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Implications of the allosteric kinetics of cytochrome P450s

William M. Atkins

Drug metabolites can uniquely contribute to therapeutic efficacy, toxicity and drug–drug interactions. Therefore, the rates of formation and clearance of each metabolite are crucially important parameters in the net therapeutic profile of new drugs. However, the recent appreciation for the importance of drug metabolism has made it apparent that the understanding of the fundamental kinetic and biophysical properties of the enzymes that are responsible for catalyzing these reactions, the cytochrome P450s, is incomplete. The need to fully comprehend the complex allosteric behavior of these enzymes has fostered increased scrutiny of cytochrome P450s, which has subsequently resulted in major changes in the way that these enzymes are perceived at the molecular level.

William M. Atkins
Department of Medicinal Chemistry
Box 357610
University of Washington
Seattle
WA 98195-7610, USA
e-mail:
winky@u.washington.edu

▼ Cytochrome P450s (CYPs) are well established as the dominant enzyme system that controls drug metabolism and clearance. In the past several years, it has become widely recognized that CYPs do not conform to typical enzymological processes; individual CYP isoforms often do not exhibit standard Michaelis–Menten kinetics and these enzymes demonstrate several types of deviation from the hyperbolic relationship between the rate of metabolism of substrate (velocity) and substrate concentration (Figure 1), even when studied as purified recombinant protein products. As a result, the consideration of CYP–drug interactions in early drug development is offset by the continuing uncertainty about the mechanism of action of CYPs and whether their atypical kinetic behavior *in vitro* is clinically relevant. The goal of this review is to provide a summary of the recent findings concerning allosterism in CYPs and, perhaps more importantly, to suggest routes of inquiry that might improve our understanding of *in vitro* drug metabolism and drug interactions. In addition, the speculation concerning other drug-metabolizing enzyme systems is intended to highlight the need to understand allosteric behavior. Several recent reviews and manuscripts have dealt with specific drug examples, which are not examined in detail here [1–6].

What is CYP 'allosterism' and why is it unique?

Many examples of non-Michaelis–Menten CYP kinetics have been collectively termed 'allosterism'. However, when applied to CYPs, the definition of allosterism deviates from the traditional definition that relates to other enzymes or proteins. Thus, in light of the rapidly increasing number of literature reports based on 'atypical' kinetics for CYP-dependent metabolism, it is useful to consider the meaning of 'allosterism'.

Allosteric effects could result from homotropic substrate interactions, which would result in a non-hyperbolic velocity versus substrate concentration curve (Figure 1) [6,7]. Homotropic effects might yield velocity versus substrate concentration curves that are either sigmoidal (also called 'autoactivation'), biphasic with continuously increasing velocity at high substrate concentrations (which implies a low-affinity second substrate site) or biphasic with a decrease in velocity at high substrate concentration after an initial hyperbolic increase (substrate inhibition) (Figure 1). In addition, apparent biphasic kinetics with a decreasing velocity at high substrate concentration might be observed with product inhibition. However, this situation does not represent allosteric kinetics because it does not require simultaneous binding of multiple ligands. In the case of sigmoidal and biphasic curves, quantitative allosteric models that account for non-hyperbolic kinetics are required. Without such models, *in vitro* kinetics will be parameterized inaccurately and *in vivo* drug clearance could be estimated incorrectly.

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To predict clearance with any degree of accuracy, homotropic effects must be understood.

Heterotropic effects occur when one drug alters the interactions of the CYP with a second drug [8,9]. In this situation, the drug that is acting as the substrate might yield classic hyperbolic velocity versus substrate concentration curves, but the second drug could change the V_{max} or K_m parameters, or induce non-hyperbolic behavior. Alternatively, if the substrate drug alone exhibits non-hyperbolic kinetics, the second heterotropic 'effector' might restore hyperbolic kinetics, or maintain hyperbolic kinetics and effect changes in the relevant parameters.

Another example of an allosteric effect is partial inhibition, which can occur through either homotropic or heterotropic interactions. A second substrate, or an effector that is bound at the same time as the substrate, might partially inhibit the enzymatic reactions. Each case is incompatible with simple competitive inhibition and requires allosteric interactions of some type. For improved predictability of drug–drug interactions, the quantitative prediction of drug clearance in heterotropic cases is a necessity.

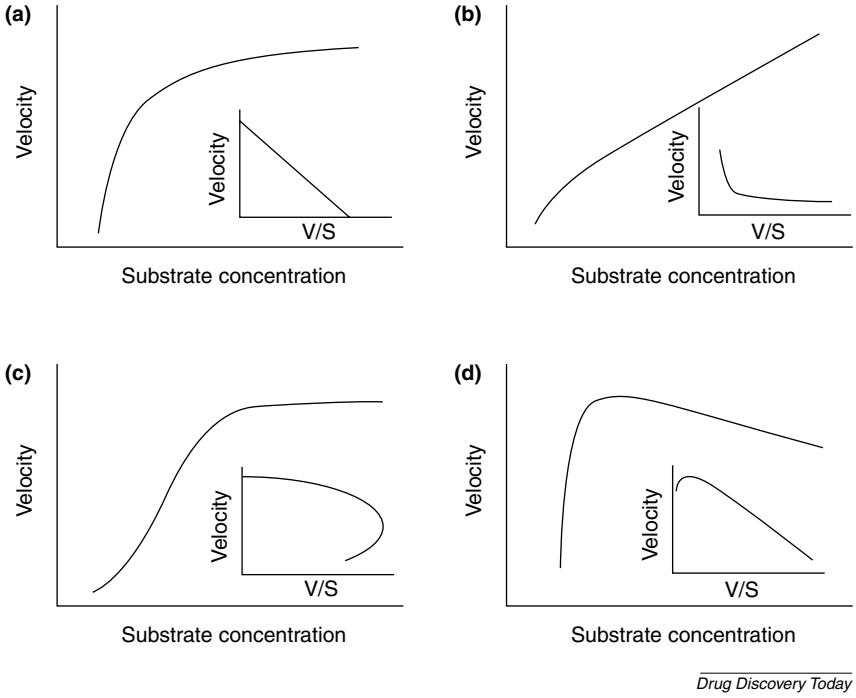

Figure 1. Plots of the rate of substrate metabolism (velocity) versus substrate concentration. **(a)** Hyperbolic, non-allosteric kinetics; **(b)** biphasic kinetics resulting from a higher K_m or lower V_{max} for CYP–substrate–substrate than for CYP–substrate; **(c)** sigmoidal kinetics resulting from positive homotropic effects in which either K_m is lower or V_{max} is larger for CYP–substrate–substrate than for CYP–substrate; and **(d)** substrate inhibition resulting from the CYP–substrate–substrate complex having a lower V_{max} than CYP–substrate. In each case the inset is the corresponding Eadie–Hoffstee plot of velocity versus velocity/substrate concentration (V/S).

Is CYP allosterism biologically useful?

Historically, biochemists have used the term 'allosterism' to signify ligand-binding sites communicating with one another in some physiologically rational way (i.e. allosteric behavior imparts a metabolic advantage). By contrast, it is not clear if communication between drugs bound to a single CYP is biochemically sensible or metabolically advantageous [10,11].

It should be remembered that CYPs are presumed to function physiologically as detoxification catalysts. Therefore, it is conceivable that an understanding of CYP allosterism in terms of a toxicological paradigm, rather than a therapeutic one, might provide a useful insight into the mechanism of CYP allosterism: is CYP allosterism a result of selective evolutionary pressure that has yielded an improved detoxification enzyme system, or is it just an inconvenient outgrowth of the 'useful' promiscuous substrate selectivity of this class of enzyme? Improved comprehension of how CYPs 'discriminate' as detoxification enzymes could potentially lead to understanding CYP allosterism with increased predictive power. However, as yet it is not obvious how this can be achieved. One possibility is through 'systems biology' or proteomics approaches; the correlation of the expression of CYPs with metabolite levels and with other protein networks could result in a better understanding of CYP allosterism.

Complexity of CYP enzymology

Another difference between CYP allosterism and 'traditional' allosterism arises from the potential of CYPs to generate multiple products from a single substrate. This can dramatically complicate experimental and theoretical analyses. For traditional allosteric systems with well-defined substrate specificity, either the rate of product formation or the substrate affinity is altered through homotropic or heterotropic effects. In these cases, allosteric effects are easily quantified as changes in V_{max} and K_m for the substrate, or K_d for the ligand. For CYPs, the operationally defined K_d, K_m and V_{max} values are subject to change by

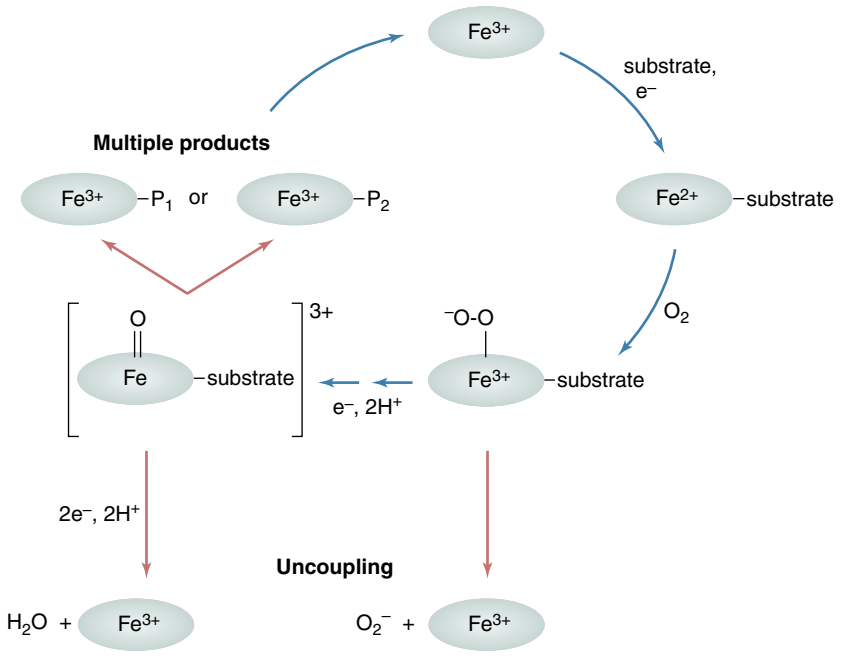

Figure 2. The proposed catalytic cycle of cytochrome P450s. The resting state of the enzyme includes a ferric heme (Fe^{3+}). Following addition of substrate and the first electron, the binding of molecular oxygen yields the ferrous dioxy intermediate, which is denoted as Fe^{3+}-O-O^{-} to reflect the additional electron density on the distal oxygen atom. The remainder of the catalytic cycle is highly 'branched', unlike other allosteric enzymes. The branch points denoted by red arrows lead to enzyme uncoupling and reduced forms of oxygen, or to formation of multiple oxidized drug products. These branching reactions decrease the apparent V_{max} of formation of any specific drug metabolite, for example, P_1 or P_2. Allosteric effects on V_{max} can arise from changes in the branching ratios, in addition to 'simple' changes in the absolute rates of the other reaction steps (blue arrows). The branching ratios change with substrate or effector concentration. Therefore, a complete characterization of CYP allosterism should address each of the products formed, including uncoupling products (red arrows).

allosteric effects, but the molecular basis of these changes could be complex and multifactorial. It is difficult to 'understand' CYP allosterism in terms of K_m and V_{max} alone. For example, many CYP–substrate complexes yield several oxidized drug products. Furthermore, the situation can be extraordinarily complex when the product distribution changes as a function of substrate concentration (homotropic effects) or on addition of effectors (heterotropic effects). Perhaps the best-documented example is midazolam metabolism by CYP3A4 in which two hydroxylated products are obtained. The ratio of 1'-hydroxy- to 4-hydroxy-midazolam produced is dependent on the initial midazolam concentration and is altered by the addition of, for example, α-napthoflavone [12,13]. Therefore, when multiple products are formed from a combination of CYP–substrate–substrate and CYP–substrate complexes, the product distribution depends on substrate and effector concentration. Indeed, on fitting the data to Michaelis–Menten models, the generation of different K_m values for the products offers proof for multiple substrate binding and a productive CYP–substrate–substrate complex. Moreover, it should be noted that when this occurs, the 'K_m' does not have a clear physical meaning. Because CYP–substrate–substrate and CYP–substrate could be catalyzing the reactions, the simple Michaelis–Menten model is not valid. Although it is inadequate to consider changes in K_m or V_{max} for only a single product when characterizing CYP allosterism and multiple products, traditional models of allosterism do not readily account for product distribution.

Conceptually, the formation of multiple products is analogous to a further complication – the degree of coupling between NADPH and oxygen consumption with substrate oxidation is altered via homotropic or heterotropic effects. Specifically, two electrons that originate from the oxidation of NADPH are consumed separately. First, the ferrous-dioxy intermediate, Fe^{2+}–O–O^{-} is generated (Figure 2). This intermediate might collapse to yield the superoxide anion and resting ferric CYP with a Fe^{3+} heme. Reduction of the Fe^{3+}–O–O^{-} species with the second electron generates the iron-oxo [$(FeO)^{3+}$] intermediate. In the next step of this catalytic cycle, the iron-oxo intermediate can either undergo further reduction to yield 'excess' water or can oxidize a substrate (e.g. a drug) to one of several possible metabolites (Figure 2). The branching of intermediates to yield alternative reduced oxygen species (uncoupling) versus substrate oxidation, combined with the partitioning of the $[FeO]^{3+}$ between different sites on a single substrate, results in a complexity that is not addressed by the standard theories of 'traditional' allosterism. For example, consider the metabolism of flurbiprofen by CYP2C9 to yield 4'-hydroxy-flurbiprofen. The addition of the effector drug dapsone results in greater coupling, a reduction in the level of superoxide and excess water formed and faster rates of flurbiprofen hydroxylation [14,15]. In effect, the ratio of the products has been altered, thus the competing non-productive pathways might dominate until a heterotropic

effector is present. The apparent V_{max} for drug oxidation increases because of a decrease in the rate of uncoupling, rather than an increase in the intrinsic rate of drug oxidation. The complexity of branching within the reaction cycle is not explicitly considered in existing allosteric models. A general quantitative treatment of this complexity could increase our predictive success for many CYP–drug combinations.

Substrate-dependent and effector-dependent interactions

A particularly interesting aspect of CYP allosterism is that an individual compound might activate CYP-dependent metabolism of one drug and inhibit or have no effect on the metabolism of a second drug that is cleared by the same CYP isoform. Equally important, a single effector molecule might change from an activator at low concentrations to an inhibitor at higher concentrations. Thus, the change in metabolism caused by an effector compound depends on the substrate that is being metabolized, as well as the concentrations of effector and substrate [7,16,17]. For example, testosterone inhibits the metabolism of terfenadine and midazolam by CYP3A4 with different K_I values, but it has no effect when the substrate is nifedipine. Moreover, testosterone itself is a CYP3A4 substrate [16,17]. In short, the behavior of a substrate or inhibitor depends on what other compounds are present. This relatively common observation makes it extremely difficult to envisage a limited battery of diagnostic *in vitro* assays that will provide a comprehensive prediction of drug interactions. One approach is to test known effectors or inhibitors with new drugs at several concentrations of each compound. A general solution to this enigma, if it exists, is likely to require multiple approaches that are based on a combination of structural methods and sophisticated kinetic modeling. A recent kinetic analysis provides a systematic description of the complex range of possible interactions, and it provides optimism that a small number of kinetic models might be sufficient to reproduce many experimental results [17].

CYP allosterism is pervasive *in vitro* and this matters *in vivo*

If CYP allosterism was apparent in a few isolated cases, it might be cost effective to ignore it. However, it is becoming increasingly clear that allosterism could be the rule, rather than the exception, in CYP-dependent metabolism. Although the majority of examples of allosterism involve CYP3A4 [16–20], it is becoming increasingly recognized that this pattern of behavior is followed by other isoforms, including CYP2C9, CYP1A2 and CYP2B4 [21–23]. All CYP isoforms are likely to exhibit allosterism with some substrates. Therefore, it is reasonable to expect that a drug that undergoes oxidative metabolism will have the potential for metabolism-based interactions and atypical kinetic profiles, at least *in vitro*. This alone might justify the continued effort to describe CYP metabolism quantitatively.

Notably, there are only a few examples that suggest that atypical kinetics occur in whole animals or even in intact microsomes [24–26], including a putative dapsone–flurbiprofen interaction [25]. Regardless of how common allosterism is *in vivo*, it is clearly a pervasive behavior *in vitro*. Because *in vitro* screens form the core of initial drug metabolism protocols, an understanding of CYP allosterism is likely to improve their reliability and accuracy of predicting *in vitro–in vivo* correlations and *in vivo* scaling of *in vitro* data.

In vitro data are frequently scaled to predict *in vivo* kinetics, thus the *in vitro* case must be defined as accurately as possible. For example, in the presence of positive homotropic (sigmoidal) kinetics *in vitro*, the *in vivo* intrinsic clearance (Cl_{int}), which approaches appropriately scaled values of V_{max} and/or K_m, might be significantly less than the Cl_{int} values predicted by the 'best-fit' Michaelis–Menten model. Similarly, with substrate inhibition or negative homotropic effects, the Cl_{int} might be modestly underestimated or overestimated and this error will be perpetuated on scaling the *in vitro* data to a predicted *in vivo* Cl_{int} [3]. In short, the most accurate prediction of *in vivo* Cl_{int} will be obtained when deviations from Michaelis–Menten kinetics *in vitro* are acknowledged. Thus, it is essential to acknowledge and cope with the complex kinetics of CYPs.

Strategies for envisaging and describing CYP allosterism

Two distinct strategies for understanding CYP complexities have already emerged. One strategy uses multisubstrate steady-state kinetic models and parameterization of each of the possible states that contribute to drug clearance (i.e. CYP–substrate, CYP–substrate–substrate and CYP–substrate–effector). With sufficiently accurate parameterization, it should be possible to scale *in vitro* steady-state data to *in vivo* clearance, even when complex allosteric kinetics are operative. Thus, in principle, this approach can be fruitful. The second approach is aimed at defining the molecular mechanisms of CYP allosterism, with the hope of learning some general rules that can be correlated with molecular characteristics.

Kinetic models and curve fitting

Several elegant kinetic models have been developed to mimic complex *in vitro* CYP kinetics [1–4,17,18]. The goal of this type of kinetic modeling is to predict accurately the clearance by a specific CYP isoform for particular drug combinations. The fundamentally important characteristics

of these models are the inclusion of singly and doubly ligated substrate complexes, as well as substrate–effector complexes. For homotropic interactions, the recovered parameters include K_{m1}, K_{m2}, V_{max1} and V_{max2}, where subscripts '1' and '2' refer to the parameters for the singly or doubly ligated species, respectively. Some models include additional fitting parameters, the factors α and β. These are proportionality constants that relate K_{m1} to K_{m2} or V_{max1} to V_{max2}. In the presence of inhibitory effectors (heterotropic interactions), recovered parameters include K_{I1} and K_{I2}, where K_{In} is the standard inhibition equilibrium constant for the *n*th inhibitor bound.

Heterotropic activators might decrease the K_m of a substrate ($\alpha < 1$) or increase the V_{max} ($\beta > 1$) for CYP–substrate versus CYP–substrate–effector complexes. The applicability of each of these parameters is limited to predicting the rate of metabolism of a given substrate at a specified concentration of drug or drug and effector. As a consequence of the complexity of the CYP cycle, the parameters K_m, V_{max}, α, β and others do not readily report on specific mechanistic details. Interestingly, more-complex models involving three bound-ligand molecules have been considered and, in some cases, are supported by indirect evidence [17,27]. The application of increasingly refined models provides an accurate prediction of clearance for each substrate; but because the parameters for a given substrate change with each different effector, and because the parameters for each effector might be substrate-dependent and concentration-dependent, each case must be parameterized individually. The situation is further complicated because these parameters can be altered by other, non-drug 'effectors', including cytochrome b_5, apo-cytochrome b_5, metal ions, specific lipids and even buffer salts [6,28–30]. Thus, the empirical modeling approach is likely to be particularly useful for *in vitro* work that is limited to well-defined assay conditions. In principle, on gaining adequate experience, the best kinetic models and parameters could be extrapolated to complex *in vitro* or *in vivo* clearance.

Molecular mechanisms

The other major area of focus within the broad context of CYP allosterism has been the application of structural and spectroscopic approaches to the determination of the molecular mechanisms of this behavior. For CYPs, it was unclear to what extent the binding sites for the individual drugs are distinct subsites within a large cavernous active site, or are overlapping spaces within a flexible continuum. Structural and spectroscopic results support the original suggestion [31] that multiple ligands can simultaneously occupy a single, large CYP active site. Most of these results have been obtained using bacterial CYP isoforms [32–34], with some direct evidence for human isoforms [35]. In addition, the available crystal structures of elegantly engineered solubilized mammalian constructs [36–38] have not directly demonstrated multiple-ligand binding. Considering the example of the warfarin–CYP2C9 complex, there is adequate room for a second ligand, in addition to the single warfarin molecule, to be accommodated at the active site of CYP2C9 [37], but the second ligand is 'conspicuously' absent in the model. Thus, although many researchers are convinced that multiple ligands bind simultaneously to the active sites of clinically relevant CYPs, structural details that confirm this proposal are rare. Furthermore, this issue was further complicated by the recent characterization of a crystal structure of CYP2C8 that revealed a peripheral binding site for fatty acids on CYP2C8 [39], which could influence substrate binding and turnover parameters. This exemplifies that structural approaches have been unable to clarify CYP allosterism.

Several conceptual models remain viable. It is possible that there are discrete and static 'subsites' within the large active site and that each subsite has its own traits. Each subsite could potentially have a characteristic affinity for each ligand and hold the ligand in a preferred orientation, which is static on the time scale of oxidative turnover [19,40,41]. In the extreme case of multiple ligands binding to discrete subsites, the ligands bind sequentially to the highest affinity subsite and then to the lower affinity subsite. From their respective binding sites, ligands might alter the metabolism of other ligands by inducing conformational changes, causing minor shifts in the distances between oxidizable sites on the drug and the heme iron-oxo species, or by altering relative uncoupling rates to non-productive formation of superoxide.

Alternatively, the large active site might be 'fluid' and, thus, multiple bound ligands could sample several subsites within the large active site, either dynamically or through a static heterogeneity. The binding site encountered by a ligand is a function of other ligands that are present – every ligand contributes to the binding site of the other ligands and specific subsites might be context-dependent. For many drugs and drug combinations the situation might best be considered as a mixture of these limiting cases. The problem with approaches that are aimed at studying these mechanisms is identical with the limitation of the steady-state kinetic models: the most appropriate model is likely to be dependent on which drug or drug combination is being considered.

Computational approaches have also been aimed at defining the molecular determinants of allosteric effects. The goal of a recent study was to define the molecular properties of allosteric activators or inhibitors of CYP2C9

[42]. This research found that there was significant overlap or similarity in the pharmacaphores of CYP2C9 activators and inhibitors. This overlap raises some doubt about the feasibility of distinguishing activators versus inhibitors and could indicate that there is significant overlap in binding sites for ligands, rather than well-defined discrete sites.

Other commonly used computational methods rely on 'docking' drugs into CYP active sites using either X-ray structures or homology models. The 'molecular' handles on each ligand that are presented to the active site might depend on what other ligand is bound in the active site. For example, docked complexes of CYP3A4 with steroids or carbamazepine suggest the possibility of inter-ligand hydrogen bonds that compete with protein-ligand hydrogen bonds [43]. The results emphasize that ligand–ligand interactions or ligand–protein interactions are almost certainly dependent on the other ligands present in the active site. Therefore, analogous to the kinetic modeling and the molecular spectroscopy techniques, the pharmacophore and docking analyses are unlikely to yield a 'consensus' of structural or behavioral features that uniquely define allosteric behavior.

Does CYP behavior predict allosterism in other drug-metabolizing enzymes?

As a result of the complexity that CYP allosterism has introduced into the drug development process, researchers might hope, perhaps optimistically, that other drug-metabolizing enzymes will have more typical enzymological characteristics. However, there is ample reason to expect other drug-metabolizing enzymes to exhibit allosteric kinetics. If the biological purpose of these enzymes is related to their detoxification function, nature would probably exploit it whenever possible – including other detoxification enzymes. Alternatively, if the allosteric properties are a physico-chemical trait that is associated with detoxification, then other substrate non-specific drug-metabolizing enzymes should exhibit similar behavior. Thus, the allosteric kinetics already observed for CYPs might be a common feature of all drug-metabolizing enzymes.

For example, the uridine-diphosphate glucuronic acid transferases (UGTs) also contribute significantly to drug metabolism. Evidence for substrate inhibition and biphasic kinetics now exists for several UGT isoforms [44–47]. Similarly, the sulfotransferases (SULTs) increase the water solubility of aryl alcohols via conjugation to sulfate, and several SULT isoforms exhibit substrate inhibition. Moreover, crystallographic models demonstrate the feasibility of two substrates binding simultaneously within a SULT active site [48,49]. It is likely that allosteric kinetics is a feature of drug transporters; drug transporters also use large promiscuous active sites and display multiple substrate binding [50,51]. Although transporters are not involved directly in metabolism, they do contribute to absorption, distribution, metabolism and excretion (ADME) profiles.

It is probable that the current knowledge of CYP allosterism will prompt more extensive searches for similar behavior in other drug-metabolizing enzymes. Furthermore, as allosterism is recognized in enzymes other than CYPs, it is also probable that the complex kinetic behavior of the enzymes will become increasingly important in predicting drug clearance and drug–drug interactions.

Conclusions

CYP allosterism is commonly observed *in vitro* and this confounds *in vitro* screens for drug–drug interactions and predictive models for clearance. Furthermore, the non-hyperbolic kinetics observed are particularly challenging because of a lack of a functional detoxification utility, the immense complexity of the CYP reaction cycle and the context-dependent behavior of each substrate and effector. In the near future, neither steady-state kinetic models and structural approaches nor computational methods alone are likely to provide generally useful models to predict *in vivo* behavior, but their combination has already begun to clarify mechanistic aspects in specific cases. It is now clear that the simultaneous binding of multiple ligands to CYP active sites might be a general phenomenon, but only a few years ago this was considered a radical proposal. In addition, the further characterization of other, non-CYP drug-metabolizing enzymes will probably indicate that these enzymes also regularly exhibit allosteric behavior. Optimistically, whatever has been learned about CYP allosterism will facilitate the understanding of other drug-metabolizing enzymes.

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