High throughput P450 inhibition screens in early drug discovery

Gregor Zlokarnik, Peter D.J. Grootenhuis and John B. Watson

This review of high throughput (HT) P450 inhibition technologies and their impact on early drug discovery finds the field at a mature stage. The relationship between P450 inhibition and drug–drug interactions is well understood. A wide variety of P450 inhibition detection technologies are readily available off-the-shelf, but what seems still to be missing is a general agreement on how much weight one should give to the various types of early discovery HT P450 inhibition data. Method-dependent potency differences are a cause of concern, and to resolve this issue the authors advocate calibration of the HT methods with a large set of marketed drugs.

Rationale for early compound profiling

Drug discovery is a multi-parameter optimization process in which compounds are optimized for interaction with the desired target and minimal off-target activities, while imparting drug-like properties on the candidate compounds. Project teams often pursue a parallel optimization approach in which multiple scaffolds are explored along multiple axes, including potency, selectivity, physicochemical properties and absorption, distribution, metabolism, excretion and toxicity (ADME–Tox) parameters [1–6]. This is to minimize the risk of making potent and target-class selective compounds in a chemical space that might be entirely incompatible with modifications needed to address other liabilities. Hence, all these molecular activities and properties need to be determined early on many compounds, to progress the overall best leads or scaffolds to the pre-clinical candidate stage. Such an approach requires a high throughput (HT) assay infrastructure, providing all relevant parameters for many, preferably all, members of privileged scaffolds at a reasonable cost and speed. Optimally, all data should be available concurrently with primary-target affinity information.

Multi-parameter optimizations are complex and project teams need to decide early upon which parameters, in addition to potency and selectivity, to focus on and to devise strategies to tackle the optimization challenges. Ideally, some liabilities can be addressed with targeted scaffold modifications that are tolerated with regard to target potency and class selectivity. Examples relating to CYP3A4 inhibition will be presented below.

Case study

As part of ADME–Tox profiling, compounds are routinely tested *in vitro* for the potential to cause drug–drug interactions [7]. These are adverse events in which a drug influences the levels of a co-administered drug, either increasing or decreasing its levels. The most prominent cases involve the inhibition of the drug's metabolism, which can raise drug levels to toxic concentrations [8]. Prominent contributors to drug metabolism are a family of oxidizing enzymes

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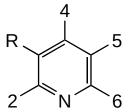
TABLE 1

CYP3A4 inhibition within a series of pyridine-containing analogues



	IC_{50} (μM) with substituent at position:			
Substituent	**2**	**4**	**5**	**6**
H	3	3	3	3
F	71	–	5	>200
OCH_3	>200	7	1	>200
CH_{2OH}	74	–	–	–
NMe_2	13	5	1	–
OH	–	–	10	–
Cl	–	15	–	–
CH_3	–	5	–	–
OCH_2Ph	–	2	–	–

Key: R, side chain; –, not tested.
Table reproduced, with permission, from Ref. [16].

called cytochromes P450 (P450s). These monooxygenases are involved in the metabolism of >60% of all marketed drugs. Cimetidine (Tagamet®, GlaxoSmithKline), a drug for the treatment of heartburn and acid reflux, quickly lost sales to competing products as its drug–drug interaction liability emerged in clinical and post-market approval studies [9]. Mibefradil (Posicor®, Roche) was withdrawn from the market in 1998 because it potently inhibits CYP3A4, the predominant drug metabolizing P450, after having led to adverse events in patients concurrently taking a variety of other commonly prescribed medicines [10]. Alerted to the dire consequences of bringing P450 inhibitors to market, the pharmaceutical industry began testing candidate drugs sooner in discovery to be able to act early and move away from unwanted interactions.

Examples of the use of P450 inhibition data in drug discovery are CYP3A4 inhibitions by two pyridine-attached scaffolds. In the first example, which relies on a small dataset generated by traditional low throughput HPLC-based methods [11], the authors investigated CYP3A4 inhibition by changing the steric hindrance of the pyridine nitrogen via 2- or 6-substitution relative to the pyridine nitrogen, as shown in Table 1. Steric bulk next to the pyridine nitrogen weakens the coordination of the nitrogen to the P450 prosthetic heme iron. This approach is very efficient in changing CYP3A4 activity and several small substituents reduced CYP3A4 inhibition by more than one order of magnitude.

The other example is from an in-house project that employed HT screens to monitor the progress of an optimization for target potency while minimizing CYP3A4 inhibition, shown in Figure 1. The medicinal chemistry effort focused on a 4-substituted pyridine series. The initial 2,6-unsubstituted 4R-pyridines showed a wide spread of target as well as CYP3A4 activities (Figure 1, gray diamonds), with the majority of compounds being sub-micromolar inhibitors of CYP3A4. Although one could argue that a few potent inhibitors of the target had a lower CYP3A4 liability, it was realized that further optimization of these series for other parameters ran the danger of creating compounds with activities closer to the center of the distribution and towards sub-micromolar inhibition of CYP3A4 (Figure 1, region indicated by the arrow). It seemed safer to substitute positions 2 or 6 of the pyridine and indeed, for most compounds, various 2-substituents (Figure 1, blue triangles, yellow circles and green square) reduced CYP3A4 inhibition potency to >1 μM, whereas, for instance, 3-fluoro substitution (Figure 1, red stars) was ineffective in dealing with the CYP3A4 liability. In both examples, the early optimization of the CYP3A4 inhibition parameter in scaffold evolution by targeted substitution removed this factor from the multi-parameter optimization matrix, allowing teams to focus on other issues, probably without the need to revisit CYP3A4 inhibition at a later stage.

In addition to pyridine moieties, terminal imidazoles are notorious for inhibiting multiple P450 isozymes, and azole antifungals as well as the antihistamine cimetidine [9] have been reported to display drug–drug interactions as a result of this activity. To ameliorate the P450–imidazole interactions, substitution patterns on imidazole derivatives have been explored. In an SAR study on imidazoles, 2- and/or 4-substitution led to compounds that are essentially inactive in inhibiting aldrin epoxidation in rat liver microsomes [12]. In humans, however, 2-methylimidazoles are known precursors to reactive metabolites, and potent P450 inhibition has been observed upon metabolism of this moiety [13]. There are several other chemical features that lead to inhibition of P450 enzymes, such as terminal olefins and acetylenes, quinolines, amines, hydrazines, hydrazones and methylenedioxy phenyls, to mention a few [14].

Prediction of potential drug–drug interactions involving lesser-studied P450 isoforms

In a recent publication, Walsky *et al.* [15] demonstrated the use of large P450 inhibition datasets in uncovering the potential of a drug to cause drug–drug interactions as a result of the inhibition of the P450 isoform CYP2C8. They tested the inhibition of CYP2C8 by 209 commonly prescribed drugs, to identify medicines with the potential to interact with other drugs that involve in their metabolism this P450 isozyme, such as paclitaxel, repaglinide, rosiglitazone and cervistatin. Although most tested drugs were not expected to cause clinically relevant drug interactions, montelukast was identified as a potent inhibitor of CYP2C8, with the potential of causing clinically relevant interactions with CYP2C8 drug substrates. Armed with such knowledge, companies should gauge the risk of inhibiting a minor P450 isoform with a new candidate drug. To establish *in vivo* relevance, companies should

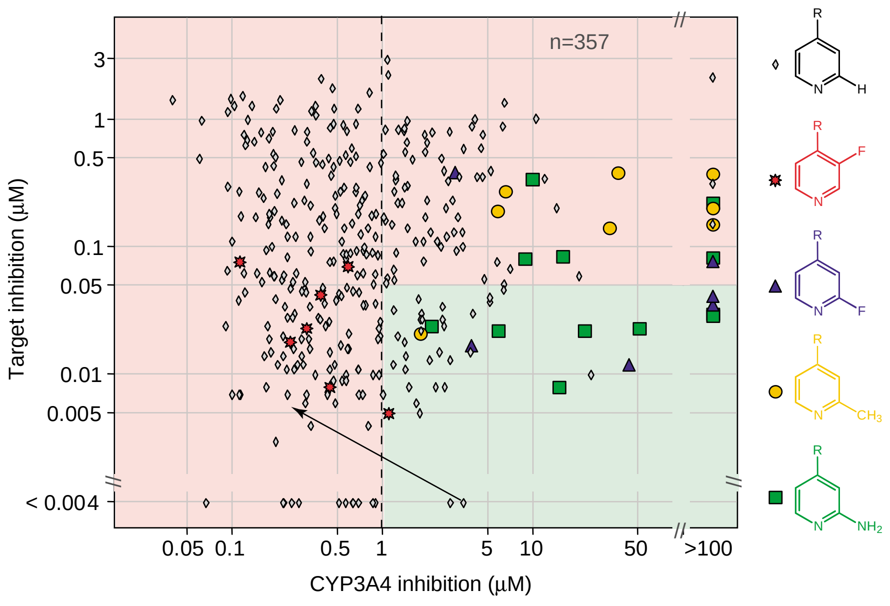


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FIGURE 1

Monitoring of a series of substituted pyridines for CYP3A4 inhibition in a fluorescence HT-CYP3A4 inhibition assay and target potency in early discovery. The fluorogenic CYP3A4 probe was Vivid™3A4 Red (aka BOMR) [38,39]. Unsubstituted pyridines (gray diamonds) show a wide spread of target and CYP3A4 inhibition. Further optimization of these series for other parameters could create compounds with activities closer to the center of the distribution and towards sub-micromolar inhibition of CYP3A4 (as indicated by the arrow). Fluoro substitution far (meta) to the pyridine nitrogen (red stars) are ineffective in dealing with CYP3A4 liability, whereas compounds with substitutions adjacent to the pyridine nitrogen (blue triangles, yellow circles and green squares) have reduced CYP3A4 inhibition potency. The preferred activity zone is colored in green.

conduct in the early clinical stage *in vivo* drug–drug interaction studies with drugs that are likely to be co-administered, therefore limiting the risk of late failures.

Rendic [16] has compiled a comprehensive list of human P450 isozymes and their known metabolic reactions and interactions, incorporating >1000 references published through 2001. Of the 57 named human P450s, only 11, including CYP2C8, have >100 references [17]. As the contribution of the lesser-studied P450s to human drug metabolism becomes clear, similar studies on their inhibition and their potential to be involved in clinical drug–drug interactions will be desirable.

Detection technologies for HT P450 inhibition testing

In the 1990s, the efforts of combinatorial and parallel synthesis required improved assay throughput to find the most potent compounds in increasingly larger compound collections. This task was much aided by the advances in HTS. HTS accelerates compound testing by providing automation and instrumentation that supports parallel processes, such as plate-handling robots, multi-channel fluidics, multi-channel analyzers, high-density assay plates and plate-scanning readers [18–20]. For assays requiring sample fractionation, multi-channel parallel separation cartridges [21], parallel mass-spectrometry (MS) analysis [22] and multiplexed assays as well as analytical sample multiplexing provide increased throughput [23]. Many of these technologies were readily adapted for increased experimentation demands in the ADME–Tox profiling of compounds [23–25].

To detect P450 inhibition, the enzymatic reaction of a P450 (plus cofactors) with the substrate is monitored and the effect of the test compound on the reaction determined. Over the past two decades, P450 inhibition assays evolved from determining substrate metabolism by HPLC-based methods in individual reactions to detecting optical signal changes indicative of substrate metabolism in parallel experiments, in plate-based formats and without the need for sample separation or purification. Traditional HPLC-based methods have also received throughput upgrades. Enhancements include multiplexing of P450 isoform probe substrates and analyses of the probe–metabolite cocktail, affording simultaneous inhibition experiments on several P450 isoforms in the same sample [26–28] and tandem liquid chromatography–mass spectrometry (LC–MS–MS) analysis, which shortens analytical run times significantly. Table 2 provides a comparison of these methods with higher throughput radioactivity and optical methods.

Radioactivity-based methods

Radioactivity-based methods rely on ^{3}H- or ^{14}C-labeled drug substrates, which are selectively oxidized by the P450 isozyme of interest [29]. The metabolite is separated from the remaining substrate by HPLC and quantified via radiochemical detection. In some methods P450-mediated oxidation causes substrate demethylation with liberation of ^{14}C-formaldehyde, which can be extracted and detected by radioluminescence in a microplate scintillation counter [26,30]. To avoid the separation step, a scintillation proximity assay version of this method has also been developed. Here, the scintillant-containing beads are impregnated with polyethylenimine to bind the ^{14}C-formaldehyde, formed in the metabolic reaction. The proximity of the bound ^{14}C-formaldehyde results in efficient excitation of the scintillant with light emission [31]. An advantage of these radioactive assays is the ability to use known drug substrates with good solubility and well-characterized kinetics, although they generate radioactive waste, which requires subsequent decontamination and disposal.

Fluorescence-based methods

Fluorogenic P450 substrates were first studied in the early 1970s. These, and more recently developed substrates,

TABLE 2

P450 inhibition screening methods

Method	**HPLC Methods**	**Radioactivity**	**Fluorescence**	**Luminescence**
Substrates	Selective drugs	^{3}H- or ^{14}C-labeled drugs	Fluorogenic probes	Luciferin derivatives
Product	Drug metabolites	Extractable radiolabel	Fluorescent dye	Luciferin (light)
Detector	Mostly UV or MS	Scintillation counter	Fluorescence reader	Luminescence reader
P450 source	Liver microsomes or recombinant	Liver microsomes or recombinant	Mostly recombinant	Recombinant
Addition only assay	No	Mostly no	Yes	Yes
Non-coupled assay	Yes	Yes	Yes	No
Throughput	Medium-high	High	Very high	Very high
Market penetration	Full	Partial	Substantial	Nascent
FDA accepted	Yes	No	No	No

combined with recombinantly expressed P450 isoforms [32], formed the basis for facile P450 inhibition testing. An early provider and vendor of this technology was Gentest Corporation (now BD-Biosciences). The assays employ low-fluorescence substrates, which are converted to more-fluorescent metabolites. The progression of the reaction is measured by an increase in fluorescence intensity during P450 metabolism of the substrate. Inhibitors of a particular P450 reduce the rate of metabolism of the substrate and this results in a reduced fluorescence signal. Advantages of these assays are the ability to use 96-well and higher-density formats, addition-only protocols, the availability of a large number of substrates and the lack of radioactive waste [33–37]. These low-cost methods mostly rely on recombinant sources of P450 enzymes, as most fluorescent probes are not sufficiently P450 isoform-selective to be used with liver microsomal preparations. For compounds that are very fluorescent at the wavelength employed in the assay, P450 inhibition measurements cannot be obtained. More recently, Aurora Biosciences (now Vertex) [38] has developed more-sensitive substrates that produce larger fluorescence signal changes and are, therefore, less sensitive to optical interference [39,40] (commercial source of Vivid™ P450 substrates: Invitrogen, Carlsbad, CA, http://gcms.invitrogen.com/downloads/vivid.pdf).

Bioluminescence-based methods

The most recent technology developed for P450 inhibition is based on substrates that release luciferin as the metabolite (commercialized by Promega, Madison WI, www.promega.com/cnotes/cn007/cn007_02.htm, www.promega.com/cnotes/cn010/cn010_06.htm) [41]. This is a coupled assay system in which addition of luciferase and ATP converts the freed luciferin to des-carboxyluciferin with light emission. The format is similar to fluorescence methods, requiring addition-only manipulations and employing a luminescence plate reader instead of a fluorescence plate reader. This assay also requires use of recombinant sources of P450 enzymes, as the probes are not sufficiently P450 isoform-selective to be used with human liver microsomes. A potential source for false positives in these assays can be compounds that interfere with light generation directly, such as compounds that interfere with luciferase enzymatic activity.

Any given organization's choice of P450 inhibition technology is driven by the work flow in discovery, the existing in-house expertise and instrumentation and the confidence in the validity and accuracy of any of the new technologies. Undoubtedly the latter is a major driver in instrument development to provide increased throughput for conventional methods, which are well established and have received FDA approval (www.fda.gov/cder/guidance/clin3.pdf) [7]. P450 screens developed for HT rely on high-density plate formats, low assay cost, little compound consumption and assay robustness. The probe substrates employed give large signal changes but typically have low P450 isoform selectivity and therefore require a preparation with individual expressed P450 isoforms. The methods readily allow routine broad P450 inhibition profiling including, if so desired, screening of entire compound libraries for P450 liabilities.

Finally, recent progress in computational methods for prediction of P450 compound interactions needs to be mentioned [42–44]. *In silico* screening has been very appealing as, with the increasing power of desktop computing screening, cost could become negligible. Virtual compounds could be screened for P450 liabilities, reducing the numbers of compounds with P450 liabilities a team would otherwise synthesize, providing additional savings [45–48]. In a recent publication [49], scientists working on a κ-opioid receptor agonist series demonstrated how molecular docking of candidate compounds into a homology model of CYP2D6 in conjunction with compound testing in a HT-CYP2D6 fluorescence inhibition assay efficiently guided medicinal chemistry efforts away from the CYP2D6 liability. As a major advance, the crystal structures of several human P450 isozymes have been solved [50–52]. These structures provide more-accurate geometries of the enzymes' active sites and should lead to better representations than previous homology models that were based on crystal structures of soluble bacterial enzymes [43,53].

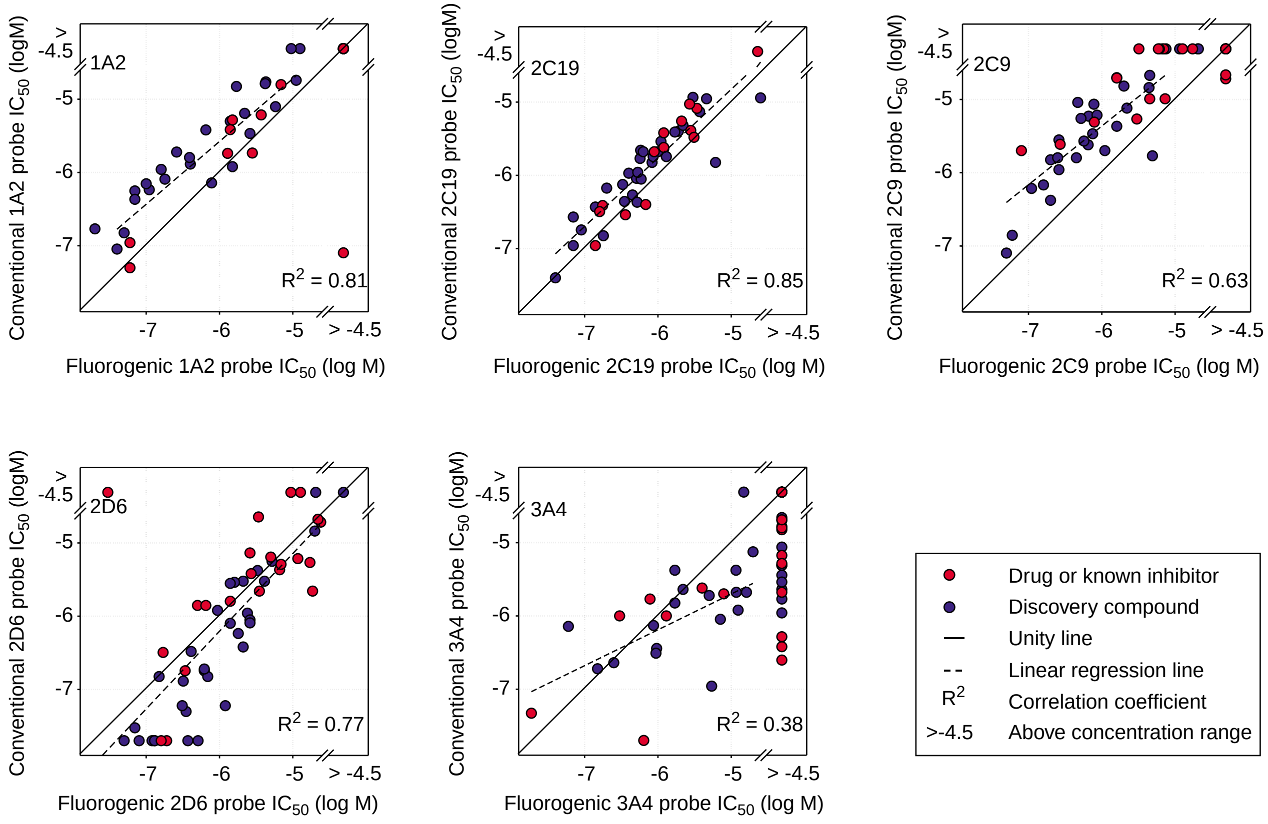

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FIGURE 2

Fluorescence HT P450 inhibition methods compared with HPLC-based methods. The Figure is based on data from Ref. [40], converted from linear to logarithmic values. It compares the log IC50 values for compounds determined with traditional drug (ordinate) and fluorogenic substrates (abscissa) for five of the human P450 enzymes. The solid diagonal line represents unity and the log linear regression (represented by the dotted line) was performed with omission of data outside the measurement range (data at >30 μM were omitted from the fit).

A staged implementation of drug–drug interaction screening methods has been proposed, in which computational models for P450 K_i prediction are deployed at the stage of virtual, *in silico* screening. During the 'hit-to-lead' stage, HT P450 inhibition screens provide guidance and teams would rely on the gold-standard HPLC-based approaches during the lead optimization phase of discovery [54].

Calibrating HT P450 inhibition screens

For several reasons, HT methods relying on fluorescence and luminescence substrates with recombinant P450 enzymes do not provide the same answers as assays based on selective drug substrates and liver microsomal preparations [55]. For P450 isoforms 1A2, 2C19 and 2D6, the differences might be attributable to enzyme preparations, whereas CYP2C9 and CYP3A4 assays also display substrate-dependent inhibition differences [36,56,57]. But differing results are an issue not only for screens using recombinant preparations. Results obtained with traditional methods, which use the same probe substrate under similar conditions, can vary substantially between laboratories [58], as a result of P450 isozyme variations in liver microsomal preparations [59,60] and differing microsomal concentrations in the assays [61].

A publication that questioned the quality of data obtainable in fluorescence P450 inhibition assays [40] compared data from several of these assays with traditional methods (Figure 2) on a series of drugs and xenobiotics (red circles) and in-house discovery compounds (blue circles). Figure 2 displays the data from this publication plotted on a log scale to show the correlation of the data, with the solid diagonals indicating unity and the dotted lines the linear fit of the log data (data at >30μM are omitted from fit). Potency trends between methods are quite similar for CYP1A2, 2C9, 2C19 and 2D6 data, whereas CYP3A4 inhibition data are less well correlated. Importantly, for the well-correlated assays, there is an average shift in potency. For CYP1A2, 2C9 and 2C19, the enzyme seems more readily inhibited in the recombinant assay using the fluorescence probe than in the liver microsomal preparations, similar to trends that have been seen with traditional probe substrates in differing enzyme preparations [6,30,55]. Hence, for calibration purposes, it is recommended to perform such an analysis on HT screens that employ

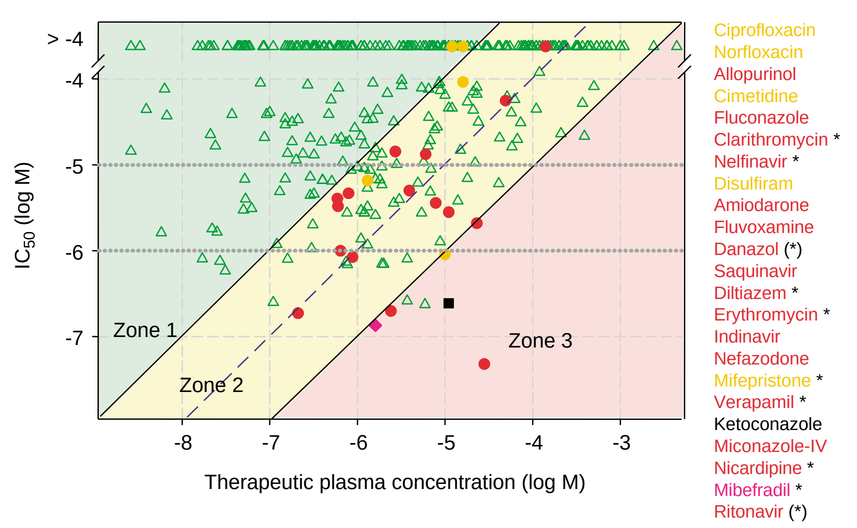

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FIGURE 3

CYP3A4 inhibition potencies of 337 drugs plotted against their highest human therapeutic plasma concentrations. The values for therapeutic plasma concentrations were taken from Ref. [65]. Inhibition of CYP3A4 was assessed with a fluorescence HT P450 inhibition screen (Vivid™3A4 Red, aka BOMR, [38,39]). Drugs without any known CYP3A4 inhibition-related drug–drug interactions are depicted with open green triangles. The remaining drugs, denoted in other symbols and colors, do display such interactions. The identities of these drugs are listed in increasing order of potency of CYP3A4 inhibition (from Ciprofloxacin >100 µM to Ritonavir at 50 nM). An asterisk next to the drug name indicates a reported contribution of a non-competitive mechanism to CYP3A4 inhibition *in vivo*. The blue dashed line represents unity, which means that drugs on the line have the same CYP3A4 IC_{50} as their maximum therapeutic plasma concentration. Zones 1–3 indicate areas in which drugs are below (1), within (2) or above tenfold (3) in potency for CYP3A4 inhibition, compared with their therapeutic concentrations.

recombinant assay preparations. The situation for the CYP3A4 inhibition assays is more complex, as it has been demonstrated that the choice of the enzyme as well as the substrate can lead to different results [62–64].

Relevance of HT CYP data to the clinical setting

An additional study was performed to demonstrate the relevance of HT P450 inhibition data as it relates to *in vivo* results in human patients. In this analysis, the assay that Vertex scientists used to determine CYP3A4 inhibition is about tenfold more sensitive to inhibitors than traditional human liver microsome-based methods with testosterone as probe substrate, similar to the findings for the fluorescence CYP1A2 and CYP2C9 assays mentioned above. The CYP3A4 inhibition potencies for 337 systemic drugs were determined and the results graphed in relation to their highest therapeutic plasma levels (C_{max} or highest therapeutic concentration reported for each drug [65]; Figure 3). Of these, 314 medications do not have any known CYP3A4 inhibition-related drug–drug interactions (Figure 3, green triangles). Of the remaining 23, one is Mibefradil (Posicor®, Roche, Figure 3, purple diamond) [15], which was withdrawn from the market. An additional 17 drugs are known to increase cyclosporine A and/or simvastatin levels due to CYP3A4 inhibition (Figure 3, red filled circles) [66,67]. The remaining 5 drugs show other CYP3A4-related drug–drug interactions (Figure 3, yellow filled circles, http://medicine.iupui.edu/flockhart/).

Figure 3 indicates that, as would be expected for a predictive screen, few drugs are administered at higher plasma concentrations than the CYP3A4 inhibition IC_{50} concentration determined. Drug candidates that have tenfold or higher plasma concentrations than the determined HT CYP3A4 IC_{50} are very likely to receive a black box warning label or could fail to reach the market (Figure 3, zone 3). Drug candidates that have therapeutic plasma levels within tenfold of the HT CYP3A4 IC_{50} have a moderate chance of interacting with other marketed drugs (Figure 3, zone 2), but drug candidates with therapeutic plasma levels of less than 10% of the HT CYP3A4 IC_{50}s are likely to be safe with respect to these types of drug interactions (Figure 3, zone 1). This shows that the HT CYP3A4 inhibition data can provide guidance in the selection of drug candidates with lower development risk.

One might wonder why in the assay drugs that display an apparent K_i ten times below their total plasma concentrations would display clinically significant drug–drug interactions. This might seem particularly puzzling for drugs that are highly bound to plasma proteins. Following are some possible explanations for the finding. For drugs administered orally, one would expect a decreasing gradient of total concentrations from the gut to the pre-hepatic circulation, the liver, the lungs, and to the systemic circulation, at which location drug plasma levels are typically monitored [68,69]. Metabolism by CYP3A4 in the gut contributes significantly to the first-pass clearance of many drugs. The concentration of a co-administered CYP3A4 inhibitor in the gut would likely be underestimated by systemic plasma concentrations. It is also known that many drugs accumulate in the liver at much higher levels than found in plasma [59,70,71]. Some drugs are metabolized to inhibitory species in the liver, which concentrations and potencies are not related to K_i values and plasma concentrations of the parent drug. Also, some drugs act as mechanism-based inhibitors of CYP3A4 [72]. Turnover of these drugs with CYP3A4 inactivates progressively and irreversibly the enzyme and the liver has to synthesize new enzyme to recover CYP3A4 activity. Such drugs can appear more potent *in vivo* than in the HT inhibition assay P450, because *in vivo* exposure to a drug lasts in the order of hours to days, whereas the enzyme in the HT assay is exposed to compound on the timescale of minutes [71]. In Figure 3, drugs that display a mechanism-based component to CYP3A4 inhibition are indicated in the list with an asterisk.

In early discovery, the likely upper limit of the therapeutic human plasma concentration of the drug candidate will not be known. Yet, to avoid late failures due to drug–drug interactions, a risk assessment needs to be performed early. Some companies suggest guidelines for CYP3A4 inhibition, which are used to assign risk [73]. Compounds with potent CYP3A4 block in traditional inhibition assays, for instance with K_i values below 1 μM, are classified as high risk, compounds between 1 and 10 μM are assigned to moderate risk, whereas compounds with K_i values above 10 μM are classified as lower risk. With calibration of the screens, HT data can be assigned to analogous risk brackets. In the analysis presented in Figure 3, 95% of drugs have CYP3A4 inhibition values of >1 μM in the recombinant HT CYP3A4 inhibition screen (above lower dotted gray line), whereas ~80% are less potent than 10 μM (above upper dotted gray line).

Conclusions

Many HT technologies are now available to detect P450 inhibitors, which should decrease the number of withdrawals of novel drugs from the market due to inhibition of the major P450 isozymes. HT P450 data can be used to guide medicinal chemistry away from these interactions in an early stage and in certain cases might entirely solve the issue by targeted modification of the P450 interacting functionality, leading to a reduced dimensionality of the drug optimization process. To be generally useful, P450 inhibition screens need to be calibrated against standard methods and preferably also tested with a large set of drugs, for which the human drug–drug interaction outcome is known.

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