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Opinion

Assembling the P450 puzzle: on the sources of nonadditivity in drug metabolism

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There is an increasing number of indications of an oversimplification in the premise that the cumulative properties of the human drug-metabolizing ensemble represent a simple aggregate of the properties of the constituting enzymes. Recent studies of the functional effects of hetero-association of multiple cytochrome P450 species and their interactions with metabolically related enzymes revealed a tight integration in the drug-metabolizing ensemble. In our opinion, the sources of interindividual variability in drug metabolism can be elucidated only when considering this ensemble as a multienzyme system, the functional parameters of which are determined by interactions between its constituents. In this article, we present a conceptual model providing a mechanistic explanation for the functional effects of the interactions between multiple P450 species and propose a clue to understanding the nonadditive behavior of the drug-metabolizing ensemble.

Highlights

There is an emerging recognition that multiple P450 species interact with the formation of heteromeric complexes, where their catalytic properties are significantly modified.

The functional consequences of these interactions are the primary sources of nonadditivity in the P450s properties that compromise the applicability of a proportional projection approach in PBPK.

The proposed mechanistic model explains the functional effects of P450–P450 interactions through positional heterogeneity in P450 oligomers.

The proposed mechanism serves for selective activation of P450 species through the reorganization of hetero-oligomers in response to the appearance of their specific substrates.

According to our hypothesis, the nonadditive behavior of the drug-metabolizing ensemble is best revealed when probed with the substrates having comparable affinity to multiple P450 species.

Interindividual variability in drug metabolism and its relevance to the composition of the P450 pool

Accurate dosing of pharmaceuticals requires knowledge of the pathways of their elimination from the human body. The crucial importance of this knowledge underlies a continuous interest in the enzymes involved in drug metabolism and stipulates their extensive exploration. The functional properties of the drug-metabolizing system are primarily determined by the properties of the ensemble of cytochromes P450 (Box 1), which is responsible for the metabolism of over 75% of all marketed drugs [1,2]. This ensemble constitutes the core of the microsomal mono-oxygenase system (MMO, Box 2) found in the endoplasmic reticulum (ER) of cells in most animal tissues. MMO can metabolize a vast majority of exogenous substances due to the presence of multiple cytochrome P450 species with different substrate specificity.

It has been long recognized that the interindividual variations in drug metabolism are largely dictated by the differences in the composition of the P450 ensemble [3]. However, despite half a century of research, much of the interindividual variation remains unexplained [4]. There are emerging indications of a significant oversimplification in the initial premise that the cumulative properties of this ensemble represent a simple aggregate of the properties of the constituting enzymes. The body of evidence suggesting a critical nonadditive contribution of mutual functional effects of P450 species continues to grow [5–7]. Gaps in our knowledge of the interrelationship between the P450 expression profile and the metabolic profile of a drug undermine the accuracy of predicting the changes in the drug metabolism profile caused by changing the P450 expression in development, aging, and under the influence of environmental and temporal factors.

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Box 1. Cytochromes P450

Cytochromes P450 (P450), the heme-thiolate enzymes found in all domains of life, are among the oldest heme-containing proteins. They probably appeared about 3.5 billion years ago [49] and may initially have played the role of NO-reductases [50]. The evolutionary development of the cytochrome P450 family intensified when green plants began to release oxygen into the atmosphere. At this stage, P450s acquired oxygen-binding capacity and became involved in the synthesis and oxidative metabolism of fatty acids and steroids [51,52]. The catalysis of oxidation of hydrophobic compounds became the primary function of P450s.

Over the course of evolution, the P450 family has become one of the largest families of enzymes with a highly conserved polypeptide chain fold and a common catalytic mechanism. Nature has utilized the P450 construct for various purposes. Known functions of P450s include synthesis of pigments, hormones, second messengers, antibiotics, toxins, as well as oxidative conversion and detoxification of foreign compounds (xenobiotics).

Present-day P450s comprise one polypeptide chain, 400–550 amino acid residues in length, which makes up a series of α-helices, β-sheets, and interconnecting regions. The buried active site houses a protoporphyrin IX (heme b) connected to the protein by a bond between the heme iron and the thiolate group of a highly conserved cysteine. Eukaryotic P450s differ from prokaryotic analogs in their cellular localization: prokaryotic P450s are water-soluble, protoplasmic enzymes, while eukaryotic P450s are associated with biological membranes. These proteins are tethered to the membrane by the hydrophobic N terminal α-helix, connected to the cytosol-exposed protein core via the polar-linking region containing several cationic residues.

In the wedge-shaped P450 molecule, the researchers discriminate between the two sides identified by their relative orientation to the heme. The distal side contains the region of F and G helices and the B/C loop that plays an essential role in substrate recognition and is partially embedded into the membrane [53–55]. The proximal side of the P450 molecule constitutes a flat area with multiple positively charged residues involved in the interactions with redox partners, CPR, and cytochrome b_5 [55,56].

P450s demonstrate prominent conformational flexibility. Many of them were found to accommodate several distinct conformations classified into open and closed structures, which differ primarily in the positions of the B-C loop and the F-G helices at the distal side of the molecule [53,57]. It is believed that, in most cases, substrates bind to or trigger the formation of the closed state [31,53,58].

Box 2. Multienzyme system of microsomal mono-oxygenase (MMO)

All known eukaryotic P450s and most of their bacterial analogs are not self-sufficient in their catalytic function. The mono-oxygenation reaction requires two electrons, which in most cases are transferred from a partner. The role of such a partner is played by non-heme, iron-containing ferredoxins (in so-called type I mono-oxygenase system present in prokaryotes and mitochondria of eukaryotes), or flavoproteins (in the type II systems that are predominant in eukaryotic cells).

In a eukaryotic cell, the most significant fraction of P450s is localized in the endoplasmic reticulum (ER) membranes, where they function as a terminal oxidase in the multienzyme system of MMO. The major electron donor in MMO is NADPH-dependent cytochrome P450 reductase (CPR), a flavoprotein. The complete MMO system also contains cytochrome b_5, an alternate electron donor and allosteric effector. The proteins constituting MMO are incorporated into the ER membrane and interact via lateral diffusion.

The MMO has been found in the ER of most animal tissues. Although the highest content of P450s is found in liver cells, they are also present in the lung, kidney, brain, intestinal epithelium, mammary gland, lymphocytes, etc.

This system catalyzes the oxidation of foreign substances, as well as endogenous substrates (hormones, steroids, fatty acids, prostaglandins, etc.). It can metabolize a wide variety of substrates due to the presence of multiple P450 species with different substrate specificities. For instance, the human genome encodes 57 functional cytochromes P450 [59]. Although the ER of hepatocytes contains around 25 distinct P450 species [60], only about a dozen of them are responsible for the majority of *in vivo* drug metabolism [3].

A very important feature of all known eukaryotic MMO systems is that they contain a significant excess of P450 versus CPR. That is, the average molar P450:CPR ratio for 150 human liver samples was found equal to 7.1:1 (range 2:1–27:1) [61]. Competition of different P450 species for CPR [41,56,62] is an essential element in the inter-P450 crosstalk, regulation of the P450 ensemble, and its coordination with the function of metabolically related enzymes [63,64]. However, the integrative connections between different P450s are not limited to this competition. Multiple P450 species coexisting in the ER membrane are known to oligomerize and interact with each other (Box 3) and these interactions have a profound impact on their functional properties [32,33,62,65,66]. Thus, the MMO system represents an integral entity, the properties of which fundamentally depend on the interactions between the constituting enzymes.

Glossary

Apparent intrinsic clearance: the parameter reflecting the intrinsic ability of a drug-metabolizing enzyme or enzymatic ensemble (drug-metabolizing ensemble of HLM) to metabolize the drug. Apparent intrinsic clearance is defined as a ratio of the values of the maximal rate of metabolism (V_{max}) and the apparent Michaelis-Menten constant (K_M) found from the fitting of the SSP of the drug to the Michaelis-Menten equation: $CL_{int,app} = V_{max}/K_M$.
P420 state: inactive state of cytochromes P450, named because of the 420 nm position of the Soret band of the CO-bound reduced protein, in contrast to the active P450 state Soret band at 450 nm. The P450-to-P420 transition may be caused by different factors, such as exposure to high hydrostatic pressure, organic solvents, or pH variations. The P420 differs from the P450 state in the nature of the proximal ligand of the heme iron. It was found that, at least in some cases, in the P420 state, this role is served by the neutral thiol group of cysteine, whereas in the P450 state, the proximal ligand is represented by a deprotonated thiolate.
Principal component analysis: a linear algebra method commonly used to reduce the dimensionality of large datasets. This method analyzes a set of M individual vectors (data sets) of the dimensionality N. It is used to construct the N × N covariance matrix, which is then transformed to find M–1 eigenvectors, each paired with M eigenvalues. The combination of each eigenvector with the corresponding set of eigenvalues is termed the principal component (PC). The PCs are sorted based on their statistical significance. The first PC represents the most typical difference between the individual datasets (individual SSPs), and the higher-order PCs contain the less significant deviations from the basis.
Spin equilibrium: an equilibrium between the high- and the low-spin states of the heme iron in ferric P450. The heme iron is hexacoordinated in the low-spin state, while it has only five axial ligands in the high-spin state. The role of the sixth ligand of the heme iron in the low-spin P450 is played by a water molecule, which dissociates from the ligand sphere upon the low-to-high spin transition. For most P450 substrates (so-called type I substrates), their binding to P450s causes a displacement

Evidence of nonadditivity of the functional properties of the individual P450 enzymes

Numerous studies published over the last decade [5,7–13] demonstrated striking interindividual variability in the activity of most drug-metabolizing P450 enzymes when studied in human liver microsomes (HLM) (*ex vivo*). Thus, over 600- and 400-fold variations in the **apparent intrinsic clearance** (see Glossary) have been reported for CYP2C19 and CYP2A6, respectively [5]. Early attempts to analyze the interrelationship between this variability and the content of the individual P450 species were performed with P450 quantification by immunoblotting [10,14]. These assays revealed notable correlations of the content of drug-metabolizing P450s with their activities [10,11,14]. However, later studies with larger sets of HLM samples and quantification of P450s with targeted proteomics [5,7] suggested that these correlations were weaker than initially implied. According to Gao and coauthors [5], who analyzed the results of analysis of the content and activity of ten P450 enzymes in 105 HLM samples [5], there are only four P450 species, CYP1A2, CYP2A6, CYP2E1, and CYP3A5, the contents of which had significant correlations with the corresponding activities. Analysis of the relevance of the observed interindividual differences to polymorphic P450 variants showed that this factor explains only a minor part of the variability. The authors concluded that the genotypes and contents of at least some of the P450 species have only limited effects on their metabolic activities, which is in turn dictated by some other, more important factors and protein–protein interactions in particular [5].

The impact of protein–protein interactions of P450s on human drug metabolism

One of the major sources of the complex interrelationship between the properties of the individual P450 enzymes and the system-wide parameters of the drug-metabolizing cascade is represented by the functional consequences of protein–protein interactions of P450s. Growing evidence of physical interactions between different P450 species and their profound functional effects (Box 3) points out their association as a principal determinant of the MMO properties and a significant source of nonadditivity. Besides their heteroassociation, P450s physically interact with other players in the drug disposition cascade, such as microsomal epoxide hydrolase [15,16] and UDP-glucuronyl transferases (UGTs) [16–18]. In addition, their interaction with some other membranous proteins, such as cytochrome b_5, the Progesterone Receptor Membrane-Associated Component 1 (PGRMC1) [19], and heme oxygenase-1 (HO-1) [20,21] may also play an important regulatory role.

Due to a complex network of protein–protein interactions in the P450 ensemble, its integral catalytic properties may significantly deviate from a simple summation of the properties of the contributing P450 species, which lies in the foundation of the current PBPK tools [22,23]. Thus, any change in the P450 expression profile, including those involved in the processes of development and aging, must alter the landscape of protein–protein interactions and induce a plethora of complex changes affecting drug metabolism in a complicated, hard-to-predict manner. Owing to these effects, the sources of nonadditivity in drug metabolism can be elucidated only when considering the human P450 ensemble as a multienzyme system, the functional parameters of which are critically affected by the interactions between its constituents.

Functional heterogeneity in P450 oligomers and its role in the effects of P450–P450 interactions

Insightful inferences about the mechanisms of the functional effects of P450–P450 interactions may be drawn from the indications of a 'persistent conformational heterogeneity' of P450s. This term refers to a 'nonequilibrating' (or, strictly speaking, slowly equilibrating) distribution of the population of membrane-bound P450 into fractions that differ in functional properties. Such

of the spin equilibrium towards the high-spin state.

Substrate saturation profiles (SSP): the dependence of the enzymatic reaction rate on the substrate concentration. In most cases, these dependencies obey Michaelis-Menten or Hill equations.

Box 3. Homo- and hetero-oligomerization of cytochromes P450

Oligomerization of P450s in the membranes was demonstrated by methods ranging from measuring the rate of rotational diffusion, crosslinking, and freeze-fracture electron microscopy to approaches using fluorescence and bioluminescence resonance energy transfer and bimolecular fluorescence complementation [31–33,66]. There is also a substantial body of evidence of intermolecular interactions between different P450s in proteoliposomes, microsomes, and living cells [32,33,62,65,66]. Heteroassociation has a striking impact on the functional properties of the interacting P450s [31,33,66]. Most frequently, it causes activation of one of them, whereas the activity of the second is either inhibited or remains unchanged. This type of interaction has been demonstrated for such pairs of human P450 enzymes as CYP3A4/CYP1A2, CYP2C19/CYP2C9, CYP2D6/CYP2C9, CYP3A4/CYP2C9 (see [31–33,66]), CYP2E1/CYP3A4 [39], and CYP2E1/CYP2D6 [44].

Most of these studies were performed with the pairs of purified P450s in reconstituted systems. Meanwhile, the effects of P450–P450 interactions in the ER remained essentially speculative. Progress in this direction was achieved by incorporating purified P450 enzymes into the membrane of human liver microsomes (HLM) and creating the preparations with augmented content of selected P450s [43,44,67]. This method was used to probe the effects of alcohol-inducible CYP2E1 on the functionalities of the P450 ensemble. These studies revealed striking effects of CYP2E1 on the function of such important drug-metabolizers as CYP3A4, CYP1A2, and CYP2D6 [39,43,44,67].

The consequences of interactions between multiple P450s appear among the primary determinants of the properties of the P450 ensemble. They can redirect the catalytic conversion of drugs metabolized by multiple P450s towards the enzymes, the metabolic role of which goes against the presumption of additivity. Examples of this kind are the activation of CYP1A2 and CYP3A4 by CYP2E1 [43,67] or redirection of the metabolism of a substrate metabolized by CYP3A4, CYP2C19, and CYP2B6 towards the latter at increasing CYP3A5 content [47].

In their recent review, Reed and Backes examined the crystal lattices of different P450 enzymes derived from X-ray diffraction data to make assumptions regarding the structure of P450 oligomers [31]. The authors classified the P450–P450 contacts on proximal-to-proximal, distal-to-distal, and side-to-side contacts. The proximal-to-proximal contacts are reluctant to catalysis as they block the CPR binding site. Importantly, these interactions were almost never observed with the closed P450 conformations that favor substrate binding (Box 1). The hypothetical trimeric unit of the oligomer is assumed to be a combination of distal-to-distal or proximal-to-proximal with side-to-side contacts [31].

stable heterogeneity has been observed both in solution and in membranes by various methods, as outlined later.

One of the early observations of this kind was obtained in the study of pressure-induced transitions in P450s. It was found that only 65–70% of CYP2B4 and CYP3A4 in solution and microsomal membranes are susceptible to pressure-induced transition into the inactivated **P420 state** [24–26]. The fact that this heterogeneity is eliminated by protein monomerization [24,27] suggests that it originates from some specific structural features of the P450 oligomers.

Persistent heterogeneity also reveals itself in the kinetics of dithionite- and NADPH-dependent reduction of microsomal P450s [28–30]. Both processes followed multiexponential kinetics in P450 oligomers, either in solution or in the membrane. However, monomerization of the heme protein by addition of detergent or incorporation into lipid-rich liposomes or nanodiscs makes the reduction kinetics monoexponential [28–30]. The most remarkable observation here is that the fractions of the oligomeric enzyme reducible in the fast and slow phases differ in the partitioning of the low- and high-spin states (position of **spin equilibrium**). In the case of dithionite-dependent reduction, the fast-reducible fraction is almost entirely represented by the low-spin enzyme. In contrast, when the electrons are supplied by the NADPH-cytochrome P450 reductase (CPR), the physiological redox partner of microsomal P450 enzymes (Box 2), the fast phase corresponds to the reduction of predominately high-spin heme protein [28–30]. These observations suggest a divergence of the P450 pool into two distinct populations that differ in the position of spin equilibrium and the ability to form complexes with electron transfer partners. These populations do not interconvert in the time frame of experiments due to the relatively slow rate of oligomer dissociation and reassociation. Thus, this feature may be better referred to as ‘positional heterogeneity’ [31]. The indications of positional heterogeneity in

P450s and their possible mechanistic explanations are discussed in detail in several review articles [31–33].

What then is the structural basis of this asymmetry in the oligomers' architecture? Our current knowledge of the structure of P450 oligomers is limited. Rotational relaxation times of CYP2B4 in the membrane suggest the enzyme to be predominately hexameric [34,35]. Electron microscopy of the oligomers of CYP2B4 and CYP1A2 in solution showed that both enzymes form hexamers organized as a two-layer dimer of planar trimers [36]. The lateral interactions observed in the trimers may take place in membrane-bound P450s as well, so that the trimeric unit may be hypothesized to be an elementary building block of the P450 oligomer.

This assumption is consistent with the persistence of a 2:1 distribution of functionally different fractions observed in reduction kinetics [28–30,37] and pressure-induced inactivation [24,26,38]. Furthermore, the experiments with crosslinking of CYP3A4 with thiol-reactive reagents also suggest a trimeric organization, both in solution and in the membrane [39]. These experiments revealed two different types of subunit–subunit interactions: if the crosslinking of the wild type enzyme yields both trimeric and dimeric crosslinks, the substitution of Cys_{239} with a serine residue virtually eliminated the trimeric aggregates. Cys_{239} residue is located at the interface between two protein molecules in the crystallographic dimer in the structure of the complex of CYP3A4 with peripherally bound progesterone (1W0F) [40]. Therefore, the mode of P450–P450 interactions observed in this structure may correspond to one of the two types of inter-subunit contacts observed in the CYP3A4 trimer. Although the trimer-based organization seems to be the most likely from our perspective, the universality of the trimeric construct as a building block of P450 oligomers remains controversial. Preferential formation of dimers and their aggregates is a plausible alternative, at least for some P450 species and their heteromeric pairs [31,41,42].

It is suggested that in the hetero-oligomers of several P450s, different enzyme species may differ in their preferences for adopting a particular position in the oligomer [31,33]. This selectivity of the individual P450 species for catalytically active and 'latent' positions in hetero-oligomers is hypothesized to bring forth selective activation of one of the two P450 in their hetero-oligomers, observed in many studies with different P450 pairs [31,33].

Hypothetical model of molecular organization of the microsomal P450 ensemble

The observations of positional heterogeneity in P450 oligomers overviewed earlier led us to a conceptual model of organization of the P450 ensemble that explains the functional effects of P450–P450 interactions [33,43]. According to this model, the predominant part of the P450 pool in the ER membrane is represented by hetero-oligomers of different P450 species. A specific quaternary organization of these oligomers gives rise to the presence of two types of subunits that differ in their competence to form functional electron transfer complexes with CPR and in the ability to interact with substrates. As a result, a significant part of the P450 pool in the ER is deposited in the inactive ('latent') positions in heteromeric complexes of multiple P450 species (Figure 1A). Due to the different abilities of the active and latent subunits to interact with substrates, the presence of a substrate that is specific for one of the interacting enzymes should cause a redistribution of P450 species between the active and latent positions in the oligomers. As a result, the P450 possessing its specific substrate becomes activated (Figure 1B). Availability of a particular P450 for interaction with substrates and CPR and, thus, its involvement in catalytic activity is presumed to be a complex function of the preferences of individual species for occupying the 'active' and 'latent' positions, the composition of the P450 pool, and the presence of selective substrates of particular P450 species.

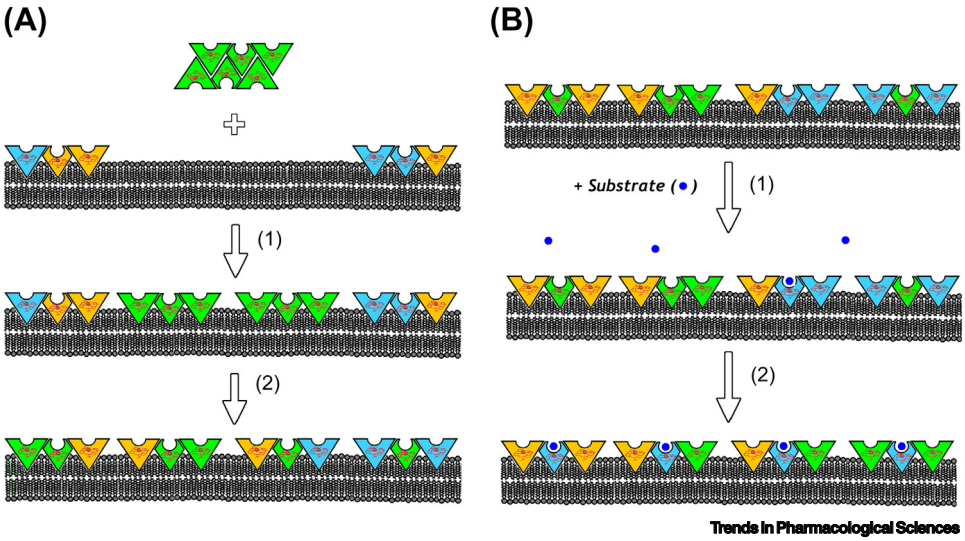

Figure 1. Isoform-selective (A) and substrate-dependent (B) activation of P450 enzymes through the positional heterogeneity mechanism. (A) The changes in the distribution of P450 species between the 'latent' (triangular subunits) and active (pentagonal subunits) positions in the oligomers resulting from an increase in the content (in this case, membrane incorporation) of one of the species. In the event (1), the added P450 protein (green subunits) incorporates into the membrane, where two interacting P450 species (blue and yellow subunits) randomly occupy the active and 'latent' positions. After dissociation and reassembly of the oligomers (2), the 'active' positions become preferentially occupied by the newly incorporated protein due to some inherent structural features of the latter. This reorganization displaces the 'yellow' and 'blue' P450s to the 'latent' positions and thereby renders them inactive. (B) The changes in the distribution of P450 species between the active and 'latent' positions caused by a substrate highly selective to one of the interacting enzymes. The interactions of three P450 enzymes present in the membrane bring forth a mixture of hetero-oligomers of various compositions, which exist in dynamic equilibrium exercised through their dissociation/reassociation (top). In this example, the 'active' positions are preferentially occupied by the 'green' enzyme due to some inherent features of the latter. A predominant part of the other two P450s is rendered inactive by depositing them in the 'latent' positions. The addition of a substrate (dark blue circles) highly selective to the 'blue' P450 results in the formation of substrate complexes of its available active subunits (1). Substrate binding stabilizes the 'blue' enzyme in the active conformation. This stabilization results in a slow redistribution of the P450 species between the hetero-oligomers of different compositions via their dissociation and reassembly (2). As a result, the 'blue' enzyme seizes most active positions and the 'green' P450 becomes inhibited.

An example of the experimentally observed manifestation of substrate-induced reorganization of P450 oligomers is provided by activation of CYP2D6 observed upon preincubation of microsomes containing co-incorporated CYP2D6 and CYP2E1 with CYP2D6-specific substrates [44]. The process of substrate-induced activation by this mechanism is relatively slow. For example, with the CYP2E1-CYP2D6 pair, the preincubation time needed for the full-range effect was as long as 20–40 minutes [44].

The hypothetical mechanism described earlier serves for selective activation of particular P450 species through the reorganization of hetero-oligomers in response to the appearance of their specific substrates or interactions with allosteric effectors. It allows for a rapid adaptation of the cell to any changes in its exposure to a varying spectrum of xenobiotics.

Additional complexity is added by apparent differences between the interacting proteins in their preferential occupation of the active or 'latent' positions in the oligomer, which can occur even in the absence of substrates [31]. Besides, oligomerization of P450s may result in the formation of effector binding sites at the subunit interface, a phenomenon that apparently occurs in the case of CYP3A4 [39,40,45]. Such sites may be involved in drug–drug interactions, allosteric regulation, and coordination of the catalytic properties of the P450 ensemble as an integrated system.

Prospective approaches for untangling the roots of nonadditivity in drug metabolism

According to the proposed model, the functional effects of interactions between P450 species would result in an essential deviation of the properties of the drug-metabolizing ensemble from the rule of simple additivity of their functional properties. Therefore, neglecting these effects in the algorithms of PBPK, *in vitro* to *in vivo* extrapolation (IVIVE), and pharmacogenetic profiling critically undermines their accuracy.

Under our model, the most critical impact of P450–P450 interactions on drug clearance is expected for the substances metabolized by multiple P450 species with comparable affinities and turnover rates. Admittedly, the degree of possible activation of a particular P450 in response to the appearance of its substrate is determined by the balance of the affinities of the interacting P450 species for this particular substrate. Therefore, the most pronounced substrate-dependent activation should be observed with the substrates specific to only one of the interacting P450 species. These substrates would activate the predominant part of the metabolizing enzyme by relocating it to the active positions. As a result, the rate of metabolism will become nearly proportional to the fractional content of the metabolizing enzyme. Consequently, probing the activities of individual P450 enzymes with these highly specific substrates may mask the functional effects of P450–P450 interactions and make the integral properties of the P450 ensemble appear as following the rule of simple additivity. In contrast, the metabolism of substrates that have comparable affinities to multiple P450 species (most drugs on the market) is expected to be most affected by the preferences of the individual P450 species for occupying 'latent' and active positions and thus reveal a pronounced nonadditivity in the properties of the system.

These considerations imply that drug metabolism studies with isoform-selective drug probes might not be instrumental enough to reveal the principles of functional integration in drug metabolism. More informative might be the studies with drugs metabolized by multiple P450 species (poly-specific substrates) and large selections of HLM samples with the known composition of the P450 pool.

An example of a sharp nonadditivity of the individual P450 functionalities revealed with a poly-specific P450 substrate is provided by our recent study with Coumarin 152, a substrate metabolized by CYP2B6, CYP2C19, CYP1A2, and CYP3A4 with comparable affinities and turnover numbers. As demonstrated in a study with specific inhibitors of these enzymes, when metabolized by HLM, this substrate is preferentially demethylated by CYP2C19, despite the expected predominant role of CYP3A4 [46].

In an attempt to establish a practical approach for these studies, we recently introduced a method based on a combination of targeted proteomics with the high-throughput screening of **substrate saturation profiles (SSP)** of poly-specific P450 substrates. In a pilot study with this technique [47], we determined the composition of the ensemble of ten major drug-metabolizing P450s in seven different HLM preparations and examined the variations in the parameters of metabolism of Coumarin 152, a model poly-specific substrate.

To delineate complex interrelationships between the composition of the P450 pool and the routes of C152 metabolism, we used **principal component analysis** of a series of SSP. The first two eigenvectors generated by this linear algebra method represent the combinations of the SSPs of the individual substrate-metabolizing P450. The corresponding eigenvalues were interpreted as being determined by the role of the individual P450s in substrate turnover. The profiles of eigenvalues for a series of HLM samples under analysis were then approximated with the profiles of the fractional content of ten P450 species in these samples. Using this strategy, we demonstrated

that the rate of C152 metabolism and the shape of its SSPs are primarily dictated by the abundances of five P450 species, namely CYP2B6, CYP3A5, CYP2C8, CYP2A6, and CYP2E1, of which only CYP2B6 possess high potency of metabolizing the probe substrate. The effects of at least four of the five enzymes pointed out in these studies (CYP2A6, CYP2C8, CYP2E1, and CYP3A5) are indirect and likely caused by their interactions with C152-metabolizing species (CYP2B6, CYP3A4, and CYP2C19) [47]. These results provide a compelling demonstration of nonadditivity in the properties of individual P450 species constituting the multienzyme system of drug metabolism. They demonstrate the high potential of the proposed approach in unveiling functional interrelationships between different P450 species and accommodating the algorithms of PBPK to the nonadditive behavior of the human drug-metabolizing ensemble.

Importance of nonadditive behavior of the drug-metabolizing ensemble for physiologically based pharmacokinetic (PBPK) modeling

PBPK modeling is emerging as a valuable approach for the prospective prediction of drug pharmacokinetics. PBPK modeling relies on physiological parameters and properties of drugs that govern their absorption, distribution, and elimination. The knowledge of the pathways of drug metabolism and its parameters is fundamental for constructing the PBPK models. This information is further integrated with the known or presumed expression levels of the individual drug-metabolizing enzymes. The current practice of PBPK implies that any increase or decrease in the metabolizing enzyme expression will proportionally affect the rate of conversion of all its substrates [48]. However, the nonadditive behavior of the drug-metabolizing ensemble makes the functional consequences of any change in the composition of the P450 pool more intricate. In our view, the next generation of PBPK models must include the algorithms accounting for the functional effects of P450–P450 interaction and, potentially, the interactions of P450s with other drug-metabolizing enzymes (such as UGTs). Introducing these new algorithms into PBPK would allow for an error-free assessment of the impact of genetic and epigenetic factors on human drug metabolism. It is also essential for using PBPK in predicting drug–drug interactions and other adverse drug effects.

Concluding remarks and future perspectives

Recent results on the interindividual variability in drug metabolism studied *ex vivo* with individual HLM preparations [5–7] substantially undermine the validity of the premise that the cumulative properties of the drug-metabolizing ensemble represent a simple aggregate of the properties of the constituting enzymes. In this article, we substantiate the decisive role of intermolecular interactions between various P450 species as an important determinant of the catalytic properties of the P450 ensemble. In our view, the functional consequences of these interactions represent the primary cause of nonadditive behavior of drug-metabolizing ensemble and an essential source of interindividual variability of drug metabolism. The effects of protein–protein interactions between multiple P450 species and their interactions with other drug-metabolizing enzymes and regulatory proteins are among the most critical factors that define the functional consequences of any genetic and epigenetic variations in the drug-metabolizing ensemble.

Lack of knowledge of the main principles of functional integration in the P450s obscures our understanding of interconnections between the composition of the P450 ensemble and human drug metabolism profile. There are many more outstanding questions than known answers in this area (see Outstanding questions). Here we propose a combination of high-throughput screening of SSP and the determination of the composition of the P450 ensemble through targeted proteomics as a potent strategy for filling this knowledge gap. Being applied to large sets of human liver samples and a rationally selected range of poly-specific drug substrates, this approach will allow elucidating the mechanisms of functional integration in the P450

Outstanding questions

What is the general architecture of P450 oligomers? Is it universal, or are there structural specifics in the interactions in certain P450 pairs?

What is the overall map of P450–P450 interactions in the ER?

Does the P450 hetero-oligomerization network include all microsomal P450s, or are there some P450s that stay aside?

Does the network of P450–P450 interactions in ER involve non-drug-metabolizing P450s? If yes, how do these interactions affect P450-dependent synthesis and metabolism of steroid hormones, cytokines, retinoic acid, and cholesterol?

How are the interactions between drug-metabolizing and non-drug-metabolizing P450s involved in the mechanisms of drug action and the side effects of drugs?

How do genetic polymorphism in P450s affect P450–P450 interactions and their functional effects?

How can the P450–P450 interactions be taken into account in predicting the effects of P450 polymorphisms?

How do the interactions of P450s with cytochrome b_5, PGRMC1, HO-1, UGTs, and other microsomal proteins affect P450–P450 interactions and their functional effects?

ensemble and obtaining the knowledge necessary for accommodating the methods of PBPK, IVIVE, pharmacogenetic profiling, and prediction of drug–drug interactions to nonadditive behavior of the drug-metabolizing ensemble.

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Declaration of interests

The authors declare no conflicts of interest.

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