of the natural logarithm of [S] against incubation time.

3. In studies where metabolic stability is measured by the metabolites formed, the total intrinsic clearance is the sum of all metabolic routes. $CL_{int} = \Sigma Vmax/Km$

Interpretation of Clearance (CL_{int}) Data

Many recent articles have proposed ways of how the *in vitro* CL_{int} can be scaled up and used to

predict *in vivo* clearances [34, 35, 31, 36]. These papers also discuss in detail the assumptions and problems with the *in vitro-in vivo* correlation models and propose possible ways to solve them. Though most metabolic studies are being done using human liver microsomes, considering the increasing awareness of the role of other non-microsomal DMEs, use of hepatocytes and liver slices which have the complete metabolic machinery is recommended. For drugs intended for short duration of action, rapid elimination is desirable, and for drugs meant to control chronic diseases, stability predictive of at most once a day dosing is desirable.

Metabolite Identification

As early as possible in the drug discovery process, efforts should be made to couple metabolic stability studies with metabolite identification. This is because some of the metabolites might turn out to have better pharmacological/safety properties than the parent compound or that they might elicit some undesirable drug-drug interactions andtoxicological effects. In retrospect, it might have been possible to avoid the terfenadine disaster if such studies had been done [37]. The traditional methods for metabolite identification involving preparation of radiolabeled and/or authentic standards, and fractionation before mass spectrometric or NMR analysis of each structure are labor intensive and incompatible with HTS. To support mass spectrometry based HTS methods for metabolite identification, an idea of potential metabolic hot spots is helpful. This information can be obtained from databases based on published *in vitro* and *in vivo* biotransformation routes of compounds [90]. Zhang *et al.*, [38] developed a rapid method for metabolite identification using Time-of Flight (TOF) LC-MS. Ferna'ndez-Metzler *et al.*, [39] and Lim *et al.* [40], Bu et al, [41] used fast LC-MS and LC-MS-MS, respectively, to identify metabolites. van Breemen *et al.* [42] developed a HTS pulsed ultrafiltration mass spectrometry method for the *in vitro* formation and characterization of microsomal drug metabolites. The same method was then used to screen xenobiotics for electrophilic metabolites that would react with glutathione [43]. Metabolite profiles using human and experimental animal tissues help rationalize interspecies differences in toxicology and pharmacokinetics.

5.0 IN VITRO HTS CYP450 INHIBITION ASSAYS

Among metabolism based drug-drug interactions, CYP inhibition seems to be the most important. Inhibition of a CYP by a test compound can lead to (a) exaggerated pharmacological effects of another drug whose elimination depends on the inhibited enzyme, and/or (b) therapeutic failure due to the inhibition of a CYP which metabolizes a prodrug to the active metabolite. These pharmacokinetic drug-drug interactions have been extensively reviewed [44, 45, 46]. The withdrawal of terfenadine, mibefradil, and furafylline from the market due to inhibition based interactions involving CYPs 3A4, 2D6 and 1A2 exemplify the medical relevance of this mechanism of drug-drug interactions [47, 44, 37]. Fatal interactions between triazolam and amitriptyline [48], fluoxetine and clozapine [49], and pentostatin and cyclophosphamide [50] also underline the need to understand the inhibitory effects of test compounds on enzymes which metabolize other drugs.

The design and interpretation of *in vitro* HTS inhibition assays has been the subject of many excellent reviews [51, 52, 53, 54, 55, 44, 56, 57]. We will, therefore, only highlight some important points about HTS inhibition assays that were not discussed in detail or omitted in these reviews. Three HTS inhibition assays have been reported to be used in the pharmaceutical industry (see Table 1): (1) the fluoresence assays developed by Crespi et al, [16], (2) the LC-MS-MS assays [17, 58] and (3) the radiometry assays [59]. These assays have been adapted to the 96 well microtiter-plate incubation formats run on automated robotic systems. These assays are associated with tremendous reduction in analysis time, from 20 min per sample by HPLC to 30 sec by LC-MS-MS [60] and from 32 hr for 4 compounds (96

Table 1. Assays for CYP Inhibition Adapted to HTS Format

CYP	Reaction			Positive control inhibitors
FLUORESCENCE BASED				
1A2	ER	→	Resorufin	α-naphthoflavone
1A2	CEC	→	CHC	furafylline
2A6	Coumarin	→	7-HC	tranylcypromine
2C8	DBF	→	Fluorescein	quercetin
2C9	7-MFC	→	HFC	sulfaphenazole
2C19	CEC	→	CHC	ticlopidine
2D6	AMMC	→	AHMC	quinidine
3A4	7-BQ	→	Quinolinol	ketoconazole
3A4	BFC	→	HFC	"
3A4	BzRes	→	Resorufin	"
3A4	DBF	→	Fluorescein	"
LC-MS/MS BASED				
1A2	Phenacetin	→	paracetamol	
2A6	Coumarin	→	7-hydroxycoumarin	
2C8/9	Tolbutamide	→	4-hydroxytolbutamide	
2C19	S-mephenytoin	→	4-hydroxymephenytoin	
2D6	Bufuralol	→	1-hydroxybufuralol	
2E1	Chlorzoxazone	→	6-hydroxychlorzoxazone	
3A4	Midazolam	→	1-hydroxymidazolam	
RADIOMETRIC BASED				
2D6	Dextromethorphan 0-demethylation			
2C9	Naproxen 0-demethylation			
2C19	Diazepam N-demethylation			
3A4	Erythromycin N-demethylation			

7-ethoxy-3-cyanocoumarin (CEC), 7-methoxy-4-trifluoromethylcoumarin (MFC), 7-benzyloxyquinoline (BQ), 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC), 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), 7-hydroxycoumarin (7-HC), resorufin benzyl ether (BzRes), dibenzylfluorescein (DBF), 3-cyano-7-hydroxycoumarin (CHC), 7-hydroxy-4-trifluoromethylcoumarin (7-HFC), ethoxyresorufin (ER).GenTest, 2000 (http://www.gentest.com/HTS/ht_summary.html, [17, 59, 61, 58]

incubations) by HPLC to 60 sec by fluoresence [52]. The recently reported HTS turbulent flow LC-MS-MS assay for assessing CYP activities has advantages, which make it attractive for adaptation for CYP inhibition studies [61].

The major advantage of the fluorescence assays is rapid generation of inhibition data comparable to those obtained using other assays and different sources of CYPs [52]. The disadvantages of these assays are: (1) most of the substrates used are not CYP-specific and hence can only be used with recombinant CYPs and not HLM, hepatocytes or liver slices, (2) a number of compounds cause apparent activation of CYPs, which is usually not observed in other assays [62], and (3) compounds that are fluorescent or metabolized to fluorescent products cannot be analyzed (Bapiro *et al.*, unpublished).

Ayrton *et al.* [17], Yin *et al*., [58] and Lim *et al.*, [61] adapted the conventional HPLC-UV CYP-specific marker reactions to 96-well microtiter plate incubations with subsequent metabolite analysis by different types of mass spectrometry without sample pretreatment. Since the CYP marker reactions are very specific, cocktail incubations can be done with human liver microsomes, hepatocytes or liver slices so that one gets inhibitory potency and CYP-selectivity simultaneously. This has led to an increase in assay sensitivity and reduction of incidences of test compound interferences which are often encountered with the less selective LC-UV assays. Unlike the fairly recent fluorometric assays [16], there are large amounts of inhibition data generated using these CYP-specific marker reactions making the method validation process easier by comparing results with those in the literature [63, 64]. The reactions are done at substrate concentrations equal to the Km value for the specific reaction to allow for the application of simple inhibition kinetic relations for competitive inhibition to estimate Ki. The major advantages of this system include, (i) use of CYP specific reactions, (ii) the ability to assess inhibitory effects on more CYPs than the other systems, and (iii) application with both human liver microsomes and recombinant CYPs. In the 'cocktail' format, all the substrates are included in the same incubation, and the test compound is simultaneously assessed for inhibitory potency against each of the CYPs as indicated by the marker reaction activity, thus providing information on both inhibitory potency and enzyme inhibition selectivity.

The radiometric assays depend on CYP-catalyzed dealkylation reactions with subsequent measurement of the radioactivity of the formed formaldehyde [59]. These assays have probably the highest detection sensitivity, but safety issues are of concern when running these assays on a HTS scale. Other disadvantages include the fact that there is an extraction step required before measurement and that the need for dealkylation reactions limits the type of CYP marker probe substrates one can use. The latter issue means that for some reactions, less specific probes are used. Since specificity is essential when using HLM, the use of less specific probes is limited to studies with recombinant (r)CYPs.

Interpretation of HTS Inhibition Data

There are different types of inhibition; reversible (competitive, non-competitive, uncompetitive and mixed-type) and irreversible (auto-catalytic inactivation and metabolite intermediate complexation). Competitive inhibition is by far the most common type of inhibition encountered in drug-CYP interactions, followed by mechanism-based inhibition [44]. This is the reason why most of the HTS inhibition assays in use are designed on the assumption of competitive inhibition. Since IC_{50} is not a constant, experimental designs must allow for estimation of Ki which can then be used to evaluate the likely impact of the inhibitor on a CYP [53]. Though the Ki of a compound for a CYP is supposed to be a constant, in practice it has been shown to sometimes vary depending on the CYP substrate used in the assay [3]. CYP3A4 is the most important CYP in drug metabolism, but it has proved to be difficult to study [62]. Kenworthy *et al.* [62] suggested the use of at least three substrates belonging to the classes of substrates for which similar kinetic behavior is observed. Since interactions with CYP3A4 are substrate dependent [62, 65], caution must be exercised in interpreting results obtained using only one substrate.

With the presently applied set-up of the inhibition assays, most mechanism-based inhibitions will be missed while uncompetitive, noncompetitive and mixed-type inhibitors are analyzed as competitive inhibitors Fig. (**2**). There are many clinically important cases of mechanism-based inhibition of CYP3A4 [13, 66, 67] which makes its consideration in HTS necessary. The assays can be easily modified to screen for irreversible inhibition by first incubating the test compound with the enzyme without the marker substrate. After a dilution step, add the substrate and measure activity after an additional incubation time, or analyze the non-linearity of a reaction with time.

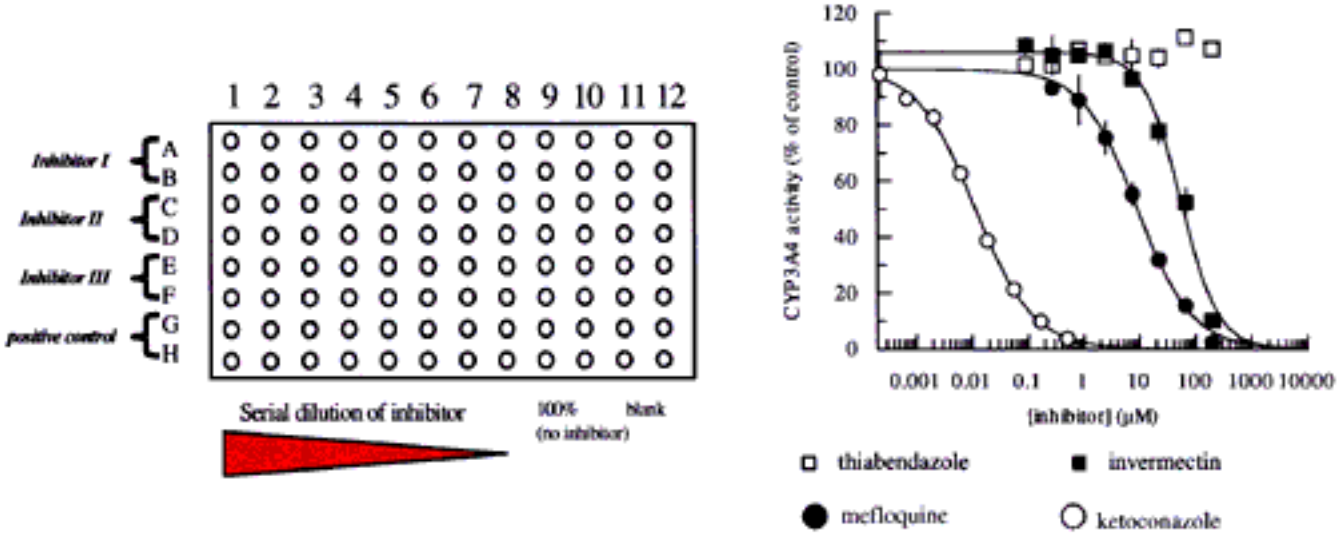

Fig. (2). Typical results of 96-well microtiter plate based HTS inhibition assays for CYP3A4 catalysed metabolism of 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) to the fluorescent 7-hydroxy-4-trifluoromethylcoumarin (HFC). Inhibitions were done at [BFC] = Km. Ketoconazole was the positive control inhibitor.

When one deals with over a hundred compounds as is the case at the hit identification stage or the primary screen during the lead optimization stage, one concentration inhibition screens using the fluorometric, radiometric assay with rCYP or the LC-MS-MS assay using either

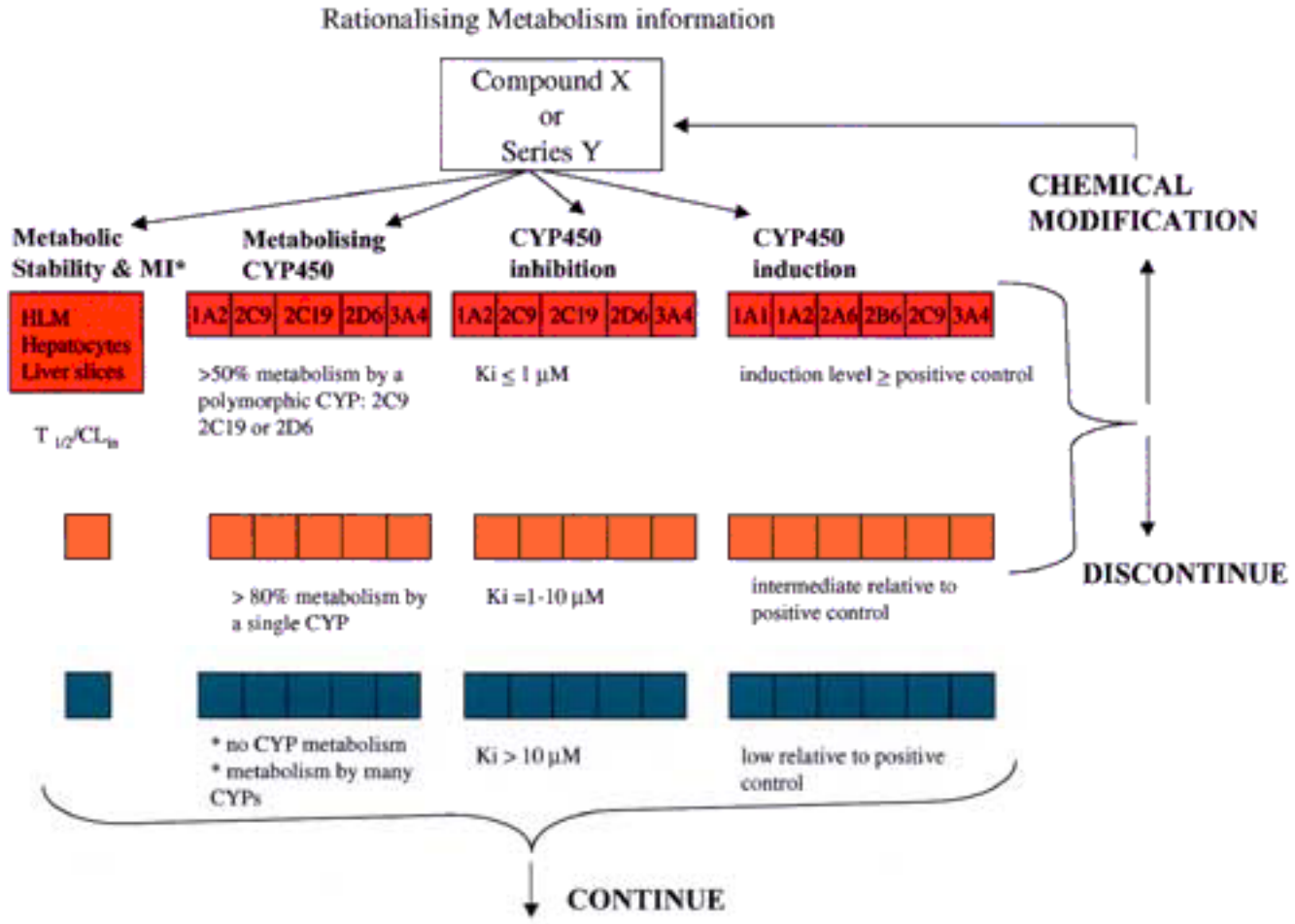

Fig. (3). A schematic guideline on how to interpret data obtained from in *vitro* metabolism screens. Note that the exact values may vary depending on the compounds and objectives of a particular project.

rCYP or HLM would be appropriate. In the lead compound/series identification and secondary screens during lead optimization, full-scale IC_{50} studies are recommended. Such data are important in making decisions to move a compound or series to lead optimization, and to medicinal chemists during lead optimization. From the IC_{50}, assuming competitive inhibition, Ki can be approximated and used to assign inhibitory properties of compounds using a flagging system. Fig. **(3)** illustrates this decision making process, with green used to indicate poor inhibitors (K>10 μM), orange for intermediate inhibitors (Ki =1-10 μM) and red for potent inhibitors (Ki <1 μM).

At the pre-CD and CD nomination stage, detailed *in vitro* inhibition studies should be carried out using non-HTS assays with both rCYPs and HLM to get accurate Ki values and to determine mechanisms of inhibition. The latter is important since all the HTS assays assume competitive inhibition, hence missing or underestimating mechanism-based inhibitors. This information is now required for the documentation of new drugs [10]. A drug that has no interaction with a CYP enzyme *in vitro* does not need to be tested for *in vivo* drug–drug interactions involving that particular isoform. If, on the other hand, a potential interaction is suggested by the *in vitro* studies, *in vivo* studies should be performed to investigate if an interaction occurs. The results from *in vitro* drug-drug interactions are, therefore, useful in the design, optimization and streamlining of clinical studies [45, 3].

6.0 IDENTIFICATION OF DRUG METABOLISING CYPs

Isoform identification screens should only be done on compounds that will have shown favorable metabolic stability properties, since identification of specific CYPs is a detail of metabolic stability. During the early phases of drug discovery, the metabolites of the compounds are usually unknown since the process to identify metabolites is still rigorous and time consuming. Knowledge of the major enzyme(s) that are likely to metabolize the compound *in vivo* is, however, important for guiding synthesis to minimize the role of polymorphic enzymes (e.g. CYP2D6, 2C19, 2C9 and CYP2A6) or to guide the design of appropriate follow-up studies to evaluate likely drug-drug interactions. The approaches outlined below are done either singly for HTS purposes or in combination for more accurate conclusions at the preCD and CD nomination stage. The three reaction phenotying approaches include (1) use of recombinant CYPs to identify CYPs playing major roles in the metabolism of the test compound; (2) use of CYP-specific chemical inhibitors and inhibitory antibodies to deduce the CYPs involved in the metabolism of the test compound using HLM; and (3) correlation analysis of the activities of a panel of HLM for CYP-specific probe substrates with the capacity of these HLM to metabolize the test compound. Results from these approaches can be combined to evaluate the relative contribution of particular CYPs in the metabolism of the test compound.

Use of a Panel of Recombinant CYPs

For screening many compounds, the metabolic stability is investigated with a fixed CYP concentration of each of the 10 or so drug metabolizing recombinant CYPs. This is done like the classical one substrate concentration, fixed incubation time, metabolic stability studies (section 4.0) or, in few cases where the metabolite is known, the rate of its formation is measured. The approach using rCYPs will give a crude inclusion/exclusion screen of the role of each CYP. The study of Suzuki *et al.* [30] on the metabolism of gallopamil on screening 10 CYPs showed that only three were involved, CYP2C8, CYP2D6 and CYP3A4, and that CYP3A4 was most responsible for substrate disappearance. A similar screen in a study by Baldwin *et al.*, [68] on rosiglitazone metabolism on 9 rCYPs showed that CYP1A2, 2C8 and 2C9 were involved with CYP2C8 being most important. Subsequent complementary studies on the metabolism of these compounds confirmed the conclusions of these primary screens. With this screen, the role of polymorphic CYPs can immediately be noticed and relevant follow-up studies done to evaluate the importance

of their contribution. A major limitation in making conclusive statements from this screen is that some of the rCYPs are used at concentrations far in excess of their relative quantities in the liver.

Use of Chemical Inhibitors and Inhibitory Antibodies on HLM

The use of inhibitors as diagnostic tools to identify CYPs involved in the metabolism of a test compound depends on their potency and selectivity for specific CYP isoforms. HLM have the advantage that CYPs are present in their natural relative proportions and can interact with other essential proteins like CYP450 NADPH reductase and cytochrome b5. For each test compound, a series of incubations are carried out each including a CYP-specific inhibitor, quinidine for CYP2D6, ketoconazole for CYP3A, sulfaphenazole for CYP2C9, α-napthoflavone for CYP1A [13], or selective inhibitory antibodies (GenTest, 2000) [69]. The incubations in which the metabolism of the test compound is reduced or stopped suggest the involvement of the CYP whose activity is affected by the inhibitor. To ensure extensive inhibition (over 80%) of CYP by a specific inhibitor, its concentration should be more than 10 times its Ki for the standard marker reaction of that CYP such that [I]/Ki $\geq$10. This seems to work well for the very potent inhibitors like ketoconazole, quinidine, α-napthoflavone and sulfaphenazole. For low potency inhibitors, selectivity becomes a problem at higher inhibitor concentrations. Even with potent inhibitors, it is not possible to completely and selectively inhibit a single CYP isoform. CYP-specific inhibitory antibodies have been produced for only a few CYPs, but progress in this area will improve the utility of this approach to identify CYPs involved in test compound metabolism [69].

Correlation of Marker Substrate Activities with Metabolism of Test Compound in Panels of HLMs

Although not HTS, this approach to isoform identification is valuable at the preCD/CD nomination stage to complement results from studies with rCYPs and selective inhibition of CYPs in HLM discussed above. This approach has been used by many researchers, and recent reports include those by Suzuki *et al.*, [30] using the substrate disappearance approach, and Baldwin *et al*., [68] using the metabolite formation approach to measure the capacity of the HLM to metabolize the test compound. Here, at least 10 HLM samples from different individualsexhibiting a large variation in the activity of each CYP as measured by the capacity to metabolize marker substrates [13] are needed. It must also be ensured that there is no co-regression between the activities for marker substrates for different CYPs. The clearance of the test compound is then determined with each of the human liver microsomes. The clearance can then be calculated as: CL_{in} = V(ml) X $0.693/T_{1/2}$ for compounds for which metabolites are unknown or CL_{in} = Vmax/Km for which metabolic routes are known. For test compounds for which metabolic routes are known, it can also be sufficient to measure reaction velocity with respect to formation of metabolites. Multiple regression analysis is then carried out on test compound CL_{in} or rate of metabolite formation against the activities of each CYP in the panel of HLM. The difficulty with this approach is to find livers which display sufficient inter sample variation and show no co-regression of activities of all the CYPs to avoid artefactual correlation results.

Relative Contribution of CYPs in the Metabolism of a Test Compound

The above assays give rather qualitative indications of the relative importance of particular CYPs in the metabolism of a test compound. There are current efforts to quantitate the relative contribution of CYPs since this would allow for better *in vitro – in vivo* extrapolations. Currently there are three approaches which have been used with varying degrees of success: (1) the relative activity factor (RAF) which is used to normalize the activity of a rCYP with its activity in HLM. This is defined as the ratio of the activity of the rCYP for a marker substrate and that of HLM for the same substrate [30]. (2) The relative substrate-

activity factor (RSF) based on the ratio of the activity of rCYP toward a test compound and a diagnostic substrate [70]; and (3) the normalized rate (NR) in which the measured rates of activity by a rCYP are normalized with respect to the specific content of the corresponding CYP in native HLM [55]. The validity of these approaches needs further validation [71].

Interpretation of Isoform Identification Data

The introduction of the HTS approach proposed above will allow for the identification of the important CYPs in the biotransformation of the many compounds encountered at the hit lead compound or series and lead optimization stages. Besides assigning the role of CYPs to a particular biotransformation, a flagging system could provide alert signals for compounds mainly metabolized (>50%) by a polymorphic CYP (e.g. CYP2D6, 2C19, 2C9 and 2A6), and compounds mainly metabolized (80%) by one CYP. Such metabolic properties are associated with large inter-individual variations in drug and metabolite concentrations in blood and tissue and drug-drug interaction incidences. Knowledge of these properties can be used to either sideline some compounds if there are better alternatives or alter the substituents which confer undesirable metabolic attributes if possible. As with inhibition studies, dogmatic actions could lead to the loss of good compounds since for some drugs, the seemingly unfavorable metabolic properties can turn out to have minimal clinical consequences. Metabolism by a polymorphic CYP is a case in mind where the clinical relevance to a large extent depends on the therapeutic index of the drug. In the absence of better alternatives and possibility for chemical modification without loss of pharmacological effect, a potentially important drug can still be developed and the knowledge used in the ever-increasing approach of individualized drug therapy [72].

7.0 CYP INDUCTION

Exposure to certain compounds may lead to an increased concentration of drug metabolizing enzymes. The increased content of a particular CYP can result in reduced therapeutic effects of a drug whose metabolism is enhanced or, when a prodrug is activated by the induced enzyme, an increased amount of a pharmacologically active metabolite. Induction is classically defined as increased protein synthesis as a result of transcriptional activation. In drug metabolism research, however, all compounds increasing the protein content independent of mechanism are usually called inducing agents. With this broader definition, induction might include mRNA or protein stabilization in addition to transcriptional activation.

The number of drugs that have been reported to cause clinically significant changes in drug metabolism due to enzyme induction is quite limited (see Table 2). Rifampicin is one of the most potent inducers known in humans and can reduce the plasma concentration of co-administered drugs up to 20-40-fold. Drugs affected by rifampicin are mainly metabolized by CYP3A4 and CYP2C9 (Table 2) [73, 74]. However, rifampicin has also been shown to increase the metabolism of S-mephenytoin in extensive metabolizers (EM) (subjects exhibiting normal levels of CYP2C19) whereas poor metabolizers (PM) (subjects lacking CYP2C19) were unaffected [75]. Since S-mephenytoin is a specific CYP2C19 substrate, this study indicates that this enzyme is induced by rifampicin. Glucocorticoids such as dexamethasone have been shown to increase erythromycin N-demethylation by 55% *in vivo* [76].

Several studies have indicated that a large part of CYP3A4 mediated metabolic clearance of drugs takes place in the intestine in addition to the liver [77, 78, 79]. The metabolism of orally administered rifampicin was suggested to result mainly by induction of gut CYP3A4 since the clearance of nifedipine, a CYP3A4 substrate, was not altered after i.v. administration whereas oral clearance increased 14-fold [80].

Induction of CYP3A and CYP2C subfamilies are probably to a large extent due to transcriptional activation of the gene. Whereas the understanding of the CYP2C9 transcriptional regulation is poor,

Table 2. ***In vivo*** **(in humans) and** ***in vitro*** **(reporter gene system) inducers of cytochrome P450s**

Clinically relevant *in vivo* induction of CYPs by therapeutics			
Inducer drug	**Plasma Conc. (μM)**	**Induced CYPs**	**Drugs affected**
carbamazepine	20 - 40	3A	praziquantel itraconazole cyclosporin Al
glucocorticoids (e.g) dexamethasone		3A	praziquantel erythromycin midazolam
phenytoin	>10	2C 3A	warfarin praziquantel quinidine cyclosporin A
phenobarbital	40-130	2C 3A	warfarin cyclosporin A
rifampin	10	2C 3A	tolbutamide, warfarin triazolam, cyclosporin, oral contraceptives, tamoxifen
troglitazone	7	3A	cyclosporin A terfenadine
EC_{50} of drugs that activate the human PXR receptor in a reporter gene assay			
Drug	**EC_{50} (μM)**		
rifampin	0.8		
lovastatin	1-5		
clotrimazole	1-5		
dexamethasone	10		
troglitazone	3		
phenobarbital	17-fold induction at 1 mM		

[73, 74, 108, 104, 76, 13, 103, 110, 109, 81, 107, 106]

the understanding of CYP3A transcriptional induction is progressing rapidly [14]. The involvement of the nuclear receptor PXR (Pregnane X Receptor) in CYP3A induction, first described in the rat, seems to be also important in human [81]. This receptor binds to the response element in the CYP3A4 promoter and is activated by a number of drugs known to induce CYP3A4 expression (Table 2). However, the affinity of the receptor for these drugs is low, since the EC_{50} values in reporter gene assays are in the μM range. This indicates that plasma concentrations of drugs must reach high levels to interact with the receptor. Indeed, drugs that clearly induce CYP3A4 *in vivo* also exhibit plasma concentrations of 10 μM or higher.

Therefore, induction of CYP3A4 may be a high dose phenomenon and should not be a problem for pharmacologically potent drugs showing plasma concentrations in the nanomolar range. A high affinity (Ki = 27 nM) PXR receptor ligand, hyperforin, was recently extracted from St. Johns wort [82]. Hyperforin was also a potent inducer of CYP3A4 expression in isolated human hepatocytes. Several reports suggest that St Johns wort increases the metabolism of HIV protease inhibitors, cyclosporin and oral contraceptives (for

references see [82]). Plants may thus contain potent CYP3A4 inducers which might affect the enzyme level. Therefore, diets containing different vegetables might be one reason for the high variation in CYP3A4 levels in the population. In addition to PXR, the nuclear receptor CAR may also be involved in CYP3A4 induction [83]. CAR is mainly associated with the induction of CYP2B genes in mammals. The importance of CYP2B6 enzyme for drug metabolism is poorly investigated, and no indication of its inducibility *in vivo* has been reported.

The transcriptionally-based induction of CYP1A via the Ah receptor is well established [84]. This receptor exhibits clear structural requirements for planar aromatic structures. However, benzimidazoles like lansoprazole and omperazole which do not conform to the structural features of the Ah receptor have been shown to induce CYP1A in human hepatocytes *in vitro* using μM concentrations. The induction mechanism is unclear but might include activation of the Ah-receptor without these compounds functioning as ligands [85]. A limited *in vivo* induction by omeprazole has only been demonstrated in high dose exposure using 120 mg in EM (induced caffeine metabolism 28%) and 40 mg in PM (induced caffeine metabolism 37%). Since these doses are 6-fold and 2-fold higher than the normal 20 mg [86], this questions the clinical significance of the findings.

Use of Cell Lines and Human Primary Hepatocyte Cultures

In vitro induction studies need an intact cell system. Immortalized cell lines would be ideal since they are easy to culture and handle. However, it has been difficult to study the induction of other enzymes than the extrahepatic CYP1A1 in established cell lines [87, 88, 89, 105]. Although HepG2 was reported to respond to CYP3A4 inducers [91] recent studies indicate that the PXR expression in HepG2 is reduced resulting in a considerably lower level of induction compared with primary cultures of human hepatocytes [92]. Human primary hepatocyte cultures retain their responsiveness to CYP1A, 2B, 2C and 3A inducing agents [93]. Even though human hepatocytes are increasingly used in induction studies, they face a number of problems which include: (1) variation in inductive response between hepatocytes from different liver sources; (2) loss of or lack of response to the induction of some CYPs, which is dependent on the media used, matrix on which culturing is done, and time since preparation; (3) cryopreservation conditions are not yet optimal such that many laboratories have to use freshly prepared primary hepatocytes; (4) cost and availability of human tissue are still limiting; and (5) the *in vivo* significance of an induction response *in vitro* is unclear [105].

For induction studies based on measuring enzyme activity, cells are exposed to the test compound for at least two days, and then the metabolic capacity of the cells is evaluated using CYP-specific marker reactions. For CYP1A, ethoxyresorufin O-dealkylation is most commonly used because it is easy to perform and rapid to measure the fluorescent resorufin formed using a 96-well plate reader [94]. Induction studies in cells should be adapted to 96-well microtiter plate assay format using CYP-marker reactions and mass spectrometric analysis as described in section 5.0.

Determination of induction by measuring mRNA is being carried out by either real-time quantitative RT-PCR or by differential gene expression [95, 18]. These approaches are associated with a reduction in exposure time to the test compound of 6-12 hr, which is sufficient to detect changes in mRNA. Using the real-time quantitative RT-PCR method, mRNA is prepared from treated hepatocytes and the cDNA of the CYP of interest prepared by RT-PCR. Quantitation is then done using fluorogenic 5'nuclease chemistry (see description in Fig. (**4**)). Genechip technology has also found utility in evaluating the differential expression of CYPs. An elegant reproduction of the effects of phenobarbital on rat CYPs and CYP450 reductase was done using this technology by Gerhold *et al.* [18]. Here, mRNA from liver of rats pre-treated with phenobarbital were hybridized with a Merck-Affymetrix DNA chip which included 28 rat CYP

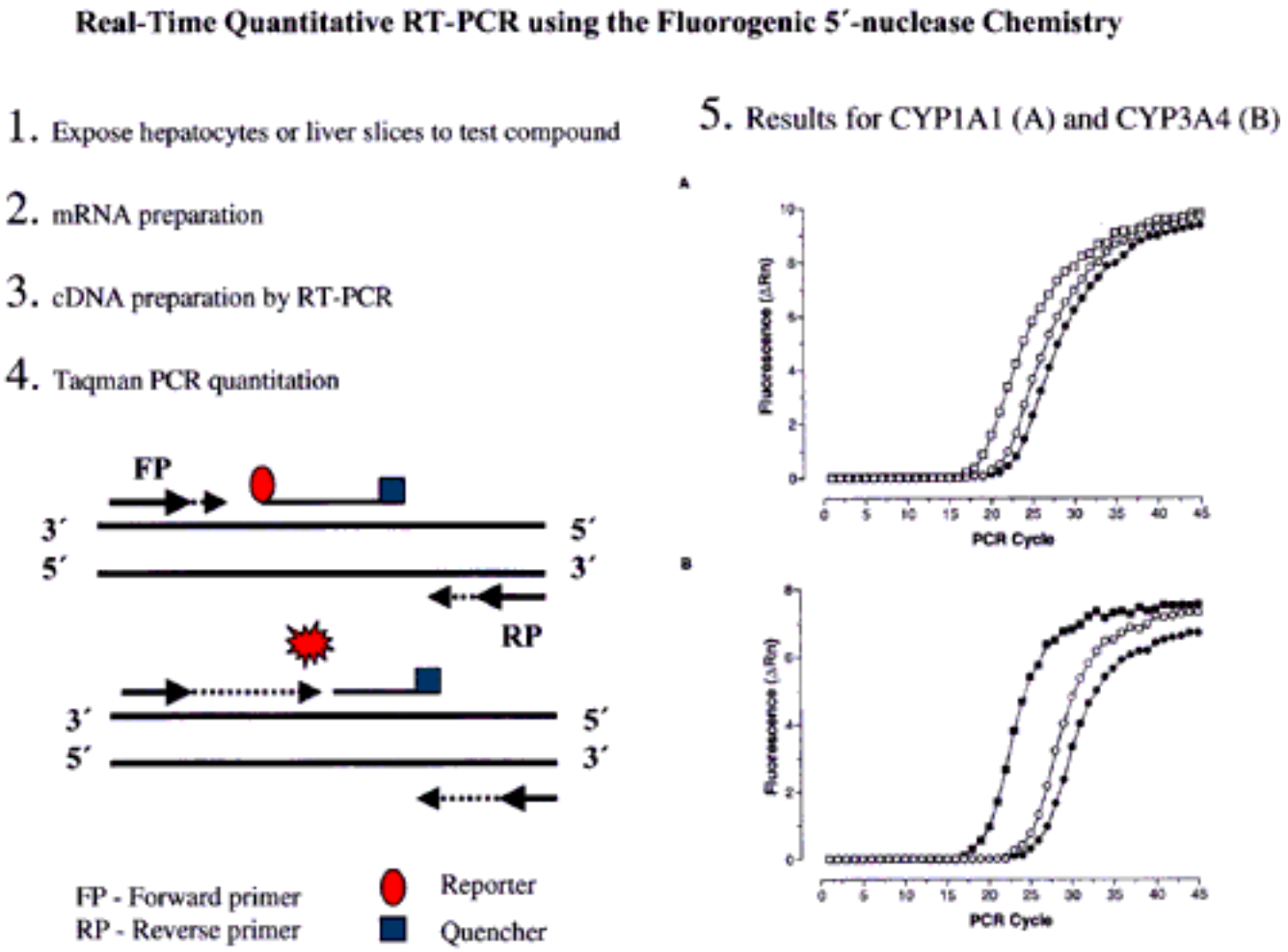

Fig. (4). Use of real-time quatitative reverse transcriptase polymerase chain reaction (real-time quantitative RT-PCR) to evaluate inductory effects of test compounds on the expression of CYPs. The results for CYP1A1 and CYP3A4 induction are reproduced from [95] with permission of the copyright holder, ASPET. **A,** CYP1A1 expression in controls (m). DMSO (l)-, and 3-MC (n)–treated cultures. **B,** CYP3A4 expression in control (m), DMSO (l)-, and rifampicin (n)-treated cultures.

genes and fluorescent molecules used to quatitate hybridization. In all assay systems, positive control inducers for each CYP should be used to assess the relative inductory effects of test compounds. Positive control inducers include β-naphthoflavone, TCCD and 3-methylcholanthrene for CYP1A, phenobarbital for CYP2B, and rifampicin and phenobarbitone for CYP2C and CYP3A.

Reporter Gene Systems

Since receptors and response elements involved in the regulation of the CYP3A and CYP1A enzymes have been described, reporter gene assays may be used for HTS. Promising reporter gene systems are now being run for CYP3A4 using upstream regulatory elements of the CYP3A4 gene coupled to either a reporter gene for secretory placental alkaline phosphatase [96] or to a reporter gene for chloramphenicol acetyltransferase [8].

8.0 IN SILICO APPROACHES TO DRUG METABOLISM RESEARCH

With many HTS *in vitro* metabolism systems in place or in advanced stages of development, most pharmaceutical companies are now able to rapidly provide empirical data on the metabolic behavior of compounds. Exciting possibilities of using computational methods to model the physico-chemical parameters that make test compounds selectively inhibit, induce or be metabolized by particular enzymes are emerging. Increased understanding of the structure and function of CYPs [15] is making it possible to model ligand-

active site interaction. Earlier attempts to use simple 2D-molecular descriptors like logD, pKa, molecular weight, and number of hydrogen-bond donors and acceptors [97] have not been successful in yielding any useful quantitative structural activity relationships (QSAR). For QSAR, 3D-molecular descriptors were found to be necessary [98]. Preliminary 3D-QSAR pharmacophore models to predict substrates and inhibitors of CYP2C9, 2D6, and 3A4 have been published [99, 100, 101]. Though they show poor predictive power, they have helped to identify problem areas such as the quality of data used to make the models, and that most available modeling software cannot cope with the chemical diversity encountered when dealing with the substrates or inhibitors of a specific CYP. The HTS assays are now being used to generate good quality data which are necessary for the modeling efforts. In our laboratory, we are applying new software which might be able to generate predictive pharmacophore models from the diverse chemicals which are substrates or inhibitors of specific CYPs (Afzelius *et al*., to be published). Combinations of CYP active site modeling with pharmacophore modeling have yielded a good model for CYP2D6 substrates [102].

It could be argued that only when we can assign chemical knowledge to our empirical data on compounds can the communication between drug metabolism scientists and medicinal chemists be fruitful (see Figs. (**1**) and (**3**)). The models will be useful during the lead optimization process by predicting inhibitory potency of compounds in the virtual libraries conjured by combinatorial chemists so that only those with predicted favorable metabolic properties are synthesized. In cases where a particular compound is good with respect to all properties except some metabolism issues, knowledge of why it bears such properties will assist chemists in implementing necessary chemical modifications (Fig. (**3**)).

9.0 CAUTIONARY NOTES ON HTS APPROACHES

Given the exciting prospects arising from HTS approaches in metabolism research, cautionary notes on important methodological and data interpretation hazards are necessary. Limitations in data interpretation for simplified assays have been highlighted in the respective subsections. The order in which different metabolism experiments are carried out and the suggested decision making values illustrated in Figs. (**1**) and (**3**) should be considered as guidelines and not as strict criteria. This is because the importance of the different studies are project dependent, i.e., the type of compounds involved, the disease to be treated, anticipated formulations, and competition at the market. Other practical problems to be considered before embarking on HTS metabolism studies are (i) cytotoxicity of compounds when using whole cell systems, (ii) purity of compounds provided by the medicinal chemist, (iii) adsorption of compounds to incubation vessels, (iv) solubility and stability of compounds under the incubation conditions, (v) effects of solvents on the assay system, and (vi) concerns raised by the FDA [3] on the design of *in vitro* metabolism experiments. Ignoring these factors can render results of the HTS useless. Knowledge of factors that explain some of the failures to make successful in vitro – in vivo correlations are also important when making decisions on what drug-interaction or special population group studies to perform in evaluating the metabolic properties of concern for a NDA [45].

10.0 CONCLUSION

In HTS metabolism studies, the bottleneck is rapidly moving away from data acquisition to data interpretation (Fig (**3**)). Efforts to improve *in vitro – in vivo* correlations [34, 35, 31, 32] will result in optimal use of the *in vitro* data to make decisions on what compounds to develop, obviate the need for or identify specific clinical studies, and reduce the need for animal experiments to predict human pharmacokinetics/toxicology. Prudent use of the *in vitro* HTS assays reviewed in this article should drastically cut costs in reagents and time and hopefully yield products with a competitive advantage on the market by either having very good pharmacokinetics, safety properties and/or informative product labels to assist the physician and patient in the use of the drug.

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