The impact of non-genetic factors on drug metabolism: towards better phenotype predictions

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Section III

In vivo tools to study alterations in drug metabolism during (inflammatory) disease

Chapter 6

Changes in plasma clearance of CYP450 probe drugs may not be specific for altered in vivo enzyme activity under (patho)physiological conditions – how to interpret findings of probe cocktail studies

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Abstract

Background and objective: CYP450 (CYP) phenotyping involves quantifying an individual's plasma clearance of CYP-specific probe drugs, as a proxy for in vivo CYP enzyme activity. It is increasingly applied to study alterations in CYP enzyme activity under various (patho)physiological conditions, like inflammation, obesity, or pregnancy. The phenotyping approach assumes that changes in plasma clearance of probe drugs are driven by changes in CYP enzyme activity. However, plasma clearance is also influenced by protein binding, blood-to-plasma ratio, and hepatic blood flow, all of which may change under (patho)physiological conditions.

Methods: Using a physiologically-based pharmacokinetic (PBPK) workflow, we aimed to evaluate whether the plasma clearance of commonly used CYP probe drugs is indeed directly proportional to alterations in CYP enzyme activity (sensitivity), and to what extent alterations in protein binding, blood-to-plasma ratio, and hepatic blood flow observed under (patho)physiological conditions impact plasma clearance (specificity).

Results: Plasma clearance of CYP probe drugs is sensitive to alterations in CYP enzyme activity, since alterations in intrinsic clearance between -75 and +150% resulted in near-proportional changes in plasma clearance, except for midazolam in case of > 50% CYP3A4 induction. However, plasma clearance also changed near-proportionally with alterations in the unbound drug fraction, diminishing probe specificity. This was particularly relevant for high protein-bound probe drugs, as alterations in plasma protein binding resulted in larger relative changes in the unbound drug fraction. Alterations in the blood-to-plasma ratio and hepatic blood flow of ±50% resulted in plasma clearance changes of less than ±16%, meaning they limitedly impacted plasma clearance of CYP probe drugs, except for midazolam. In order to correct for the impact of non-metabolic determinants on probe drug plasma clearance, an R script was developed to calculate how much the CYP enzyme activity is actually altered under (patho)physiological conditions, when alterations in the unbound drug fraction, blood-to-plasma ratio and/or hepatic blood flow impact probe drug plasma clearance as well.

Conclusions: As plasma protein binding can change under (patho)physiological conditions, alterations in unbound drug fraction should be accounted for when using CYP probe drug plasma clearance as a proxy for CYP enzyme activity in patient populations. The tool developed in this study can support researchers in determining alterations in CYP enzyme activity in patients with (patho)physiological conditions.

Introduction

CYP450 (CYP) phenotyping is an important tool to characterize an individual's CYP enzyme activity (1). It involves the quantification of an individual's plasma clearance upon administration of a CYP-specific probe drug, as a proxy for individual in vivo CYP enzyme activity. Various probe drugs for evaluating the activity of different CYP isoforms have been utilized, administered either individually or combined in a 'phenotyping cocktail' (2,3). Among the human CYP enzymes, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 metabolize more than 85% of drugs administered to patients and the assessment of their activity is therefore an integral component of phenotyping assays. In this approach, CYP enzyme activity is derived from the estimated plasma clearance of a probe drug that is primarily metabolized by the respective CYP enzyme, such as midazolam for CYP3A4 or dextromethorphan for CYP2D6. Alternatively, the metabolic ratio, representing the ratio between the CYP-specific metabolite and the unaltered parent compound, is determined, as it is a less resource-intensive surrogate marker for plasma clearance. Findings on changes in plasma clearance or metabolic ratios are subsequently used to quantify how the factors that are studied, i.e. drug–drug interactions or genotype, impact drug metabolism. This methodology is now also increasingly used to explore the impact of (patho) physiological conditions such as inflammation, obesity, or pregnancy on in vivo CYP enzyme activity (4–6).

The implicit assumption made when utilizing the CYP phenotyping approach is that differences in plasma clearance of the CYP probe drugs are sensitive and specific to changes in the enzyme activity of the CYPs they represent. This enzyme activity is generally quantified as intrinsic clearance (CL_{int}). This means that changes in plasma clearance of the CYP probes are proportional to changes in CL_{int} of the CYP of interest, and that plasma clearance is insensitive to alterations in other physiological parameters. In traditional pharmacokinetic interaction studies investigating drug–drug and/or drug–gene interactions in healthy volunteers, this assumption may be reasonable; however, when studying the impact of (patho) physiological conditions on enzyme activity, this assumption is challenged by the fact that besides the CYP enzyme activity (CL_{int}), plasma clearance of probe drugs may also be influenced by other non-metabolic determinants, including the fraction of drug that is not bound to plasma protein (fu), the blood-to-plasma ratio

(B/P), and the hepatic blood flow (Qh) (7). Indeed, alterations in the abundance of drug binding plasma proteins that impact protein binding, hematocrit levels that impact B/P, and the cardiac output (CO) that drives Qh have been observed in a range of pathophysiological conditions such as cancer, rheumatoid arthritis (RA) and coronavirus disease 2019 (COVID-19) infection (8–10). As a result, CYP phenotyping studies that aim to assess the consequences of (patho)physiological conditions on CYP enzyme activity could be compromised by simultaneous changes in fu, B/P, and/or Qh, in addition to changes in CL_{int}. In this context, CL_{int}, fu, and B/P are composite parameters that are impacted by both drug-specific and system-specific factors (Figure 1). When studying conditions for which (patho)physiological changes in these non-metabolic determinants may occur, an important question is to what extent clinical CYP phenotyping results from probe drug studies reliably reflect changes in CYP enzyme activity, and to what extent (patho)physiological changes in fu, B/P, and Qh affect the clearance of probe drugs used to phenotype CYP enzyme activity. It is conceivable that (patho)physiological conditions may impact the phenotyping probe drug metrics by mechanisms beyond changes in CL_{int} only.

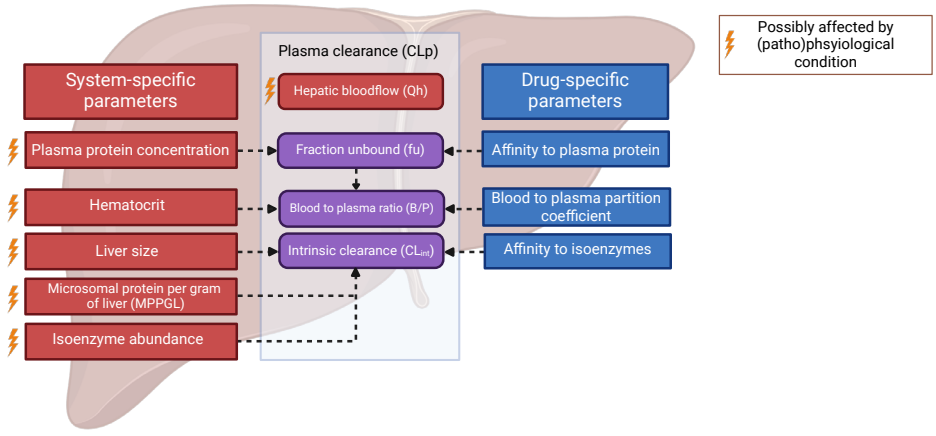

Figure 1 Illustration of how system-specific parameters (red boxes) and drug-specific parameters (blue boxes) drive hepatic plasma clearance (CLp) (white square). Plasma clearance (CLp) of probe drugs is driven by the four parameters presented in the white center square. Purple parameters are influenced by both system-specific and drug-specific parameters. During (patho)physiological conditions, alterations may occur in one or more system-specific parameters, symbolically depicted by a lightning bolt. All these alterations have the potential to affect the plasma clearance of probe drugs.

In this study, we investigate whether the plasma clearance of commonly used CYP phenotyping probe drugs is directly proportional to alterations in CYP enzyme activity, CL_{int} (i.e., probe sensitivity), and to what extent fu, B/P, and Qh impact the plasma clearance (i.e., probe specificity). As delineating and quantifying the impact of alterations in either CL_{int}, fu, B/P, or Qh on plasma clearance is not possible in human subjects, physiologically based pharmacokinetic (PBPK) modeling principles are applied, enabling a univariate analysis of how changes in each parameter separately, i.e. CL_{int}, fu, B/P, or Qh, affect the plasma clearance of CYP probe drugs. As an illustration, we explore how changes in fu, B/P, or Qh observed in three inflammatory conditions, including chronic inflammation during RA, surgery-associated acute inflammation, or acute COVID-19 infection, impact the plasma clearance of probe drugs. We thereby provide insights and tools that are necessary to interpret the results of CYP phenotyping studies obtained in (patho)physiological conditions correctly, and can help clinicians and clinical pharmacologists to conclude whether a probe drug is suitable to predict altered CYP enzyme activity in conditions that might impact fu, B/P, and Qh.

Materials and methods

The PBPK-based workflow illustrated in Figure 2 was used to simulate the plasma clearance of CYP phenotyping probe drugs.

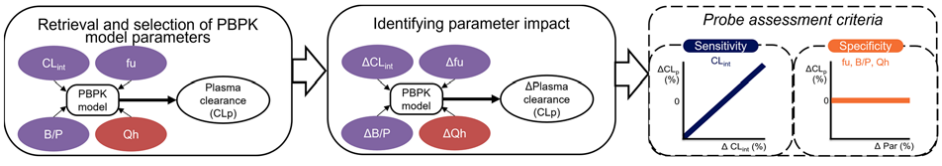

Figure 2 Applied PBPK-based workflow. Parameter values of CL_{int}, fu, B/P, and Qh of the 13 studied probe drugs were obtained for the PBPK model to predict hepatic CLp. Each parameter value was subsequently changed univariately to assess the impact of each parameter change on the predicted plasma clearance. Probe sensitivity was defined as a change in plasma clearance that is proportional to a change in CL_{int}. Probe specificity was defined as an absence of change in plasma clearance with changes in fu, B/P, and Qh. System-specific parameters are shown in red; parameters that are influenced by both drug- and system-specific parameters are shown in purple. PBPK physiologically based pharmacokinetic, CL_{int} intrinsic clearance, fu fraction unbound, B/P blood-to-plasma ratio, Qh liver bloodflow, CLp plasma clearance.

Cytochrome P450 (CYP) probe drugs

Thirteen probe drugs commonly used in phenotyping drug cocktails, representing selective substrates for the six most clinically relevant CYP enzymes, were selected (Online Resource 1 Table 1). This yielded the following CYP enzyme–probe drug combinations for the analysis: CYP3A4: midazolam and quinine; CYP2D6: dextromethorphan and metoprolol; CYP2C19: omeprazole; CYP2C9: diclofenac, flurbiprofen, losartan, s-warfarin and tolbutamide' CYP2B6: bupropion and efavirenz; and CYP1A2: caffeine.

Physiologically based pharmacokinetic (PBPK) model for plasma clearance

Hepatic plasma clearance (CLp) was calculated using the dispersion model (Eqs. 1–4), which has been shown to predict plasma clearance well for drugs with both high and low hepatic extraction ratios (11,12). This model was implemented in R version 4.4.1 (The R Foundation for Statistical Computing, Vienna, Austria).

$$CLp = (Qh \cdot E_H) \cdot B/P \tag{1}$$

$$E_H = 1 - \frac{4a}{(1+a)^2 \cdot e^{\frac{a-1}{2D_N}} - (1-a)^2 \cdot e^{-\frac{a+1}{2D_N}}} \tag{2}$$

$$a = \sqrt{1 + 4R_N \cdot D_N} \tag{3}$$

$$R_N = \frac{fu}{B/P} \cdot \frac{CLint}{Qh} \tag{4}$$

where E_H is the hepatic extraction ratio; D_N is the axial dispersion number, which is set to 0.17 (13); R_N is the efficiency number, which quantifies how effectively a drug is extracted from the blood as it flows through the liver; and CL_{int}, Qh, and CLp are expressed in the same units (mL/min).

Retrieval and selection of PBPK model parameters

The systems-specific parameter Qh and the composite drug- and system-specific parameters CL_{int}, fu, and B/P were extracted from published sources. CL_{int} values describing CYP-specific metabolite formation were obtained from in vitro studies in human liver microsomes (HLMs), either by extracting the reported microsomal CL_{int} ($CL_{int,mic}$) values or calculating $CL_{int,mic}$ by using the kinetic parameters Vmax (in pmol/min/mg) and Km (in μM), as described in Eq. 5.

$$CL_{int,mic} = \frac{Vmax}{Km} \quad (5)$$

For each probe drug, the average $CL_{int,mic}$ (in mL/min/mg microsomal protein) was calculated from multiple studies. Subsequently, $CL_{int,mic}$ values were scaled to whole liver CL_{int} (in mL/min) by using the average weight of a human liver and the milligram protein per gram of liver (MPPGL), as described in Eq. 6.

$$CL_{int} = MPPGL \cdot Weight_{liver} \cdot CL_{int,mic} \quad (6)$$

The fu values were obtained from either the Summary of Product Characteristics (SmPC), available from the European Medicines Agency (EMA), or from the US Food and Drug Administration (FDA) drug label. Due to the considerable variability and experimental challenges associated with accurately determining fu values, an fu range is often reported. The average of this range was initially selected. B/P ratios were obtained from the literature or assumed to be 1 when unavailable.

To ascertain that the clearance predictions by the PBPK model (Figure 2) based on the retrieved parameters for CL_{int}, fu, and B/P were in line with clinically observed plasma clearance values, reported plasma clearance values from studies in healthy volunteers were extracted from the literature for comparison. Since the PBPK model exclusively predicts hepatic metabolic clearance by the primary metabolizing isoenzyme, the comparison involved multiplying the reported clinical plasma clearance by the fraction of the drug eliminated through the main CYP-specific metabolic pathway. In case the initially retrieved parameters yielded a fivefold difference or more in prediction of plasma clearance, changes were made to the parameter values with most uncertainty and with the reported range of values in the literature. This meant that fu was adapted first to a value within the range reported in the SmPC/FDA drug label that yielded a plasma clearance prediction that aligned with the clinically reported plasma clearance that was adjusted for the potential presence of additional clearance routes as described above. If alignment between observed and predicted plasma clearance could not be achieved by changing the fu within the reported range, the $CL_{int,mic}$ values were adapted within the reported range in HLMs to yield accurate PBPK-predicted plasma clearance.

The final parameter values obtained, including their references, and the evaluation of the predicted plasma clearance can be found in Online Resource 1 Tables 2–5, and Online Resource 2.

Identifying parameters that impact the plasma clearance of probe drugs

To examine the percentage change in plasma clearance (ΔCLp) as a function of univariate changes in each input parameter, CL_{int} and fu were varied over a range of -90% to +150%, and B/P and Qh over a range of -50% to +50%. These ranges were chosen to reflect clinically relevant changes in the parameters. Because the fu cannot exceed 100%, the fu values of metoprolol and caffeine were only increased with a maximum of 7% and 56%, respectively (i.e. fucaffeine = 0.64, therefore the maximal percentual increase is $(\frac{1-0.64}{0.64}) * 100 = 56\%$

For a probe drug to be considered sensitive to changes in CL_{int}, plasma clearance ideally changes proportionally with changes in CL_{int}, as illustrated in the 'Sensitivity' panel of Figure 2. On the other hand, to be considered specific, an ideal probe should be insensitive to changes in fu, B/P, or Qh, yielding a desired sensitivity criterion of ΔCLp being close to zero across the full range of parameter changes, as illustrated in the 'Specificity' panel of Figure 2.

Assessment of probe specificity in the context of inflammatory diseases

We used three inflammatory conditions, i.e. chronic inflammation (RA), acute inflammation (surgery), and acute infection (COVID-19) to assess the suitability of phenotypic probe drugs for the quantification of alterations in enzyme activity under pathophysiological changes in fu, B/P, or Qh. Alterations in parameters fu, B/P, and Qh during RA, surgery, and COVID-19 were extracted from the literature, or, alternatively, when no reported values could be retrieved from the literature, assumptions were made to derive inflammation-induced changes in parameter values. For fu, it was assumed that changes in protein binding of all probe drugs during inflammation were fully dependent on changes in human serum albumin (HSA) concentrations and not on changes in any other drug binding proteins (Eq. 7).

$$fu_{inflammation} = \frac{1}{1 + \frac{(1 - fu_{healthy}) \cdot [HSA_{inflammation}]}{[HSA_{healthy}] \cdot fu_{healthy}}} \quad (7)$$

Inflammation-induced change in B/P were assumed to be only dependent on changes in hematocrit and assessed independently from the change in fu (Eq. 8).

$$B/P_{inflammation} = 1 + Hematocrit_{inflammation} \cdot \left(fu_{healthy} \cdot k_p - 1\right) \quad (8)$$

To calculate Qh alterations during inflammation, it was assumed that the fraction of cardiac output (CO) directed to the liver is similar as for healthy volunteers (Eq. 9).

$$Qh_{inflammation} = 0.25 \cdot CO_{inflammation} \quad (9)$$

When multiple parameter values of HSA, hematocrit, or CO were found, the most extreme reported value was selected to reflect worst-case scenarios. The retrieved and derived pathophysiological changes in fu, B/P, and Qh during chronic inflammation in RA, surgery-related acute inflammation, and acute COVID-19 infection are listed in Online Resource 1 Tables 6 and 7.

Results

Parameters that impact the plasma clearance of probe drugs

The PBPK workflow was used to identify which parameters affect plasma clearance of CYP phenotyping probe drugs. Plasma clearances of quinine, omeprazole, diclofenac, flurbiprofen, losartan, s-warfarin, tolbutamide, efavirenz, and caffeine are highly sensitive to alterations in CL_{int}, showing a proportional change in plasma clearance when decreasing or increasing CL_{int} values over a range of -90% to +150% (Figure 3a). For midazolam, dextromethorphan, metoprolol, and bupropion, decreasing CL_{int} leads to a proportional decrease in plasma clearance, but when the CL_{int} increases more than 50%, these probe drugs exhibit a disproportionally smaller increase in plasma clearance. This disproportional relationship between CL_{int} and plasma clearance is most evident for midazolam, considering an increase of 150% in CL_{int} results in an increase in plasma clearance of only 41%.

Plasma clearance of all probe drugs was also found to be sensitive to both increases and decreases in fu (Figure 3b). On the contrary, alterations in B/P and Qh had minimal impact on plasma clearance of most probe drugs, as indicated by a horizontal line for quinine, omeprazole, diclofenac, flurbiprofen, losartan, s-warfarin, tolbutamide, efavirenz and caffeine (Figure 3b). For midazolam, dextromethorphan, metoprolol, and bupropion, alterations in B/P or Qh led to slight changes in plasma clearance, with a maximum decrease in plasma clearance of -35% at a -50% parameter change for midazolam. Thus, our results highlighted

that plasma clearance of probe drugs is sensitive to alterations in CL_{int}, but might not be specific to alterations in CL_{int} only, considering alterations in the fu also impact plasma clearance.

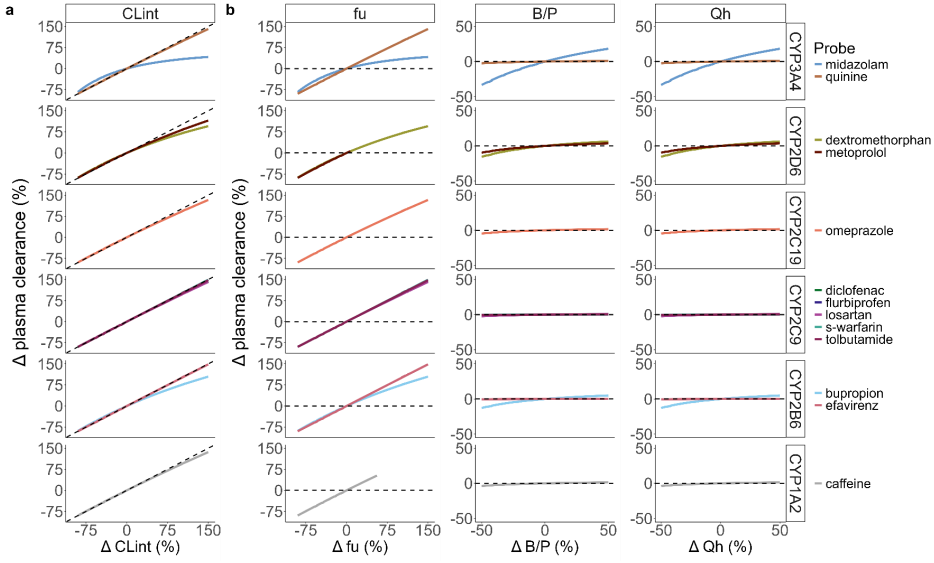

Figure 3 PBPK-predicted change in plasma clearance resulting from the univariate changes in each of the indicated parameters. As absolute parameter values differ for each drug, relative changes are depicted, with a value of 0 reflecting no change compared with the value representing a healthy state. Results are presented per parameter (columns) and per cytochrome P450 isoenzyme (rows). The dotted lines indicate the patterns for ideal probe drugs for phenotype assessment [i.e., **(a)** plasma clearance changes that are proportional to change in CL_{int} (sensitivity), and **(b)** no plasma clearance changes with changes in fu, B/P, and Qh (specificity)]. Please note the different axes between panels for CL_{int} and fu compared with B/P and Qh.

Probe specificity in the context of inflammatory diseases

Alterations in fu that occur during inflammation might impact the specificity of probe drug plasma clearance as a proxy for CYP enzyme activity. Figure 4 shows the probe-specific relative changes in fu for the three selected inflammatory diseases. Evidently, relative changes in fu are dependent on the initial degree of protein binding of the probe drug, as well as the inflammatory condition. The alterations in fu were highest for acute COVID-19 infection, followed by chronic inflammation in RA, and least for surgery-related acute inflammation. The relative changes in fu are smaller for probe drugs with low protein binding, i.e., metoprolol and

caffeine, with Δfu between 3 and 4% for metoprolol and between 16 and 21% for caffeine, depending on the inflammatory condition. The fu alterations were larger for the intermediate protein bound drugs dextromethorphan and quinine, with a maximal fu change of 64% for quinine and 46% for dextromethorphan during acute COVID-19 infection. Relative changes in fu during chronic inflammation (RA), acute inflammation (surgery), and acute infection (COVID-19) were largest for the high protein bound drugs midazolam, omeprazole, diclofenac, flurbiprofen, losartan, s-warfarin, tolbutamide, bupropion, and efavirenz.

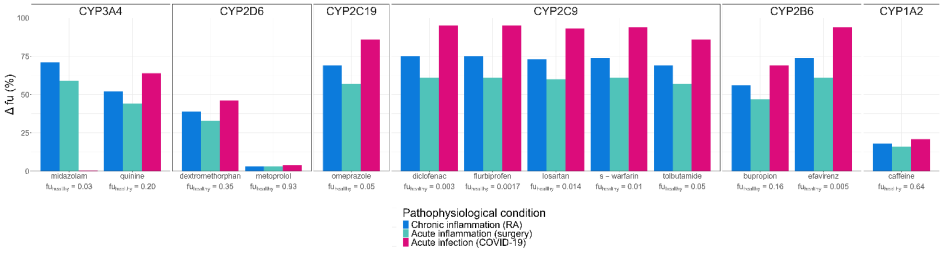

Figure 4 Percentage change in fu during three inflammatory conditions: chronic inflammation (RA), acute inflammation (surgery), and acute infection (COVID-19) for all probe drugs per CYP isoenzyme. Indicated below the probe drug is the fu in a healthy state, showing which probe drugs are low (< 0.3), intermediate (0.3–0.7), or high (> 0.7) protein-bound. Due to the absence of reported changes in fu for all drugs and all conditions, except for midazolam during acute infection, the changes in fu are derived from reported changes in albumin concentration.

Figure 5 highlights how the univariate changes in fu observed during RA, surgery, and COVID-19 impact the plasma clearance of either a low-protein bound, intermediate protein-bound, or high protein-bound probe drug. The impact of alterations in fu on plasma clearance of a probe drug is dependent on its initial degree of protein binding. The plasma clearance of the low protein-bound drug metoprolol is limitedly affected by the fu changes occurring in these inflammatory conditions, with a maximum plasma clearance change of 4%. For intermediate and high protein-bound probe drugs such as caffeine and dextromethorphan, alterations in fu that occur during acute COVID-19 infection result in plasma clearance changes of 21% and 46%, respectively. As such, plasma clearance of probe drugs with high protein binding are predicted to be most impacted by alterations in fu observed in inflammatory and other (patho)physiological conditions, limiting the specificity of plasma clearance as a proxy for enzyme activity.

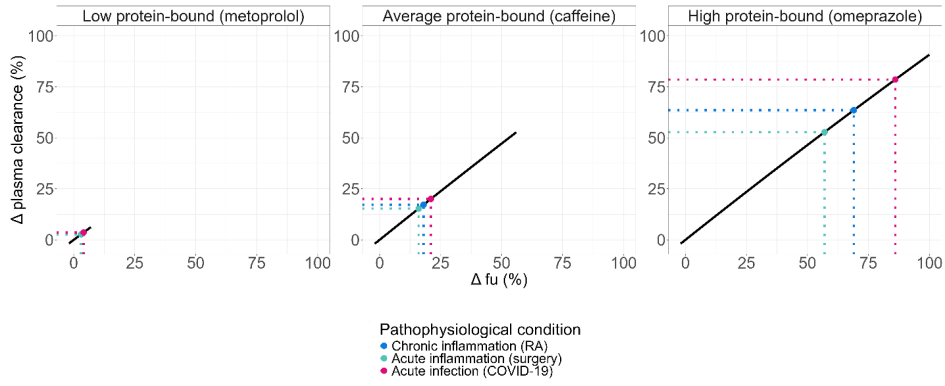

Figure 5 The impact of univariate alterations in fu observed during chronic inflammation (RA), acute inflammation (surgery), and acute infection (COVID-19) on plasma clearance, for the low, intermediate, and high protein-bound drugs metoprolol, caffeine and omeprazole, respectively.

Tool to interpret phenotyping study results under (patho)physiological conditions

The impact of alterations observed in fu, B/P, and Qh in these three inflammatory conditions on plasma clearance of all probe drugs is outlined in Online Resource 2 Figure 1. Analog to the graphs in Figure 5, these graphs can be used to assess the impact of a univariate change in the three non-metabolic determinants on plasma clearance for each of the probe drugs. When, for a studied (patho)physiological condition, it is evident from Figure 3 that fu impacts the plasma clearance of a probe drug, alterations in this parameter should be accounted for to derive true alterations in CL_{int} from measured CLp. There is no analytical solution for this calculation, therefore the R script provided in Online Resource 3 can be used to iteratively derive alterations in CL_{int} based on CLp and fu in healthy and (patho) physiological conditions. As an example, if a phenotyping study in diseased patients with caffeine showed a decrease in plasma clearance of 10% and, additionally, an increase in fu of 25% as compared with a healthy population, Figures 3 and 5 can be used to conclude that the change in fu will impact the plasma clearance of caffeine. To derive the change in in vivo CYP1A2 activity from these results, the changes in CLp and fu need to be included in the dispersion model and solved for CL_{int}, according to the R script in Online Resource 3. In this example, this would

lead us to conclude that the CL_{int} (CYP1A2 activity) decreased 28%. The script also allows for the calculations of CL_{int} in scenarios of changed B/P or Qh, or a combination of changes in the three non-metabolic variables.

Discussion

In this study, we used a PBPK-based workflow to investigate how sensitive and specific changes in plasma clearance of commonly used CYP probe drugs are in detecting alterations in in vivo CYP enzyme activity, quantified as CL_{int}. This is particularly relevant when the probe drugs are used to assess changes in enzyme activity in the context of (patho)physiological conditions, such as inflammation, obesity, or pregnancy (4–6).

Plasma clearance of all probe drugs was sensitive to alterations in CYP enzyme activity, as changes in plasma clearance demonstrated a close to proportional relationship with alterations in CL_{int}, except for midazolam. One of the validation criteria for phenotyping metrics states that the metric should not depend on factors beyond enzyme activity, meaning the metric should not be sensitive to, amongst others, the non-metabolic variables fu, B/P, and Qh that were studied in this work (2). Importantly, our results highlight that plasma clearance of all probe drugs was equally sensitive to alterations in protein binding as to alterations in CL_{int}, highlighting that alterations in fu might impact the phenotyping metric. This will not affect the use of cocktail approaches to assess differences in enzyme activity in scenarios where fu remains unchanged, for example in traditional pharmacokinetic interaction studies investigating drug–drug and/or drug–gene interactions, since these interactions are linked to changes in the levels or activity of CYP enzymes only and studied in healthy volunteers. However, it does indicate that additional methodological approaches are required to account for changes in fu, if the cocktail approach is applied to study scenarios where fu might change, as illustrated in this work for inflammatory conditions. Taking the alterations in fu into consideration as a confounder that can impact the phenotyping metrics will improve the validity of the metric during (patho)physiological conditions. Practically, this would involve the measurement of fu through measuring both total and unbound drug concentrations in patient populations to evaluate how this parameter is impacted as compared with a healthy population. Subsequently, these values can be used as

input for the dispersion model to derive changes in enzyme activity (CL_{int}) from changes in plasma clearance. An R script was provided in Online Resource 3 for the practical implementation of this method.

Midazolam is a widely acknowledged probe for phenotyping in vivo CYP3A4 activity. Interestingly, our results for midazolam show that changes in its plasma clearance are not proportional to changes in CL_{int}, when CL_{int} increases more than 50%. This might not have implications for, for example, inflammatory conditions, since CYP3A4 activity is known to decrease under these conditions (14). However, midazolam is often utilized to study CYP3A4 induction in clinical DDI studies, when a disproportional relationship between increased CL_{int} and midazolam plasma clearance could become relevant, because with high induction the observed increase in midazolam plasma clearance will be less than the increase in in vivo CYP3A4 activity. We found that midazolam clearance is also sensitive to alterations in fu, B/P, and Qh, which is in line with its intermediate extraction ratio (15). The importance of this sensitivity towards non-metabolic determinants was recently highlighted in an obese study population (16). This study showed that midazolam clearance in obese patients was higher as compared with controls, contrary to what would be expected given the decrease in hepatic CYP3A activity with increasing body weight. Given that patients with severe obesity have a higher Qh (17), this study suggested that increased Qh may have a more significant role than CL_{int} changes on plasma clearance of midazolam in this patient population. Particularly for midazolam, it may therefore be required to measure and account for alterations in Qh and B/P, as well as for changes in fu, when using it as a phenotyping probe for measuring in vivo CYP3A4 enzyme activity in (patho)physiological conditions.

Our findings on probe sensitivity and specificity are in line with the general notion that clearance of low extraction ratio drugs is limited by both fu and CL_{int}, while drugs with a high extraction ratio are mainly limited by Qh. It is therefore unlikely that probe drugs that are sensitive to changes in CL_{int}, but not sensitive to changes in fu, will ever be identified. One way to circumvent issues with changes in fu, is to select probe drugs with low protein binding, as our results show that low protein binding probe drugs are likely less impacted by alterations in fu as compared with probe drugs that are highly protein bound. Given that information on alterations in fu during inflammatory or other (patho)physiological conditions is scarce, inclusion of fu measurements in study designs will increase our

understanding of the magnitude of alterations in fu during disease and whether they impact clearance of probe drugs. In the absence of (literature) data on disease-driven changes in fu, assumptions can be made. We assumed that all probe drugs exclusively bind to HSA and calculated fu changes based on reported alterations in HSA levels in RA, surgery, and COVID-19 patients. This is a limitation considering inflammation is also characterized by upregulation of α-1-acid glycoprotein (AGP) and other proteins that may impact fu (18). However, the affinity of probe drugs to AGP is unclear, as is the exact value of upregulation under various (patho) physiological conditions. The effects of alterations in drug binding proteins on fu may contradict and counterbalance each other, potentially clarifying why the fu of midazolam remained unaffected in COVID-19 patients, contrary to reported HSA alterations (19). Therefore, measured fu values should be considered to be the golden standard in this context.

Incorporation of the impact of (patho)physiological conditions on drug exposure to advance personalized medicine is a long-standing goal. Several phenotyping cocktail studies have been conducted to characterize how (patho) physiological conditions such as non-alcoholic fatty liver disease, obesity, diabetes or inflammatory conditions can affect drug clearance (4,16,20–24). Incorporation of phenotyping results into clinical guidelines has indeed been demonstrated in special patient groups, e.g. pregnant women, where results from phenotyping studies were utilized to estimate alterations in CYP-mediated drug clearance (25). One important consideration here is that the phenotyping methodology assumes that drug exposure can be predicted by the pharmacokinetics of a relevant probe drug with a shared metabolic pathway. Indeed, the EMA guidelines on DDIs state that results of cocktail studies can be extrapolated to other drugs and can be used to support treatment recommendations in the SmPC (26); however, these results may not always be directly translatable between drugs. Our result highlighted that plasma clearance of probe drugs might also be impacted by alterations in fu, and to a lesser extent B/P and Qh, with the extraction ratio of the drugs determining which parameters are most influential. When findings from phenotyping cocktails in (patho)physiological conditions are used to make inferences about plasma clearance of other drugs, potential differences in fu, B/P, and Qh need to be accounted for, particularly when the extraction ratio of the drug that the finding is extrapolated to is higher. To achieve this, the equations of the dispersion model can be used.

Our results suggest that alterations in fu observed during inflammatory conditions such as COVID-19, RA, or surgery may influence CL_{int} predictions derived from differences in plasma clearance. Of note, our research did not aim to study the impact of inflammation on CL_{int}, but rather to assess the accuracy of the cocktail approach used to quantify such impact. Inflammation is known to affect liver function, induce oxidative stress, and modulate CYP enzyme activity through the release of proinflammatory cytokines (27). While the broader question of how inflammation impacts drug clearance and plasma concentrations has been well-studied by others (28,29), our focus is on providing tools and insights to improve the interpretation of clinical phenotyping studies that assess the overall effects of these inflammation-related mechanisms on CYP enzyme activity.

Our approach focused on the plasma clearance of probe drugs, but the primary metric used in phenotyping studies is the metabolic ratio in plasma or urine at a specific time point. The ratio of metabolite to parent concentrations in plasma indeed increases when plasma clearance increases, however this ratio also changes continuously over time within each individual, causing the method to be sensitive to deviations in sampling time. Moreover, the plasma concentration of both the metabolite and parent drug may be impacted by alterations in distribution volume or equilibration to peripheral tissue, while, in addition, the metabolite concentration may also be impacted by alterations in its elimination rate (26). Especially in diseased study participants, these processes may all be altered. When employing urine sampling, variations in urinary pH and glomerular filtration rate may further impact the measured metabolic ratio (30,31). Considering that all these disease-driven alterations could lead to changes in the metabolic ratio that are independent of changes in enzyme activity, taking multiple plasma samples and calculating plasma clearance may be more appropriate to study in vivo enzyme activity under (patho)physiological conditions. Finally, our approach focused on systemic plasma clearance of probe drugs, while the probe drugs are typically administered orally. Disease-related changes in pre-systemic clearance (i.e., gut and first-pass metabolism) would impact oral bioavailability (F) and thereby the apparent oral clearance (CL/F) that is obtained in cocktail studies. As quantitative information on disease-related changes in pre-systemic clearance pathways is limited, this could not be included in our PBPK assessment.

A limitation that applies to clinical plasma clearance values is that probe drugs are never exclusively eliminated through metabolism by a single isoenzyme and that minor elimination pathways will impact the concentration of the parent compound. The PBPK approach applied in the current analysis allows for studying the major elimination routes in isolation, which would be impossible to do in vivo and which is an advantage for establishing sensitivity and specificity of the drugs in phenotyping cocktails. Due to limited information on intrinsic hepatic transporter activity for most probe drugs, the applied PBPK framework does not incorporate the influence of hepatic influx or efflux transporters, but as far as we know, hepatic transporters are not major contributors to the plasma clearance of the studied probe drugs, which reduces the impact of this limitation on our findings. Finally, we describe the influence of univariate changes in CL_{int}, fu, B/P, and Qh on plasma clearance. Multiple parameters could change simultaneously under (patho) physiological conditions, leading to additive, synergistic or antagonistic effects due to the non-linearities in the dispersion model between plasma clearance and the model parameters. Conducting a multivariate analysis investigating all possible combinations of changes in all four variables would exponentially increase the number of scenarios to be evaluated and further challenges the interpretation of the results. Despite the fact that univariate changes in B/P and Qh limitedly impact probe drug plasma clearance, a combination of changes in parameters might have a more significant impact. Specifically, changes in Qh could become more important in scenarios where CL_{int} increases, while changes in B/P will be more relevant when the fu is considerably impacted by altered partitioning of drugs into red blood cells. With the provided R script in Online Resource 3, the reader can derive changes in CL_{int} for any drug, based on the difference in CLp and any possible combination of changes in fu, B/P, and Qh.

Conclusion

The PBPK-based simulation workflow utilizing mechanistic equations defining hepatic plasma clearance allowed us to unravel that plasma clearance of 13 commonly used drugs in CYP phenotyping cocktails is highly sensitive to alterations in enzyme activity, except when capturing > 50% CYP3A4 induction with midazolam. However, plasma clearance of all these drugs is also sensitive to

changes in unbound drug fraction, which reduces the specificity of probe drug plasma clearance as a proxy for CYP enzyme. As drug-binding plasma protein levels can change under (patho)physiological conditions, alterations in protein binding should be considered when using probe drug plasma clearance as a proxy for CYP enzyme activity in these patient populations. The provided R script can be used to accurately determine changes in CYP enzyme activity in patients under (patho)physiological conditions by accounting for these alterations.

Key points

- CYP450 (CYP) phenotyping entails quantifying an individual's plasma clearance of CYP-specific probe drugs to estimate in vivo CYP enzyme activity. It is increasingly applied to study alterations in CYP enzyme activity under different (patho)physiological conditions.
- Plasma clearance of 13 commonly used CYP probe drugs is sensitive to changes in enzyme activity, but is also affected by variations in the unbound drug fraction, which reduces the specificity of probe drug plasma clearance as a proxy for CYP enzyme activity under (patho) physiological conditions.
- To facilitate the interpretation of results from phenotyping studies, an R script is provided that allows for calculating the alterations in enzyme activity under (patho)physiological conditions by accounting for alterations in the unbound drug fraction, blood-to-plasma ratio and/or hepatic blood flow that might occur.

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Supplemental information

Supplemental information can be found online at https://doi.org/10.1007/s40262-024-01426-8.