Multifold Activation of CYP3A4 Caused by Alcohol-Induced Increase in the Content of CYP2E1 in Human Liver Microsomes

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Abstract

In order to probe the effect of alcohol-induced increase in the content of CYP2E1 in human liver microsomes (HLM) on the function of other P450 species we established a model that implements enrichment of HLM samples with CYP2E1 through membrane incorporation of the purified protein. Enrichment of HLM with CYP2E1 results in a considerably increased rate of metabolism of 7-benzyloxyquinoline (BQ) and eliminates the homotropic cooperativity observed with this CYP3A-specific substrate. Furthermore, incorporation of CYP2E1 also eliminates the activating effect α-Naphthoflavone (ANF) on BQ metabolism seen in untreated HLM. To probe the physiological relevance of these effects we compared three pulled preparations of HLM from normal donors (HLM-N) with a preparation obtained from heavy alcohol consumers (HLM-A). The composition of the P450 pool in all four samples was characterized with mass-spectrometric determination of isoform-specific peptides of 11 cytochrome P450 species. The molar content of CYP2E1 in HLM-A samples was from 2.5 to 3.3 times higher than that found in HLM-N preparations. In contrast, the content of CYP3A4 in HLM-A was the lowest among all four studied HLM samples. Despite the lower content of CYP3A4, HLM-A exhibited much higher rate of metabolism and lower degree of homotropic cooperativity with BQ, similar to that observed in CYP2E1-enriched HLM-N. Our results demonstrate that the catalytic activity and allosteric properties of CYP3A4 are fundamentally dependent on the composition of the cytochrome P450 ensemble in human liver and imply a profound impact of chronic alcohol exposure on the pharmacokinetics of drugs metabolized by CYP3A4.

***Keywords*:** cytochrome P450; CYP2E1; CYP3A4; oligomerization; membrane protein; protein-protein interaction; alcohol-drug interactions, drug metabolism

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Introduction

The core of the drug-metabolizing ensemble in human liver is the ensemble of multiple P450 species co-localized in the membrane of endoplasmic reticulum (ER). There is an emerging recognition of the fact that multiple P450 enzymes interact with each other with the formation of heteromeric complexes where the functional properties of individual P450 enzymes are largely modified (Davydov, 2011; Reed and Backes, 2012; Davydov, 2016; Reed and Backes, 2016). Resent findings point out the association of dissimilar P450s as the major cause for poor correlation between the composition of the P450 ensemble and its drug metabolizing profile demonstrated with the studies in a large set of normal human liver microsomes (Gao et al., 2016; Zhang et al., 2016).

Of particular importance are the changes in P450-P450 crosstalk induced by alcohol consumption. Although the multi-fold increase in the content of CYP2E1 in liver observed in alcohol consumers is well documented (Cederbaum, 1998; Dupont et al., 1998; Lieber, 1999; Cederbaum, 2006), the involvement of CYP2E1 in alcohol-drug interactions is commonly considered insignificant due to a minor role of this enzyme in drug metabolism (Jang and Harris, 2007). However, the effects of induction of CYP2E1 may stretch beyond the changes in CYP2E1-dependent drug metabolism and involve the effects of CYP2E1 on the functional properties of other drug-metabolizing enzymes.

In order to probe the crosstalk between CYP2E1 and other P450 species and elucidate its role in alcohol-drug interactions we established a model of alcohol-induced increase in CYP2E1 content that implements enrichment of HLM samples with CYP2E1 through membrane incorporation of the purified protein (Davydov et al., 2015; Davydov et al., 2017; Davydova et al., 2019). We demonstrated that the adopted CYP2E1 becomes a fully-functional member of the drug-metabolizing ensemble and interacts with other P450 enzymes with the formation of heteromeric complexes (Davydova et al., 2019). Studying the effect of enrichment of HLM with CYP2E1 on the function of other P450 enzymes we demonstrated a CYP2E1-induced activation of CYP1A2 and the associated re-routing of the metabolism of 7-ethoxy-4-cyanocoumarin (CEC), the substrate concurrently metabolized by CYP2C19 and CYP1A2, towards the latter enzyme (Davydova et al., 2019).

The present study continues our efforts on revealing the impact of alcohol-induced increase in CYP2E1 content on drug metabolism. Here we explore the effects of CYP2E1 on the functional properties of CYP3A4, the enzyme that metabolizes about 50% of drugs on

the market (Guengerich, 1999). As a probe substrate we used 7-benzyloxyquinoline (BQ), a fluorogenic substrate highly selective for CYP3A4 (Stresser et al., 2002). In addition to studying the effect of CYP2E1 on the parameters of BQ metabolism in a series of microsomal preparations with thoroughly characterized composition of the P450 pool, we also probed its impact on the effect of α-naphthoflavone, a prototypical allosteric effector of CYP3A4. In order to assess the relevance of the results obtained with CYP2E1-enriched microsomes to the changes caused by the effects of chronic alcohol exposure we also compared the parameters of BQ metabolism in a pulled HLM preparation obtained from 10 alcoholic donors with those exhibited by three pulled HLM preparations obtained from donors with no history of alcohol dependence. Our results demonstrate that the catalytic activity and allosteric properties of CYP3A4 are fundamentally dependent on the composition of the cytochrome P450 ensemble in human liver and imply a profound impact of chronic alcohol exposure on the pharmacokinetics of drugs metabolized by CYP3A4.

Materials and methods.

Chemicals

7-benzyloxyquinoline (BQ) was a product of CypEx (Dundee, UK). 7-hydroxycoumarin was obtained from Acros Organics, a part of Thermo Fisher Scientific (New Jersey, NJ). 7,8-benzoflavone (α-naphthoflavone, ANF) was a product of Indofine Chemical Company (Hillsborough, NJ). All other reagents were of ACS grade and used without additional purification.

Protein expression and purification

N-terminally truncated (Δ3-20) and C-terminally His-tagged CYP2E1 (Spatzenegger et al., 2003) was expressed in *E. coli* TOPP3 cells and purified as described earlier (Davydova et al., 2019).

Pulled human liver microsomes and their characterization with mass-spectrometry

The preparation of Human Liver Microsomes (HLM) obtained from 10 donors (mixed gender) with a history of chronic alcohol exposure (lot FVT) was purchased from BioIVT corporation (Baltimore, MD). This preparation is referred hereinafter as HLM-A. We also studied three different preparations of pulled human liver microsomes from 50 donors (mixed gender) without reported history of alcohol exposure. The preparation obtained by differential centrifugation of pulled human liver S9 fraction (the product of BD Gentest, lot number 3212595) is referred to as HLM-N1. The HLM samples referred here as HLM-N2

and HLM-N3 are InVitroCYP™ M-class 50-donor mixed gender pooled HLM preparations, lots LBA and LFJ respectively obtained from BioIVT corporation (Baltimore, MD).

The composition of the cytochrom P450 ensemble in these preparations was characterized by mass-spectrometric analysis with a triple quadrupole mass spectrometer using the method of multiple reaction monitoring (MRM) as described previously (Davydova et al., 2019). Content of NADPH-cytochrome P450 reductase and cytochromes P450 1A2, 2A6, 2B6, 2D6, 2C8, 2C9, 2C18, 2C19, 2E1, 3A4 and 3A5 was determined based on the concentration of protein-specific tryptic peptides in trypsin-digested samples with the use of the stable isotope labeled internal standards (SIS) (Davydova et al., 2019). For each protein, one standard peptide with three transitions was used. The peptides, which sequences are provided in Table 1, were arranged into one Selected Reaction Monitoring (SRM) assay. Mass-spectrometry measurements of P450 content were performed using the equipment of "Human Proteome" Core Facilities of the Institute of Biomedical Chemistry (Moscow, Russia).

Characterization of the content of NADPH-cytochrome P450 reductase and cytochromes P450 in HLM

The concentration of NADPH-cytochrome P450 reductase in microsomal membranes was determined based on the rate of NADPH-dependent reduction of cytochrome *c* at 25 °C and the effective molar concentration of CPR was estimated using the turnover number of 3750 min^{-1} (Davydova et al., 2019). The total concentration of cytochromes P450 in HLM was determined with a variant of "oxidized CO versus reduced CO difference spectrum" method as described earlier (Davydova et al., 2019).

Preparation of CYP2E1-enriched HLM samples.

Incorporation of CYP2E1 into HLM was performed by incubation of undiluted suspensions of HLM (20-25 mg/ml protein, 10-13 mM phospholipid) in 125 mM K-Phosphate buffer containing 0.25M Sucrose with purified CYP2E1 for 16 - 20 hours at 4°C at continuous stirring. CYP2E1 was added in the amount ranging from 0.25 to 2 molar equivalents to the endogenous cytochrome P450 present in HLM. Following the incubation the suspension was diluted 4-8 times with 125 mM K-Phosphate buffer, pH 7.4 containing 0.25 M sucrose and centrifuged at 53,000 rpm (150,000 g) in an Optima TLX ultracentrifuge (Beckman Coulter Inc., Brea, CA, USA) with a TLA100.3 rotor for 90 min at 4 °C. The pellet was resuspended in the same buffer to the protein concentration of 15-20 mg/ml. The amount of incorporated cytochrome P450 was calculated from the difference

between the heme protein added to the incubation media and the enzyme found in the supernatant. According to the results of this assay, our procedure resulted in incorporation of 96 -98% of the added protein into the microsomal membrane.

Fluorimetric assays of BQ metabolism.

The rate of debenzylation of 7-benzyloxyquinoline was measured with a real-time continuous fluorometric assay using a Cary Eclipse fluorometer (Agilent Technologies, Santa Clara, CA, USA) or a custom-modified PTI QM-1 fluorometer (Photon Technology International, New Brunswick, NJ (Davydov et al., 2017). In the experiments with Cary Eclipse the excitation was performed with a monochromatic light centered at 405 nm with 5 nm bandwidth. In the case of PTI QM-1 the excitation light centered at 405 nm was emitted by a CPS405 collimated laser diode module (Thorlabs Inc, Newton, NJ). The emission wavelength was set at 516 nm with a 20 nm slit. The rate of formation of 7-hydroxyquinolyne was estimated by determining the slope of the linear part of the kinetic curve recorded over a period of 2 – 3 min.

All kinetic assays were performed in 0.1 M Na-HEPES buffer, pH 7.4, containing 60 mM KCl. In the case of the use of Cary Eclipse the total volume of the incubation mixture was equal to 300μl and a 5 x 5 mm quartz cell was used. In the experiments with PTI QM-1 fluorometer we used a 3 x 3 mm quartz cell and the volume of the sample was equal to 60μl. With both instruments the kinetic assays were carried out at continuous stirring and the temperature was maintained at 30 °C with a circulating water bath. An aliquot of 15-20 mM stock solution of BQ in acetone was added to attain the desired substrate concentration in the range of 0.5 - 250 μM. The reaction was initiated by addition of 20 mM solution of NADPH to the concentration of 200 μM. Fitting of the dependencies of the reaction rate on the substrate concentration to Michaelis-Menten and Hill equations was performed with a combination of Marquardt and Nelder-Mead non-linear regression algorithms as implemented in our SpectraLab software (Davydov et al., 1995; Davydov et al., 2016).

Results.

Characterization of the composition of the cytochrome P450 pool in HLM samples.

The preparations of HLM used in this study were characterized by determining the content of NADPH-cytochrome P450 reductase and 11 major cytochrome P450 species, namely CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2E1, CYP3A4 and CYP3A5, with mass-spectrometry. The total content of cytochromes P450 and the

concentration of NADPH-cytochrome reductase (CPR) were also quantified with absorbance spectroscopy and determination of the rate of NADPH-dependent reduction of cytochrome *c* respectively.

Results of our analysis of the composition of the P450 pool in HLM are summarized in Table 1. The values presented in this table for HLM-N2, HLM-N3 and HLM-A are the mean values averaged over the results of 12, 17 and 11 individual measurements respectively. The values shown for HLM-N1 are the results of a single measurement. As expected, the fractional content of CYP2E1 in HLM-A was from 2.5 to 3.3 fold higher than that detected in the HLM-N samples.

Parameters of BQ metabolism exhibited by four HLM-preparations

In order to probe the effect of the composition of the P450 ensemble on the functional properties of CYP3A4 we determined the parameters of O-debenzylation of BQ by all four microsomal preparations under study. We also probed the effect of ANF, the prototypical allosteric effector of the enzyme. Results of these studies are summarized in Table 2 and illustrated in Fig. 1. It should be noted that the rate of metabolism of BQ in HLM in this study was normalized on the concentration of CPR in the microsomal membrane (see Materials and Methods). In view of a high excess of cytochromes P450 over CPR in HLM, this normalization was considered as the most appropriate approach for comparing the rates of metabolism in the HLM preparations with different composition of the cytochrome P450 ensemble.

In all three samples of HLM-N the metabolism of BQ revealed a prominent homotropic cooperativity characterized with the Hill coefficient of 1.9 – 2.2., in a good agreement with the previous reports (Stresser et al., 2002; Davydov et al., 2008). In HLM-N samples the maximal rate of reaction increases concomitant with increasing fractional content of CYP3A4 in microsomes, although the relationship between these two parameters is markedly nonlinear (Table 2). Strikingly, despite the low content of CYP3A4 in HLM-A, this preparation is characterized with the highest maximal rate of BQ metabolism over all four HLM samples studied. V_{max} value exhibited by HLM-A is almost 10 times higher than the value obtained with HLM-N1, where the fractional content of CYP3A4 (28%) is comparable to that observed in HLM-A (21%). The values of V_{max} obtained with HLM-N2 and HLM-N3, where the fractional content of CYP3A4 is around 40%, are respectively 2.2 and 3.5 times lower than that characteristic to HLM-A. This multifold activation of BQ

metabolism in HLM-A is associated with attenuation of homotropic cooperativity, which is revealed in a considerable decrease of the Hill coefficient (Table 2).

The effect of ANF.

Consistent with the previous observations (Domanski et al., 2001; Davydov et al., 2008; Davydov et al., 2013) the addition of 25 mM ANF completely eliminated homotropic cooperativity observed with BQ and considerably increased decreased the S_{50} values in all four HLM samples studied (Table 2). However, the effect of ANF on the maximal rate of reaction differs dramatically between the HLM samples. Whereas in the case of HLM-N1 the addition of ANF increased the maximal rate of reaction almost two times, it resulted in over two-fold inhibition of BQ metabolism in HLM-N2 while having no statistically significant effect in on V_{max} in HLM-N2 and HLM-A (Table 2). The effect of ANF on substrate saturation curves in four HLM samples is illustrated in Fig. 1 (curves in black).

Effect of incorporation of CYP2E1 into HLM on BQ metabolism

To probe if the loss of homotropic cooperativity with BQ and a multifold increase in the rate of its metabolism in HLM-A is caused by alcohol-induced increase in the fraction of CYP2E1 in the P450 pool we studied the effect of incorporation of CYP2E1 into HLM membrane. The dependencies of the reaction rate on substrate concentration with and without addition of ANF obtained with HLM-A and three samples of HLM-N enriched with CYP2E1 are exemplified in Fig. 1 (curves shown in red). The parameters of BQ metabolism in these preparations may be found in Table 2. As seen from these data, incorporation of CYP2E1 essentially eliminates the heterotropic cooperatvity in all four microsomal samples. Furthermore, in HLM-A and two of the three HLM-N samples – HLM-N1 and HLM-N3 – this incorporation results in a significant increase in the maximal rate of BQ turnover. Therefore, in some sense, the effect of incorporation of CYP2E1 into HLM-A is analogous to the effect of the addition of ANF. Interestingly, incorporation of CYP2E1 into HLM-N results in an increase in S_{50} value, where is HLM-A the effect of incorporation on S_{50} is opposite .

Importantly, addition of ANF to CYP2E1-enriched HLM preparations largely eliminates their differences from the untreated HLM samples in the parameters of BQ metabolism. With all three CYP2E1-enriched HLM-N samples the addition of ANF considerably inhibits BQ turnover at substrate saturation, while decreasing S_{50} values and eliminating heterotropic cooperativity. In the case of CYP2E1-enriched HLM-A the effect of ANF on V_{max} is marginal, while the effects on S_{50} and the Hill coefficient are retained.

The effect of increasing amounts of incorporated CYP2E1 on the parameters of BQ metabolism in HLM-N is illustrated in Fig. 2 and Fig. 3. The dependencies of S_{50}, V_{max} and the Hill coefficient on CYP2E1 content obtained in the absence of ANF reveal a striking contrast between the "normal" HLM samples (Fig. 2) and the sample obtained from the alcohol-exposed donors (Fig. 4). Enrichment of HLM-N in CYP2E1 resulted in a stepwise decrease in the values of S_{50} and the Hill coefficient, while increasing V_{max} (Fig. 2). In contrast incorporation of CYP2E1into HLM-A, while also increasing $V_{max,}$, resulted in a significant decrease in S_{50} at virtually unchanged Hill coefficient (Fig 4). It is extremely unlikely that these changes may result from the activity of CYP2E1 itself or be due to participation of any other P450 enzyme, as BQ metabolism is known to be almost entirely selective to CYP3A4. CYP2E1 itself lacks any potential to metabolize this substrate (Stresser et al., 2002; Davydov et al., 2015). According to the data of Stresser and co-authors (Stresser et al., 2002), the only other enzymes present in adult HLM and capable of BQ metabolism are CYP1A2 and CYP3A5. However, the turnover numbers exhibited by these enzymes with BQ are respectively 8.5 and 4.3 times lower than that characteristic to CYP3A4 (Stresser et al., 2002). Therefore, due to relatively low abundance of CYP1A2 and CYP3A5 in HLM their sizable participation in BQ metabolism is unlikely.

Our results on the effects of CYP2E1 incorporation demonstrate that the increased rate and decreased homotropic cooperativity in BQ metabolism seen in HLM-A are attributable, at least in a part, to direct effects of increased CYP2E1 content in this sample. However, while the differences between the HLM-N and HLM-A preparations in the degree of homotropic cooperativity are essentially eliminated by CYP2E1 incorporation, large contrast between them in the values of V_{max} and S_{50} values is retained, despite a pronounced activation of BQ metabolism in HLM-N samples by their enrichment in CYP2E1 (Fig. 2b).

In contrast to what is observed at no effector added, the dependencies obtained in the presence of ANF (Fig. 3) show that the addition of this allosteric effector eliminates most of the effects of CYP2E1, although the increased activity of CYP2E1 in HLM-A preparation remains to be seen (Fig. 4). This observation suggests that the grounds of heterotropic cooperativity in CYP3A4 and the origins of its activation by CYP2E1 may involve some common mechanistic elements.

Discussion.

Changes in the composition of the cytochrome P450 ensemble under influence of chronic alcohol exposure.

Analysis of the content of 11 drug-metabolizing cytochrome P450 species in pooled HLM preparation obtained from alcoholic donors revealed a 2.5 – 3.3 fold increase in the fractional content of CYP2E1 as compared to the microsomal samples unaffected by alcohol exposure (Table 1). This observation is in a good agreement with the previous reports (Cederbaum, 1998; Dupont et al., 1998; Lieber, 1999; Cederbaum, 2006). Our data also indicate 1.5 – 2 fold elevation of the fractional contents of CYP2D6, CYP2C18 and CYP2C8 (Table 1). Although the results of our analysis of a single pooled preparation of HLM from alcohol-exposed donors cannot serve as a proof of alcohol-induced increase in the content of these P450 species, this observation may provide a clue to further identification of changes in P450 expression induced by alcohol exposure. Importantly, our results reveal no alcohol-induced increase in the contents of CYP3A4 and CYP2A6, notwithstanding with the existing reports on induction of expression of these enzymes by ethanol in model animals and in cell culture (Feierman et al., 2003; Jin et al., 2012).

Another notable difference of the HLM-A preparation from HLM-N samples is low degree of coverage of the total P450 concentration with the sum of the contents of 11 analyzed P450 species. The averaged degree of coverage of 61±12 % calculated for three HLM-N samples is in a reasonable agreement with the estimate of 78%, calculated for the fractional content of the 11 analyzed P450 species based on the data of Wang and co-authors, who determined the content of 25 P450 species in a series of 102 individual HLM samples (Wang et al., 2020). In contrast, the degree of coverage calculated for HLM-A is as low as 18±8 %. This difference may reveal an alcohol-induced increase in the content of some major P450 enzyme(s) which were not included in our assays, such as CYP4F2 and/or CYP4A11.

Multifold activation of CYP3A4 by alcohol exposure is stipulated by the effect of CYP2E1 on the landscape of P450-P450 interactions

The most striking result of this study is a demonstration of a multifold activation of CYP3A4-dependent metabolism of BQ in HLM sample from alcohol-exposed donors. Although the fractional content of CYP3A enzymes in HLM-A was the lowest among all four studied preparations, these microsomes exhibited much higher rate of metabolism and

lower homotropic cooperativity with BQ as compared to HLM-N samples. Notably, in contrast to two of the three HLM-N preparations, HLM-A does not show any ANF-induced increase in V_{max} of debenzylation of BQ. Incorporation of additional CYP2E1 into HLM-A results in a further increase in the rate of BQ metabolism and makes this rate over an order of magnitude faster than that exhibited by the HLM-N sample with a similar CYP3A content.

The finding that the activation of BQ metabolism and attenuation of its cooperativity may be reproduced in HLM-N samples via incorporation of the exogenous CYP2E1 into their membranes suggests that these effects, at least in a part, represent direct results of the alcohol-induced increase in CYP2E1 content. The striking effects of one P450 protein on the functional properties of another P450 enzyme observed in this study and evidenced in earlier reports from our group and by others (Backes et al., 1998; Kelley et al., 2006; Reed et al., 2010; Davydov et al., 2015; Davydov et al., 2017) may be best explained with a concept of "positional heterogeneity" in P450 oligomers (Davydov, 2011; Davydov et al., 2015; Davydov, 2016; Reed and Backes, 2017; Davydova et al., 2019). This concept is based on considering heteromeric complexes of multiple P450 species as a predominant state of the P450 enzymes in the microsomal membrane. According to our hypothesis the P450 subunits forming these oligomers are not identical in their conformation and orientation, but are characterized by different abilities to be reduced, bind substrates and interact with redox partners (Davydov et al., 2015; Davydova et al., 2019). In effect, oligomerization results in abstracting an important part of the P450 pool from the catalytic activity. A discussion of possible structural basis for positional heterogeneity in P450 oligomers may be found in a comprehensive review by Reed and Backes (Reed and Backes, 2017).

From the standpoint of the above concept, the activation of one P450 enzyme by its interaction with another one might reveal a difference between the two proteins in their propensities for occupying the positions of two different types. In particular, it might be hypothesized that, when CYP3A4 interacts with CYP2E1, the former preferentially occupies the "active" positions, whereas CYP2E1 fills the "restrained" ones. Resulting re-distribution of CYP3A4 between the "active" and "restrained" positions increases the active fraction of this enzyme in HLM.

Variability of heterotropic cooperativity of CYP3A4 in HLM and its modulation by P450-P450 interactions

Four preparations of HLM examined in this study revealed notable differences in their response to the addition of ANF, a prototypical allosteric effector of CYP3A enzymes.

Although the attenuation of the homotropic cooperativity with BQ and increase in the affinity to this substrate were revealed with all four samples, the effect of ANF on the maximal rate of BQ turnover was contrastingly different. Whereas in the case of HLM-N1 the addition of ANF substantially increased the maximal rate of reaction, it resulted in over two-fold inhibition in HLM-N2 while having no statistically significant effect in on $V_{\max}$ in HLM-N2 and HLM-A (Table 2).

Importantly, increase in the fractional content of CYP2E1, either as a result of chronic alcohol exposure or through incorporation of the exogenous protein, resulted in a substantial decrease in the degree of heterotropic cooperativity seen with BQ (Table 2). Moreover, incorporation of additional CYP2E1 into HLM-A causes a decrease in the S_{50} value, similar to that observed upon the addition of ANF. Therefore, the effect of enrichment of the microsomal membrane with CYP2E1 on BQ metabolism reveals some parallelism with the effect of ANF.

The mechanisms of heterotropic cooperativity in CYP3A4 became a subject of extensive studies and a vigorous discussion over the last two decades. The initial interpretation of all known examples of CYP3A4 cooperativity, either homo- or heterotropic, is that the large substrate-binding pocket of the enzyme sometimes requires simultaneous binding of several substrate molecules to assure a productive orientation of at least one of them (see (Davydov and Halpert, 2008; Denisov et al., 2009; Denisov and Sligar, 2012) for review). The presence of at least two molecules of some substrates in the binding pocket of CYP3A4 is well established (Roberts et al., 2005; Fernando et al., 2006; Denisov et al., 2007). Although this model provides a reasonable explanation for most cases of homotropic cooperativity, it fails to explain complex instances of heterotropic activation in CYP3A4 (Davydov et al., 2013).

More recent studies compellingly demonstrated the presence of a separate allosteric effector binding site and revealed its role in the instances of heterotropic activation of CYP3A4 by various effectors, including ANF (Davydov et al., 2012; Davydov et al., 2013; Polic and Auclair, 2017; Denisov et al., 2018; Marsch et al., 2018; Denisov et al., 2019; Ducharme et al., 2019). This allosteric site is formed at the vicinity of the *F'* and *G'* helices and located at the interface between the two CYP3A4 molecules in the crystallographic dimer of CYP3A4 complex with peripherally-bound progesterone (PDB 1W0F (Williams et al., 2004)). As we discussed earlier (Davydov et al., 2013), the high-affinity interactions at this site require the protein to be oliogomeric – ligands are unlikely to bind with high

affinity at this site in the monomeric enzyme due to small size and low degree of "buriedness" of the pocket formed by *F – F'* and *G – G'* loops. Therefore, if this peripheral site is involved in the mechanism of heterotropic activation of CYP3A4, the allosteric properties of the enzyme are expected to be determined by the degree of its oligomerization. This close interconnection between the allosteric properties of CYP3A4 and the degree of its oligomerization we demonstrated in our study of the activating effect of ANF in model microsomes with variable surface density of CYP3A4 in the membrane (Davydov et al., 2013).

Location of the allosteric site at the interface between two subunits of the P450 oligomer suggests a critical dependence of allosteric properties of the enzyme in HLM on the formation of mixed oligomers between multiple P450 species. Although in some pairs of CYP3A4 with interacting partners this allosteric site may be retained, its affinity to the ligands and the effect of their binding on the functional properties of CYP3A4 must be considerably modified by heterooligomerization. Even though we are far away from understanding the mechanisms of P450 heteroassociation and their relationship to the allosteric properties of the P450 ensemble, the proposed model provides a reliable explanation for a remarkable connection between CYP3A4 allostery and the composition of the P450 pool revealed in our studies.

Concluding remarks

Our study with human liver microsomes obtained from alcoholic donors demonstrated that chronic alcohol exposure results in a multifold activation of CYP3A4, the enzyme responsible for metabolism of wide variety of drugs currently on the market. This activation and the associated attenuation of homo- and heterotropic cooperativity of CYP3A4 can be reproduced in model HLM preparations, where the content of CYP2E1 was elevated by incorporation of the purified enzyme. These results reveal a profound effect of alcohol-induced increase in CYP2E1 content on the functional properties of CYP3A4 and suggest an important role of CYP2E1-CYP3A4 interactions in the mechanisms of the effects of alcohol exposure on drug metabolism. Furthermore, our findings compellingly demonstrate that the profile of human drug metabolism cannot be unambiguously determined from a simple superposition of the properties of the P450 species constituting the drug-metabolizing ensemble. This profile rather reflects a complex, "non-linear" combination of the functionalities of the individual enzymes which is largely affected by their intermolecular interactions and the formation heteromeric complexes.

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Authorship Contributions.

Davydov and Zgoda designed the study; Davydov wrote the paper; Davydova, Dangi, Vavilov and Davydov conducted the experiments; Davydov, Dangi, Vavilov and Zgoda analyzed the results.

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Table 1. Composition of the cytochrome P450 pool in HLM preparations used in this study*

Cytochrome P450 Species	Peptide used in the assay	HLM-N1		HLM-N2		HLM-N3		HLM-A		A-to-N ratio[b]
		Content, pmol/mg	Specific fraction, %[a]	Content, pmol/mg	Specific fraction, %[a]	Content, pmol/mg	Specific fraction, %[a]	Content, pmol/mg	Specific fraction, %[a]	
CYP1A2	ASGNLIPQEK	38.1	15.6	12.6 ±2.4	5.44 ±0.38	12.7 ±2.6	5.64 ±0.79	4.1 ±0.8	8.16 ±1.47	0.92
CYP2A6	GTGGANIDPTFFLSR	43.1	17.6	46.8 ±6.7	22.4 ±2.4	40.7 ±9.4	17.8 ±1.9	8.7 ±3.5	15.2 ±2.8	0.79
CYP2B6	GYGVIFANGNR	1.13	0.46	2.69 ±0.70	1.24 ±0.14	2.33 ±0.75	1.08 ±0.19	0.29 ±0.06	0.58 ±0.11	0.63
CYP2C8	GLGIISSNGK	5.13	2.10	2.55 ±3.55	0.56 ±0.69	4.46	1.34	0.91 ±0.23	1.93 ±0.33	1.5
CYP2C9	GIFPLAER	33.7	13.8	33.9 ±7.1	17.0 ±0.3	31.4 ±9.5	15.2 ±2.4	5.6 ±0.9	10.7 ±2.3	0.70
CYP2C18	IAENFAYIK	2.61	1.07	0.50 ±0.06	0.28 ±0.03	0.97 ±0.36	0.36 ±0.04	0.53 ±0.22	0.93 ±0.21	1.6
CYP2C19	ICVGEGLAR	7.81	3.19	0.52 ±0.12	0.27 ±0.07	0.69 ±0.14	0.34 ±0.07	0.25 ±0.12	0.59 ±0.29	0.47
CYP2D6	LLDLAQEGLK	1.07	0.44	1.26 ±0.49	0.49 ±0.06	2.00 ±1.24	0.61 ±0.18	0.75 ±0.56	0.98 ±0.37	1.9
CYP2E1	GIIFNNGPTWK	37.6	15.4	26.0 ±6.8	11.9 ±1.3	34.7 ±10.5	14.5 ±1.4	22.6 ±8.4	38.9 ±2.3	2.8
CYP3A4	LSLGGLLQPEKPVVLK	67.3	27.5	109 ±54	39.9 ±7.0	122 ±54	40.6 ±5.9	16.3 ±12.3	21.4 ±6.2	0.59
CYP3A5	DVEINGVFIPK	6.89	2.82	1.69 ±1.61	0.47 ±0.31	7.69 ±5.65	2.55 ±1.28	0.38 ±0.11	0.71 ±0.12	0.36
Total of all species		208	85.2	236 ±66	53.5 ±14.6	259 ±76	65.6 ±19.1	60.0 ±25.5	17.6 ±7.5	
P450 content[c]		244		405 ±95		394 ±53		341 ±70		
CPR (by LC-MS/MS)	NPFLAAVTTNR	58.7	101	34.4 ±8.1	55 ±12.8	37.6 ±9.3	73 ±18.1	44.3 ±17.3	127 ±50	
CPR content[c]		57.8		63.0 ±12.2		51.4 ±11.6		34.9 ±4.3		

* The values given in the table are the averages of 11 -17 individual measurements, except for the measurements with HLM-N1 and the determination of CYP2C8 in HLM-N3, which were performed only once. The"±" values are the confidence intervals calculated for p=0.05.

[a] The values of the specific content of P450 species are the averages of the values calculated for each individual dataset. The fractions shown for the total P450 content and the content of CPR are the ratios of the amounts detected with LS-MS/MS to the amounts determined with absorbance spectroscopy (P450) and activity (CPR) assays.

[b] "A-to-N ratio" is the ratio of the fractional content of a given P450 species in HLM-A to an average of the respective contents in HLM-N samples. This ratio characterizes the direction and the amplitude of apparent alcohol-induced changes in the content of P450 enzymes.

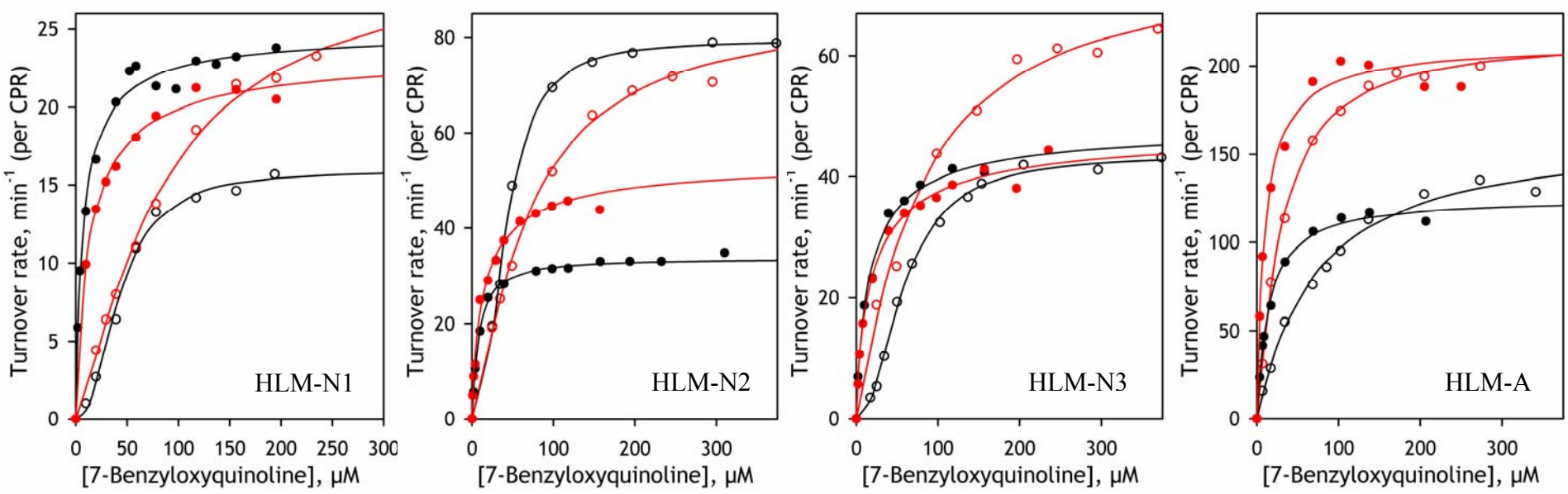
[c] The values of P450 and CPR content were determined with absorbance spectroscopy (P450) and activity (CPR) assays and expressed in pmol per mg of protein.

<u>Table 2.</u> Parameters of BQ metabolism obtained with four HLM preparations and their modulation by α-naphthoflavone*

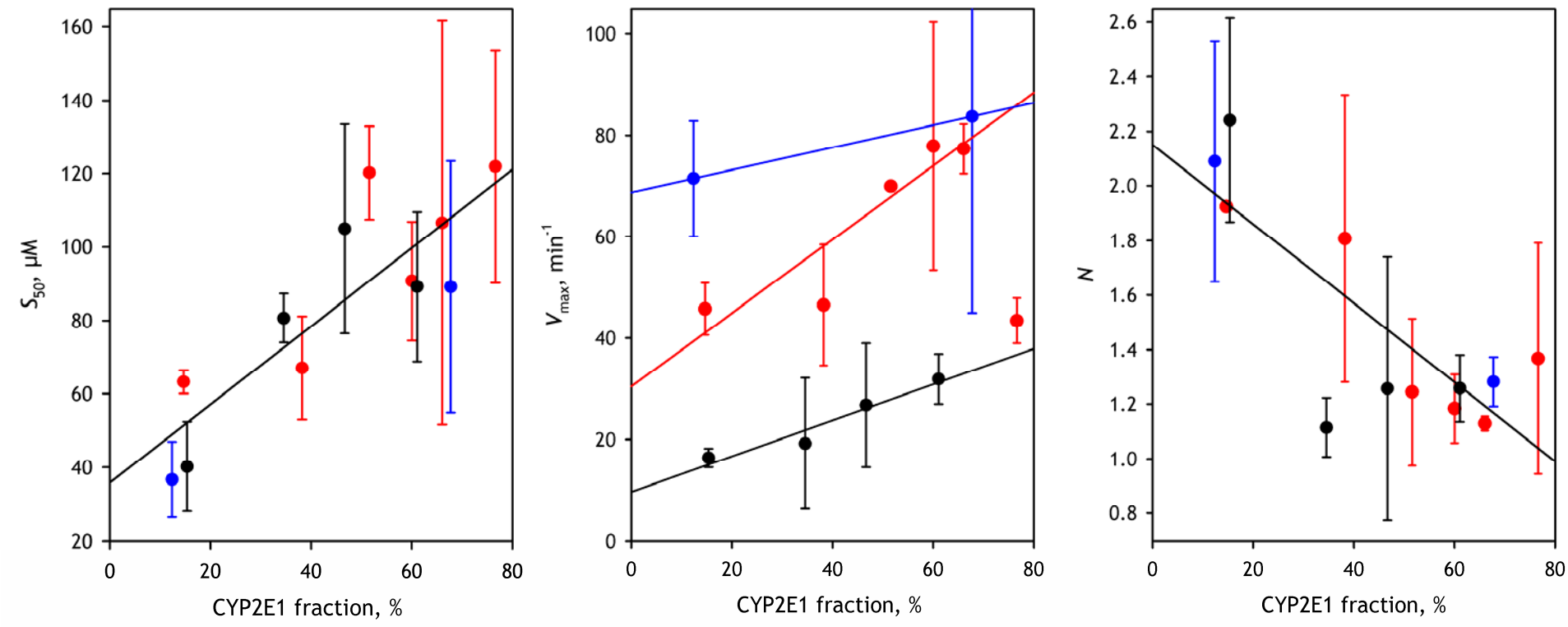
HLM preparation[a]	S_{50}, μM				N				V_{max}, min^{-1}			
	No effector		+ANF		No effector		+ANF		No effector		+ANF	
HLM-N1	40.2 ±12.2		**8.1 ±1.3**	**(0.007)**	2.24 ±0.37		**0.89 ±0.11**	**(0.002)**	16.4 ±1.8		**22.6 ±5.0**	**(0.033)**
HLM-N1+2E1	**89.2 ±20.4**	**(0.012)**	**12.4 ±4.3**	**(0.012)**	**1.26 ±0.12**	**(0.009)**	**0.87 ±0.16**	**(0.020)**	**31.9 ±5.0**	**($<10^{-3}$)**	**19.9 ±2.7**	**(0.014)**
HLM-N2	36.7 ±10.3		**7.4 ±3.8**	**(0.006)**	2.09 ±0.44		**1.22 ±0.30**	**(0.033)**	71.4 ±11.4		**32.6 ±3.5**	**(0.003)**
HLM-N2+2E1	**89.1 ±34.3**	**(0.031)**	**12.0 ±4.7**	**(0.014)**	**1.28 ±0.09**	**(0.011)**	1.02 ±0.24	(0.068)	83.7 ±38.8	(0.573)	46.1 ±11.1	(0.131)
HLM-N3	63.3 ±3.2		**19.7 ±6.7**	**($<10^{-3}$)**	**1.92 ±0.02**		**1.02 ±0.37**	**(0.010)**	45.7 ±5.1		49.3 ±7.9	(0.528)
HLM-N3+2E1	107 ±55	(0.248)	**16.3 ±6.9**	**(0.019)**	**1.13 ±0.03**	**($<10^{-6}$)**	**0.86 ±0.08**	**(0.003)**	**77.3 ±4.9**	**(0.001)**	46.8 ±16.6	(0.082)
HLM-A	55.4 ±12.8		**22.9 ±9.5**	**(0.010)**	1.30 ±0.13		**0.99 ±0.20**	**(0.021)**	123 ±32		118 ±50	(0.779)
HLM-A+2E1	28.8 ±19.0	(0.105)	7.5 ±3.3	(0.162)	1.16 ±0.16	(0.336)	0.99 ±0.05	(0.178)	**233 ±91**	**(0.015)**	204 ±102	(0.725)

* The values given in the table are the averages of 2 - 6 individual measurements. The"±" values are the confidence intervals calculated for p=0.05. The values in parentheses represent the p-values of Student's t-test for the hypothesis of equality of the respective values with the value obtained with untreated microsomes (for the values in "No effector" columns) or at no ANF added (for the values in "+ANF" columns). The values characterized with p-values ≤0.05 are shown in bold to emphasize the effects with high statistical significance.

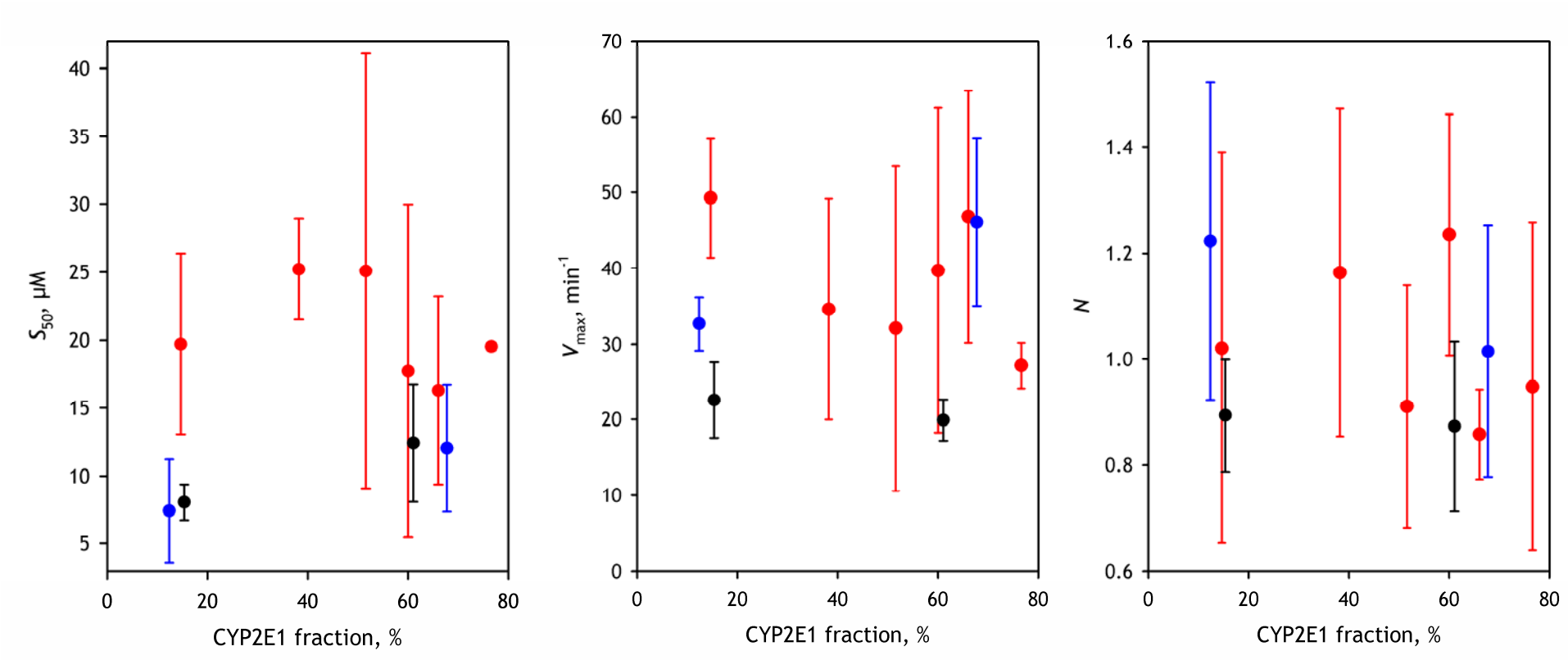
[a] The CYP2E1-enriched samples represented in this table are the samples where the content of added CYP2E1 was equal to the total P450 content in untreated HLM.



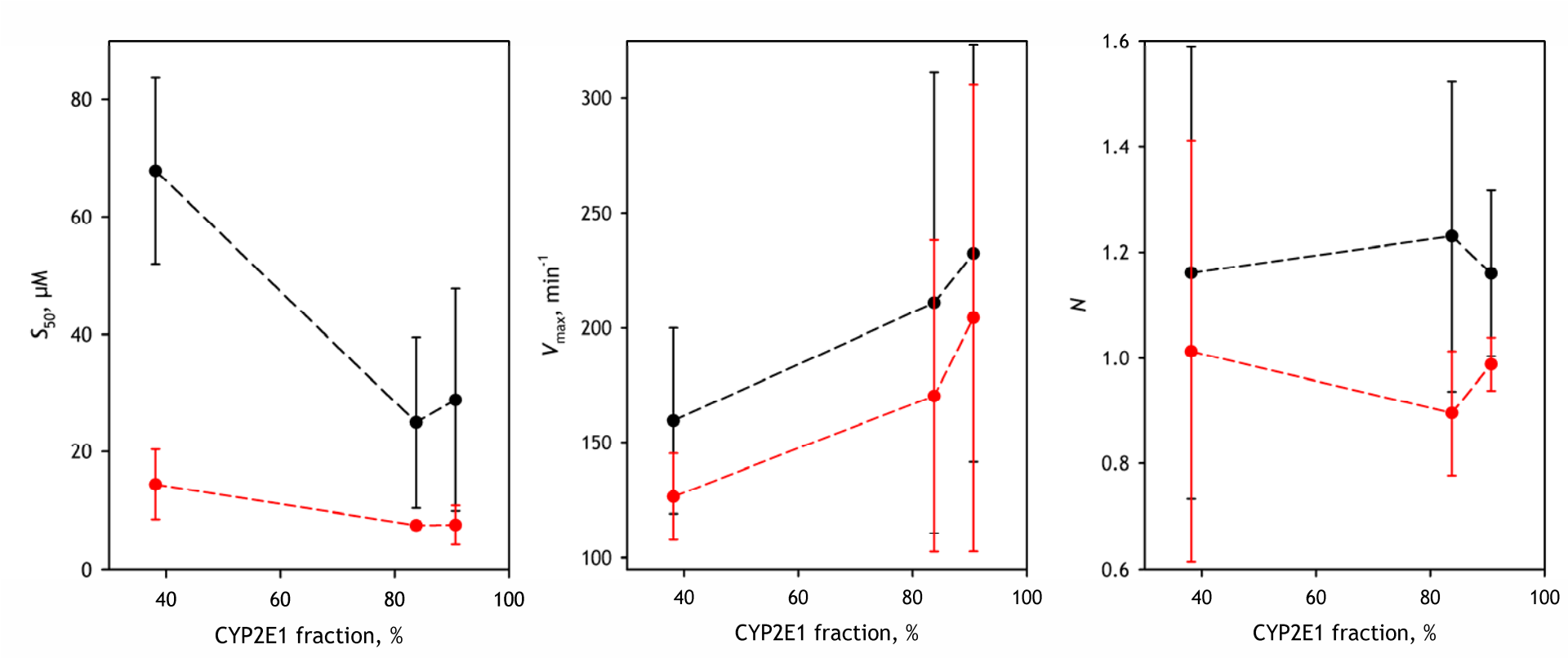
<u>Fig. 1.</u> BQ metabolism in intact and CYP2E1-enriched HLM samples. The figure show the dependencies of the rate of BQ turnover on substrate concentration in intact (black) and CYP2E1-enriched (red) HLM preparations. The dependencies obtained in the absence of added effector are shown in open circles, while the data obtained in the presence of 25 μM ANF are designated by closed circles. The CYP2E1-enriched samples exemplified in this figure are the samples where the content of added CYP2E1 was equal to the total P450 content in HLM ("1:1 incorporation"). The solid line show the results of fitting of the datasets with the Hill equatrion.



<u>Fig.2.</u> Effect of incorporation of CYP2E1 into HLM-N on the parameters of BQ metabolism at no added effector. Dependencies of S_{50}, V_{max} and N on the fractional content of CYP2E1 in the total of 11 analyzed P450 species. The datasets obtained with HLM-N1, HLM-N2 and HLM-N3 are shown in black , blue and red respectively. The error bars show the confidence intervals calculated for p=0.05. Solid lines show the linear approximations of the data.



<u>Fig.3.</u> Effect of incorporation of CYP2E1 into HLM-N on the parameters of BQ metabolism in the presence of ANF. Dependencies of S_{50}, V_{max} and N on the fractional content of CYP2E1 in the total of 11 analyzed P450 species. The datasets obtained with HLM-N1, HLM-N2 and HLM-N3 are shown in black , blue and red respectively. The error bars show the confidence intervals calculated for p=0.05.



<u>Fig.4.</u> Effect of incorporation of CYP2E1 into HLM-A on the parameters of BQ metabolism. Dependencies of S_{50}, V_{max} and N on the fractional content of CYP2E1 in the total of 11 analyzed P450 species. The datasets obtained at no substrate added and in the presence of ANF are shown in black and red respectively. The error bars show the confidence intervals calculated for p=0.05.