The complexities inherent in attempts to decrease drug clearance by blocking sites of CYP-mediated metabolism

Michael B Fisher[1]*, Kirk R Henne[2] & Jason Boer[3]

Addresses
[1]Boehringer Ingelheim Pharmaceuticals Inc
900 Ridgebury Road
Ridgefield
CT 06877
USA
Email: mfisher2@rdg.boehringer-ingelheim.com

[2]Amgen Inc
1120 Veterans Boulevard
South San Francisco
CA 94080
USA

[3]Pfizer Inc
Pharmacokinetics, Dynamics and Metabolism
Pfizer Global Research and Development
Eastern Point Road
Groton
CT 06340
USA

*To whom correspondence should be addressed. Michael B Fisher was employed at Pfizer Inc[3] at the time of writing.

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Oxidative metabolism by the cytochromes P450 (CYPs) is the most common metabolic pathway of drug clearance. Medicinal chemists in drug discovery often synthesize analogs of lead molecules to reduce clearance due to metabolism. One method generally used when attempting to reduce CYP metabolism is to identify the site of modification to 'block' it. Substituting fluorine in the place of hydrogen at metabolically labile positions, for example, is a common approach, although deuterium can also be considered here for simplicity. In this case, the rate of metabolism via a specific pathway is attenuated, but the rate of overall substrate consumption or overall clearance is not significantly altered, due to a compensatory increase in the rate of formation of an alternate metabolite. The concepts and evidence behind this phenomenon as it relates to complexities in blocking metabolic clearance are presented herein.

Keywords ADME, CYP, cytochrome P450, deuterium, metabolism, metabolite

Abbreviations

ADME	Absorption, distribution, metabolism and excretion
CYP	Cytochrome P450
ES	Enzyme-substrate
GSH	Glutathione
PET	Positron-emission tomography

Introduction

The last two decades have seen a steady evolution in the drug discovery and development process, with drug metabolism studies gradually increasing in importance, and departments working in this field becoming integrally involved earlier in drug discovery [1]. This migration has resulted from the recognition of unacceptable pharmacokinetics as one of the main causes of compound attrition [2]. Discovery screening strategies now include medium- and high-throughput absorption, distribution, metabolism and excretion (ADME) assays, performed in parallel with compound potency screening [3]. Drug discovery groups now consist of medicinal chemists, pharmacologists, and drug metabolism and pharmacokinetic scientists working together to achieve both appropriate predicted ADME properties and biological potency in the selection of lead compounds for further development.

Unfortunately, this combination of attributes is usually difficult to achieve, due to the fact that the physicochemical properties that lead to improvements in potency often incorporate ADME liabilities [4•]. For example, it has been known for some years that increasing lipophilicity often results in increased potency for a receptor or enzyme target. These same increases in lipophilicity will often have detrimental effects on the pharmacokinetic profile of the compound, usually attributable to increased metabolism and hepatic clearance due to more efficient interaction with the cytochromes P450 (CYPs) [5•,6]. Additionally, changing lipophilicity will often modulate individual pharmacokinetic parameters in opposite directions, resulting in no net improvement [7]. For example, decreasing logD often leads to decreased hepatic intrinsic clearance but also decreased plasma protein binding, yielding no net change in the observed clearance, or results in increased intestinal solubility but decreased membrane permeability, again providing no net change in overall oral bioavailability [8•].

Metabolism by the CYP superfamily of enzymes is a major mechanism of drug clearance. Thus, human liver microsome lability assays are usually conducted in the first tier of ADME assays incorporated in a lead development program, and strategies to improve the pharmacokinetics of a series usually involve decreasing the predicted hepatic clearance [3,8•]. Modulation of gross physical properties is a strategy that could alter hepatic intrinsic clearance, but could be complicated by other effects, for the reasons cited above. Another strategy that has been attempted is to block a major site of metabolism, often via substitution of hydrogens at metabolically labile positions with fluorine or deuterium [9-11]. Since CYPs catalyze a net insertion of oxygen into a C-H bond via hydrogen abstraction followed by radical recombination, substitution with fluorine or deuterium is predicted to block or attenuate metabolism, respectively. For simplicity, deuteration is considered first herein, since this is the most conservative substrate alteration and the least likely to affect physical properties; however, deuterium isotope effect theory and the mechanism of CYP enzymes taken together suggest that this strategy will usually not

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result in significant alterations in overall metabolic clearance of the substrate [12].

Drug-metabolizing enzyme evolution and properties

Unlike enzymes that have evolved to efficiently catalyze the anabolism or catabolism of some crucial endogenous molecule, drug-metabolizing CYPs have evolved to process a diverse and unexpected milieu of xenobiotics to which an organism may be exposed [13•]. Thus, any specific isoform is usually able to process a considerable range of xenobiotics in chemical space. The repercussions of this evolutionary process introduce many relatively unique observations and characteristics that differentiate the CYPs from classical enzymes. Gene duplication and genetic mutations have resulted in multiple family members with broad and overlapping substrate selectivities, and common and multiple allelic variants [14]. Also, the propensity to convert one substrate molecule into multiple various metabolites is common among CYPs, and appears to be beneficial to the organism [13•,15]. Specifically, the production of low levels of multiple products (ie, 'distributive catalysis') provides less probability of producing a toxic metabolite at toxic levels than would the production of a single metabolite.

The mechanism of substrate processing by the enzyme is the result of this distributive catalysis, and it affects the outcomes of blocking strategies (described below). The interaction of substrates in the CYP active site is usually not simply a ligand-receptor, lock-and-key-type interaction, but rather, it appears that several rapidly interconverting enzyme-substrate (ES) complexes exist in the active site [13•]. Moreover, catalysis only occurs in the generation of the reactive iron-oxo species of CYPs (similarly to hydroperoxyflavin for flavin-containing monooxygenases and deprotonated glutathione (GSH) for GSH transferases). For CYPs, it is more appropriate to consider oxygen as the actual substrate for catalysis, and active-site amino acids are involved in the binding and catalysis of heterolytic dioxygen cleavage to the oxene species. This oxidant has sufficient energy to abstract a hydrogen atom from any position in a drug, even a non-activated aliphatic methyl group, and has led to the enzyme being called a 'biological blowtorch' [16]. Thus, the rapidly interconverting ES complexes eventually result in collisions with reactive species and formation of metabolite.

Active-site mobility

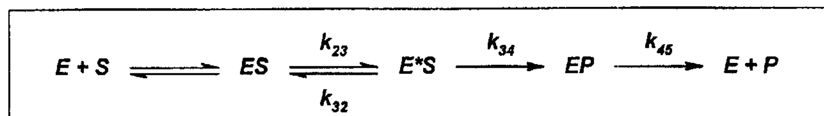
The considerable mobility of substrates in the CYP active site often allows the active oxygen to sample multiple sites in a molecule. This phenomenon is highly consistent with the large, open nature associated with the CYP3A4 active site, although it has also been observed with other isoforms. There is substantial evidence for this active site mobility and rapidly interconverting ES complexes. For example, deuterium magic angle spinning studies of substrate in the active site of CYP101 resulted in quadrupole interactions that were averaged due to conformational mobility on the time scale of enzyme turnover, which is a similar observation as for substrate in free solution [17]. Also, time-resolved fluorescence anisotropy measurements were indicative of significant substrate active-site dynamics [18]. Spectral studies of ligand binding to CYP-carbon monoxide complexes under high pressure demonstrated volume differences that were interpreted as substrate mobility [19]. In addition, paramagnetic relaxation studies have been used to determine proton distances from the heme iron. For 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in CYP2D6, in the presence of reductase, the data are consistent with two interconverting ES complexes that result in the two observed metabolites [20]. Interestingly, for caffeine and CYP1A2 [21], flurbiprofen and CYP2C9 [22], and multiple ligands and CYP2C9 [23], all protons were essentially equidistant from the heme iron; this was consistent with several different ES complexes at various positions relative to the heme, averaged over the time scale of the measurement as equidistant. Docking/molecular modeling efforts also provided evidence for significant substrate mobility [24-26]. However, as described below, most evidence directly demonstrating substrate mobility in the active site of CYPs on a time scale of catalysis is derived from deuterium isotope effect experiments.

Isotope effect theory for CYPs

Consideration of purely chemical aspects suggests that if C-H bond cleavage were rate determining in the biotransformation of substrate, then the turnover of substrate would decrease upon deuterium substitution. 'Normal' isotope effects on chemical or enzymatic reactions are typically in the range of k_H/k_D = 6 to 10 (where k is the rate constant), although under some conditions they can be higher or lower than this range [27]. For example, other steps, such as product release, may be rate limiting and result in the apparent isotope effect being suppressed, or masked, relative to the intrinsic isotope effect [27]. A few fundamental and unique aspects of the CYPs, however, complicate the expression and interpretation of their isotope effects. Firstly, the rate-limiting step in the enzymatic reaction likely occurs prior to the actual bond-breaking step [28,29]. Secondly, the presence of an irreversible step prior to C-H bond breaking, namely heterolytic cleavage of dioxygen bound to heme iron, adds complexity to the isotope effect expression [12], as described below.

In most chemical or enzymatic reactions, a decrease in metabolite formation is observed with deuterated substrate due to the reversibility in ES complex formation, as described by Equation 1.

Equation 1.



$$E + S \rightleftharpoons ES \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} E^{*}S \xrightarrow{k_{34}} EP \xrightarrow{k_{45}} E + P$$

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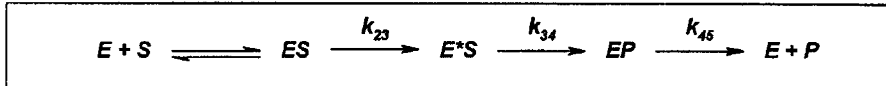
Equation 2.



In Equation 1, * indicates an ES complex with activated enzyme, P is a metabolite, and k_{23}, k_{32}, k_{34} and k_{45} are the rate constants for the indicated steps. A build-up in ES complex with deuterated substrate, due to $k_{34H} > k_{34D}$, is prevented by reverse breakdown to free enzyme and substrate. E^*S_H and E^*S_D do not accumulate, and an isotope effect is observed because $k_{34H}[E^*S_H] > k_{34D}[E^*S_D]$. However, with the CYPs, k_{23} actually provides an irreversible step prior to the isotopically sensitive k_{34} step (E*S to EP), preventing reverse breakdown of E*S, as described by Equation 2.

With no ability of the enzyme to revert to a resting state when encountering a deuterium atom, theory suggests that no isotope effect should be observed. This is because the greater stability, and thus a lower rate constant for C-D bond cleavage relative to C-H bond cleavage leads to a build-up of E^*S_D, resulting in no net difference in rate, as described by Equation 3.

Equation 3.

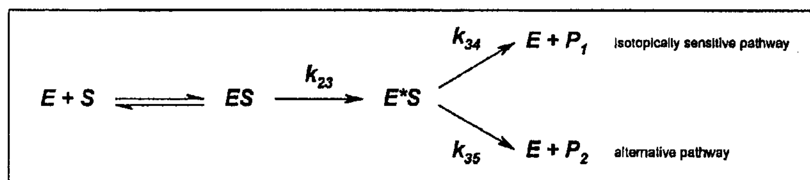
$$k_{34H}[E^*S_H] = k_{34D}[E^*S_D]$$

The key to expression, or 'unmasking', of a deuterium isotope effect on a CYP-mediated metabolic pathway is the presence of a branching pathway to an alternative metabolite [30•]. The active site mobility described above is responsible for the ability of the enzyme to undergo metabolic switching, or isotopically sensitive branching, to an alternative metabolite. This alternative pathway acts as a shunt to prevent build-up of E*S and allows the observed isotope effect to approach the ratio of rate constants, or the intrinsic isotope effect k_{34H}/k_{34D}, as described by Equation 4.

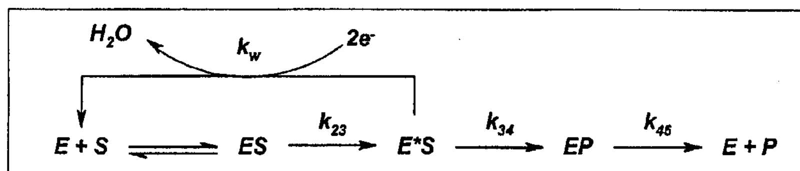
In Equation 4, P_1 and P_2 represent different metabolites. If the alternative pathway is simply a result of metabolism at another position, there will be an isotope effect on the distribution of metabolites (ie, P_1 decreases and P_2 increases upon deuteration), but the overall rate of metabolism will likely be unchanged (ie, $P_1 + P_2$ is unchanged). Only if the alternative metabolic pathway is the two-electron reduction of the iron oxene (from $Fe^{(V)}$=O) to water (k_w in Equation 5), will the overall rate of metabolism of the substrate be decreased. In this case, the shunt to prevent build-up of the ES complex due to deuterium substitution is water production, and not substrate consumption to an alternative metabolite, as described by Equation 5.

Additionally, the magnitude of the isotope effect will be directly dependent on whether the alternative metabolite is usually a major or minor pathway. In other words, if deuterium substitution results in switching from a major to a minor metabolite (as would be observed upon deuteration of the site of major metabolite formation on a drug), the magnitude of the isotope effect will be low, or masked; alternatively, if the metabolism is switched from a minor to a major metabolite, the isotope effect will approach its theoretical maximum, or be unmasked. This result is described in Equations 6A and 6B, as reported by Korzekwa *et al* [31] and others.

Equation 4.



Equation 5.



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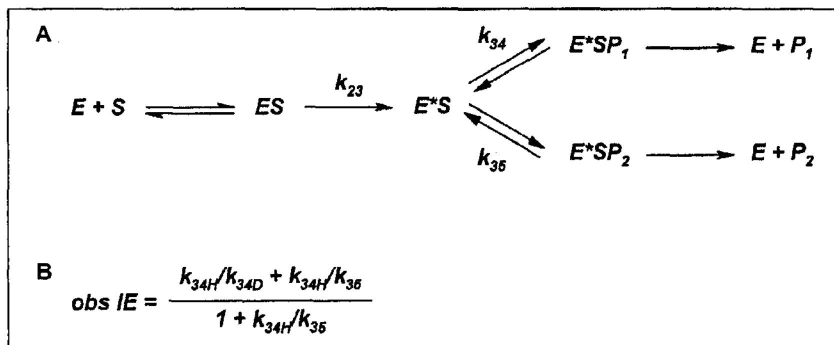
Equation 6.



In Equation 6B, obs IE is the observed isotope effect. When P_1 is the major metabolite (ie, $k_{34H} > k_{35}$) and the position of metabolism to P_1 is deuterated, isotopically sensitive branching to P_2 is not favored ($k_{34H}/k_{35} > 1$), and the isotope effect is masked. However, when P_2 is the major metabolite ($k_{34H} < k_{35}$) and the position of metabolism to P_1 is deuterated, switching to P_2 is favored ($k_{34H}/k_{35} < 1$) and the observed isotope effect approaches the intrinsic isotope effect. Interested readers are directed to the seminal paper on octane metabolism for further discussion of this mechanism [32•]. The masking explained by this equation is accounted for in the 'commitment to catalysis' observed in earlier studies as a more generic masking factor accounted for in previous kinetic equations [33].

While the experiments described above are indicative of active site mobility, there are a couple of reports of isotope effect experiments designed to quantitatively determine the extent of substrate dynamics in the CYP active site. Substrates were designed and utilized (namely 2-xylene, 4-xylene, 2,6-dimethylnaphthalene and 4,4'dimethyl-biphenyl, each with one methyl group labeled as CD_3) with increasing distance between the methyl groups where the isotope effect was measured and the degree of masking was determined. It was demonstrated that for the xylenes, the isotope effect was completely unmasked, indicating rapid rotation to ensure equilibration of the two (protium- and deuterium-containing) methyl groups. However, depending upon the enzyme, larger distances led to partial or complete masking [34•,35,36], suggesting that substrate mobility was not sufficient to equilibrate the methyl groups to fully express the isotope effect.

A couple of reports have quantitated the extent of total metabolism for protio and deuteron substrates. Obach performed substrate depletion measurements for ezlopitant isotopomers, and demonstrated no net isotope effect with CYP3A4, while there was an effect, albeit small, on specific metabolite formation [37]. Wust and Croteau reported the rates of formation of several metabolites from specifically deuterated analogs of limonene, and while significant effects were observed on specific metabolites, overall metabolism was essentially unchanged [38].

It is therefore apparent that deuterating a site of major metabolite formation will often result in metabolic switching to an alternate metabolite, with no net change in substrate consumption (Figure 1). The only situation in which blocking a site of metabolism via deuterium substitution will result in decreased substrate clearance is when metabolite P_2 (Equations 4 and 6) is actually a reduction of oxene to water [12]. While it is impossible to measure the proportion of enzyme turnover that occurs through this pathway relative to metabolite formation *in vivo*, several *in vitro* studies have attempted this measurement. In some cases, water can be a major 'metabolite', although in one side-by-side comparison of various isoforms and substrates it appeared that water formation was not a major pathway in most cases [39].

Deuterium studies

A recent paper described an attempt at using deuteration as a blocking strategy and observed no significant effect on total clearance [11]. Some examples exist of modest decreases in the formation of a single CYP-catalyzed major metabolite upon deuteration; Helfenbein *et al* [11] cited *in vitro* studies with 1-benzyl-4-cyano-4-phenylpiperidine [40] and lidocaine [41] that reported observed k_H/k_D values of ~ 1.5, but no alternate metabolites were monitored. A further study was referenced in which no isotope effect was observed *in vivo* [42]. However, again no alternate metabolites were monitored and, in fact, per-deuterated substrate was utilized, effectively blocking all preferred switching pathways and essentially ensuring that minimal or no isotope effect would be observed [32•]. Clearly, when this strategy is attempted, either no isotope effect is observed, or the effect has a k_H/k_D value < 1.5, significantly less than the completely unmasked k_H/k_D value of 9 for aliphatic hydroxylation [32•]. Experimental assessment should always, but usually does not, include the assessment of metabolic switching in addition to changes in substrate clearance. A recent comprehensive review has summarized the use of deuterium in probing CYP mechanisms and elucidating the formation of reactive metabolites [43•]. Several examples of significant attenuation of specific pathways are described, and the interested reader is directed to that review for more examples of specific deuteration [43•].

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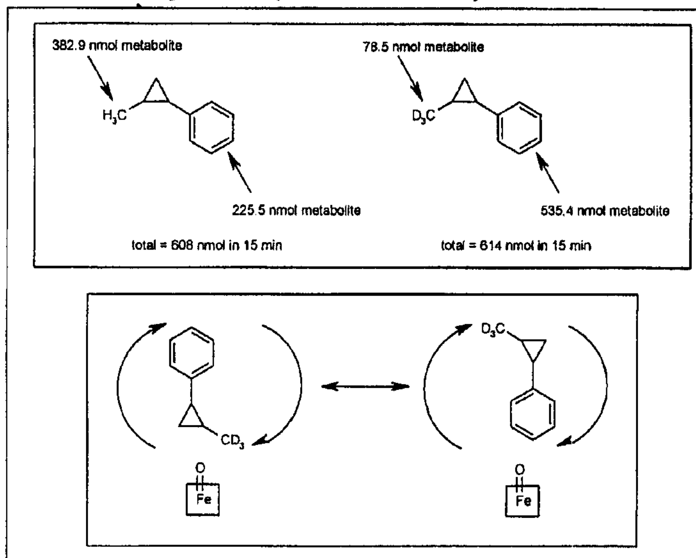
Figure 1. Schematic of metabolic switching observed upon deuteration of a major site of metabolism.



Note that there is no net change in total metabolism, only a change in metabolite distribution. Upon metabolization, mostly aliphatic methyl hydroxylation occurs in the protio compound, while mostly aromatic hydroxylation occurs when the methyl group is deuterated. Metabolic switching occurs via active site rotation on the time scale of catalysis (bottom panel). Data adapted from reference [55].

Fluorination

Similarly to isotopic substitution using deuterium in place of hydrogen, the strategic placement of fluorine atoms within a new chemical entity is an approach intended to lend metabolic stability to a given molecule. Although the issue associated with deuterium incorporation is generally one of potentially slower rates of metabolism (ie, lower clearance rate) versus metabolic switching (ie, unchanged clearance rate), fluorine atoms are generally believed to be inert to commonly anticipated metabolic pathways and, thus, reduce metabolism potential overall. One way in which fluorine atoms may reduce the potential for oxidative metabolism is by virtue of their electron-withdrawing properties, making oxidation more difficult. A confounding factor, however, is that fluorine substitution in a molecule notably affects physicochemical properties, such as logP and logD, which may indirectly affect metabolism. Whether by design or serendipity, the use of fluorine atoms to reduce metabolism may be successful [44], but there are numerous reports in which fluorine atoms have proved to be labile and, in rarer cases, demonstrated to be involved in bioactivation pathways.

When positioned on aromatic rings, fluorine atoms are potentially labile as a result of CYP oxidation via ipso-substitution reactions [45•]. Other substituents on the ring system may influence the course of this reaction, but typically dehalogenation of aromatic rings is thought to be enhanced electronically by the presence of ortho and para electron-donating atoms (ie, nitrogen and oxygen). For example, rats dosed with 4-fluoroaniline (1, R = H; Figure 2A) excreted ~ 10% of the dose in urine as 4-acetomido-phenol, or its sulfate and glucuronide conjugates 3 (Figure 2A) [46]. In the same study, investigators dosed a separate group of rats with 4-fluoroacetanilide (1, R = $C(O)CH_3$; Figure 2A) and measured free fluoride levels in urine, which suggested that a similar defluorination mechanism occurs with the acetylated molecule. Oxidative defluorination of gefitinib (4; Figure 2B) an anilino-quinazoline inhibitor of epidermal growth factor receptor, also occurs as a minor metabolic pathway *in vitro* [47] and *in vivo* [48] in human. The aniline moiety in this compound is substituted at the 4-position with fluorine and at the 3-position with chlorine. Although chlorine might be thought of as a better leaving group, it is the fluorine that is actually more susceptible to removal.

Positron-emission tomography (PET) imaging agents require appropriate radioligands to study targets of interest, and one such ligand for a serotonin transporter in the brain is *N,N*-dimethyl-2-(2-amino-5-[^{18}F]fluoro-phenylthio)benzylamine (6, 5-[^{18}F]-ADAM; Figure 2C) [49]. Studies to validate the use of this PET ligand in rat demonstrated marginal utility for its use, with one limitation being the propensity for 5-[^{18}F]-ADAM to lose its aromatic fluorine atom (as measured by uptake of radioactivity in the femur). The placement of the ^{18}F atom para to the aniline nitrogen atom likely contributed to loss of the radioisotope since the closely related analog 4-[^{18}F]-ADAM, in which the ^{18}F atom is positioned meta to the aniline nitrogen atom, tended to be more stable [50]. This finding is consistent mechanistically with the notion that electron-donating substituents located ortho or para to fluorine (or other halogen) atoms increase the potential for metabolism via ipso-substitution.

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