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Effect of ethanol on spectral binding, inhibition, and activity of CYP3A4 with an antiretroviral drug nelfinavir

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ABSTRACT

Cytochrome P450 3A4 (CYP3A4) is the most abundant CYP enzyme in the liver and metabolizes approximately 50% of the drugs, including anti-retrovirals. Although CYP3A4 induction by ethanol and impact of CYP3A4 on drug metabolism and toxicity is known, CYP3A4-ethanol physical interaction and its impact on drug binding, inhibition, or metabolism is not known. Therefore, we studied the effect of ethanol on binding and inhibition of CYP3A4 with a representative protease inhibitor, nelfinavir, followed by the effect of alcohol on nelfinavir metabolism. Our initial results showed that methanol, ethanol, isopropanol, isobutanol, and isoamyl alcohol bind in the active site of CYP3A4 and exhibit type I spectra. Among these alcohol compounds, ethanol showed the lowest K_D (5.9 ± 0.34 mM), suggesting its strong binding affinity with CYP3A4. Ethanol (20 mM) decreased the K_D of nelfinavir by >5-fold (0.041 ± 0.007 vs. 0.227 ± 0.038 μM). Similarly, 20 mM ethanol decreased the IC_{50} of nelfinavir by >3-fold (2.6 ± 0.5 vs. 8.3 ± 3.1 μM). These results suggest that ethanol facilitates binding of nelfinavir with CYP3A4. Furthermore, we performed nelfinavir metabolism using LCMS. Although ethanol did not alter k_{cat}, it decreased the K_m of nelfinavir, suggesting a decrease in catalytic efficiency (k_{cat}/K_m). This is an important finding because alcoholism is prevalent in HIV-1-infected persons and alcohol is shown to decrease the response to antiretroviral therapy.

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1. Introduction

Cytochrome P450 3A4 (CYP3A4) is the most abundant CYP enzyme in the liver and metabolizes approximately 50% of the drugs, including anti-retroviral therapeutic (ART) drugs, such as non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) [1]. In addition to substrates, NNRTIs and PIs are also the inducers and inhibitors of CYP3A4 [2,3]. Since ART regimens consist of multiple drugs, there are several reports of drug–drug interactions as a result of CYP3A4 induction or inhibition [2,4]. Although most drug–drug interactions are toxic, some of these interactions are beneficial. For example, ritonavir increases the bioavailability of most NNRTIs/PIs by inhibiting CYP3A4, and therefore, it is included in NNRTIs/PIs regimens [5].

Ethanol is known to interact with many medications (e.g., antibiotics, antidepressants, and anti-inflammatory agents) thereby altering drug metabolism and causing toxicity [6]. Ethanol-mediated altered drug metabolism occurs through the induction of CYP3A4 by ethanol [7,8]. Recently, we have shown that ethanol also induces CYP3A4 in monocyte-derived macrophage [9], which is one of the targets of ART drugs [10]. Thus, individuals who consume ethanol and take ART drugs are at high risk of deleterious alcohol–drug interaction. Although CYP3A4 induction by alcohol is well documented [7–9], there is no report on alcohol-CYP3A4 physical interaction and its impact on ART–CYP3A4 interaction.

Ethanol is known to interact with proteins through H-bond and stabilize hydrophobic interactions by removing water molecules [11]. CYP3A4 contains several water molecules in the active site; some of which are expelled out upon binding of a hydrophobic drug [12,13]. It is plausible that ethanol replaces water molecules from the active site and provides additional H-bonds and hydrophobic interactions that strengthen drug-CYP3A4 binding. Our hypothesis is that the binding of ethanol in the active site of CYP3A4 facilitates ART–CYP3A4 interaction and alters ART drugs metabolism. To test the hypothesis, we studied the spectral binding of several alcohol compounds with CYP3A4 followed by the effect of ethanol on binding, inhibition, and activity of CYP3A4 with an ART drug, nelfinavir. Nelfinavir is one of the PIs used for HIV-1 treatment [14]. In addition, it is gaining future prospects for the treatments of malaria, tuberculosis, SARS, and cancer [15].

Abbreviations: CYP3A4, cytochrome P450 3A4; CPR, cytochrome P450 reductase; b_5, cytochrome b_5; ART, antiretroviral therapy; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; 7-BFC, 7-benzyloxy-4-trifluorom ethylcoumarin; 7-HFC, 7-hydroxy-4-trifluoromethylcoumarin; LCMS, liquid chromatography mass spectroscopy; MRM, multiple reactions monitorings.

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2. Materials and methods

2.1. Materials

7-Benzyloxy-4-trifluoromethylcoumarin (7-BFC) and 7-hydroxy-4-trifluoromethylcoumarin (7-HFC) were purchased from Molecular Probes, Inc. (Eugene, OR). Nelfinavir was bought from Toronto Research Chemicals (North York, On, Canada). NADPH was from Sigma Chemical Co. (St. Louis, MO). All the alcohol compounds (methanol, ethanol, isopropanol, isobutanol, and isoamyl alcohol) were obtained from Fisher scientific Inc. (Waltham, MA). Ni–NTA affinity resin was purchased from Qiagen (Valencia, CA). All other chemicals were of the highest grade available and were obtained from standard commercial sources.

2.2. Enzyme preparation

CYP3A4 was expressed as His-tagged proteins in *E. coli* TOPP3 and purified using a Ni-affinity column as described previously [16]. Recombinant cytochrome P450 reductase (CPR) and cytochrome b_5 (b_5) from rat liver were prepared as described previously [17] at Protein production core laboratory, KU Higuchi Bioscience center, The University of Kansas, Lawrence, KS. Protein concentrations were determined using the Bradford protein assay kit (BioRad, Hercules, CA). The specific content of CYP3A4 was 12 nmol of P450 per mg protein.

2.3. Spectral binding

For binding studies, difference spectra were recorded on a 6800 UV/VIS JENWAY spectrophotometer at 25 °C as described earlier [18]. Briefly, buffer (Hepes 0.1 M, pH 7.4) containing 0.5 μM CYP3A4 was pre-incubated in sample and reference cuvettes in the spectrophotometer for 3 min. The difference in absorbance between the maxima and minima (δA) was recorded 2 min after the addition of compounds (alcohols or nelfinavir) to the sample cuvette. The same amount of methanol (in the case of nelfinavir) or water (in the case of alcohols) was added to the reference cuvette. The final methanol concentrations in the case of nelfinavir titration were kept at 1%. To examine the effect of ethanol on nelfinavir spectral binding, CYP3A4 was pre-incubated with 20 mM ethanol in both the cuvettes prior to titration with nelfinavir. The spectral dissociation constants (K_D) were obtained by fitting the data (δA vs. ligand concentration) using non-linear regression analysis to the equation for ligand binding using Sigma plot 11 (Jandel Scientific, San Rafael, CA).

2.4. CYP3A4 inhibition

Inhibition of CYP3A4 by nelfinavir was determined using 7-BFC debenzylation reaction essentially as described earlier [19]. In brief, 100 μl reaction mixture contained 75 μM 7-BFC (1% methanol) and varying concentrations of nelfinavir (0.5–50 μM) in 50 mM Hepes, pH 7.4, 15 mM MgCl2, and 10 μg DOPC in the standard reconstitution system (P450:CPR:b_5, 1:4:2) at 5 pmol P450. The reaction was performed at 37 °C for 5 min using 1 mM NADPH. The activity in the absence and presence of inhibitors was determined by measuring 7-HFC at λ_{ex} = 410 nm and λ_{em} = 535 nm. Non-linear regression analysis was performed to fit the data to ligand binding equation to derive the IC_{50} values using Sigma plot 11. To accurately determine the relative IC_{50} values and minimize experimental errors, experiments at all the nelfinavir concentrations were done simultaneously.

2.5. CYP3A4 activity assay

The standard NADPH-dependent assay for nelfinavir metabolism by CYP3A4 was essentially carried out as described above. The reaction was performed at varying concentrations of nelfinavir (0.25–12.5 μM) in 100 μl reaction volume. After terminating the reaction, 850 μl of Tris–HCl (0.1 M, pH 9) was added to the reaction mixture prior to isolating and measuring nelfinavir and its metabolites using a liquid chromatography mass spectroscopy (LCMS) as described below. The k_{cat} and K_m values were calculated using Michaelis–Menten non-linear regression parameters using Sigma Plot 11. To accurately determine the relative kinetic parameters and minimize experimental errors, experiments with and without 20 mM ethanol were done simultaneously.

2.6. Analysis of nelfinavir and metabolites

A selective and sensitive LCMS/MS method was developed for the quantitative analysis of nelfinavir in multiple reactions monitoring (MRM) electrospray positive ionization mode using saquinavir as an internal standard. Nelfinavir and metabolites were extracted with liquid–liquid extraction using acidified ethyl acetate. The extracted samples were chromatographed with isocratic mobile phase (70:30, acetonitirle:water, 0.05% acidified with formic acid) followed by separation on Xterra MS RP C-18 column (50 × 4.6 mm, 5 μm). The lower limit of quantization was found to be 1 ng/ml, and therefore, the method was developed over a linear range of 1–1000 ng/ml. Nelfinavir and saquinavir parent and daughter ions, obtained by direct infusion mode at m/z [M + H]+ 568.4, 330.5 and 671.3, 570.6, respectively, were optimized. 25 μl of 30% trifluroacetic acid (v/v) was added to 200 μl reaction samples and 25 μl nelfinavir and saquinavir (10 μg/ml) and vortexed vigorously for 2 min. Then 950 μl of ethyl acetate was added to the samples followed by centrifugation at 14,000 rpm for 25 min at 4 °C. The supernatant (850 μl) was then separated and evaporated to dryness. The dry residues were reconstituted in a 200 μl of HPLC mobile phase and then 10 μl was injected onto LCMS/MS for its quantitative analysis.

3. Results and discussion

3.1. Spectral binding of alcohol compounds

We examined the effect of several alcohol compounds (methanol, ethanol, isopropanol, isobutanol, and isoamyl alcohol) on spectral binding with CYP3A4. We selected these compounds because they are some of the most common alcohol solvents of CYP3A4 substrates, and the effect of these solvents on drug-CYP3A4 interaction is not well studied. The results are presented in Fig. 1 and Table 1. All the alcohol compounds showed type I spectral change, suggesting the interaction of these compounds with the heme and inducing low-to-high spin transition. The results showed an increase in the magnitude of spectral change ($\delta A_{390-418}$) with increasing size of the compounds (methanol < ethanol < isopropanol < isobutanol < isoamyl alcohol). Ethanol showed the lowest K_D (5.9 ± 0.3 mM), while smaller (methanol) as well as bulkier (isopropanol, isobutanol, and isoamyl alcohol) compounds showed higher K_D than ethanol (Fig. 1, Table 1). The results suggest that ethanol has the optimal size to fit the active site of CYP3A4. However, smaller compound (methanol) may fit loosely in the active site and bulkier compounds may bind the heme weakly due to steric hinderance.

Although, the effect of common solvents, such as dimethylsulfoxide, on the activity of P450 enzymes is known [20,21], the

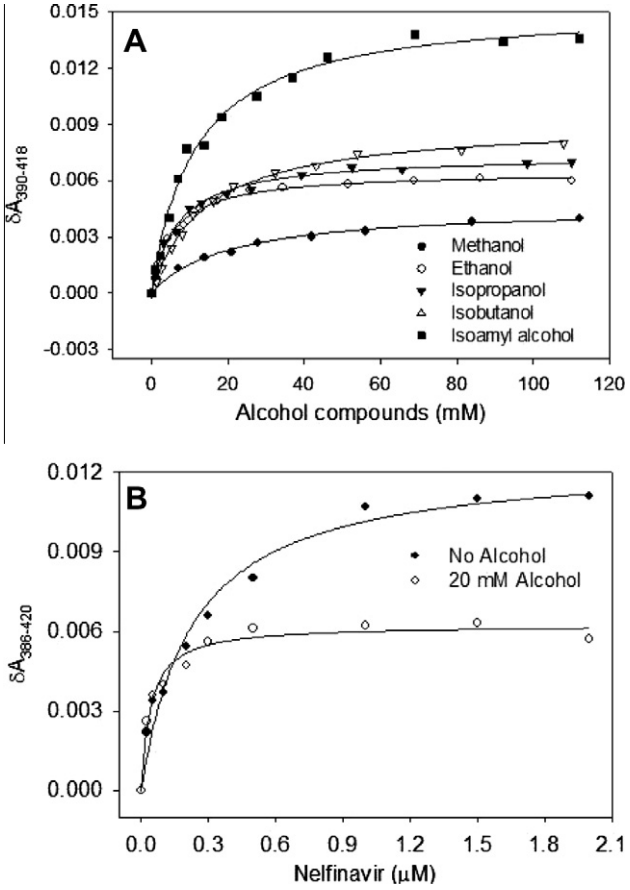

Fig. 1. (A) The type I spectral binding of CYP3A4 by alcohol compounds. (B) The type I spectral binding of CYP3A4 by nelfinavir at 0 and 20 mM ethanol. The spectral binding was performed at varying concentrations of alcohols and nelfinavir as described in Section 2. The data were fit to a single site ligand binding equation using Sigma plot 11. Spectra induced by alcohols and nelfinavir showed slightly different maximum and minimum absorbance. The δA and K_D are presented in Table 1.

Table 1
Type I spectral binding of commonly used alcohols and nelfinavir with CYP3A4.

Sample	$\delta A_{max,\ 390-418}$	K_D (mM)
Methanol	0.0046 ± 0.00001[a]	20.0 ± 3.5
Ethanol	0.0065 ± 0.00006	5.90 ± 0.34
Isopropanol	0.0074 ± 0.00013	6.74 ± 0.54
Isobutanol	0.0091 ± 0.00015	14.1 ± 0.75
Isoamylalcohol	0.0155 ± 0.00043	11.8 ± 1.04
Sample	$\delta A_{max,\ 386-420}$	K_D (μM)
Nelfinavir (0 mM ethanol)	0.012 ± 0.001	0.227 ± 0.038
Nelfinavir (20 mM ethanol)	0.0062 ± 0.0002	0.041 ± 0.007

Results are the representative of at least two independent determinations. The variation between the experiments is <15%.
[a] Standard errors for fit to ligand binding are shown as ±.

mechanism by which these compounds inhibit P450 activity is not well understood. In general, these compounds at relatively high concentrations (>2%) inactivate or inhibit P450 enzymes through non-specific physical interactions (hydrophobic, polar, H-bond). However, P450-substrate interaction at physiological ethanol concentrations (⩽0.10% or 20 mM) is unknown. Therefore, we further investigated the effect of ethanol on CYP3A4–ART interaction at physiological concentration, and the mechanism by which it occurs.

3.2. Effect of ethanol on spectral binding of nelfinavir

To examine the effect of alcohol on ART–CYP3A4 affinity, we performed CYP3A4 spectral binding with a representative ART drug, nelfinavir. The results are presented in Fig. 1 and Table 1. The K_D of nelfinavir was very low (0.227 ± 0.038 μM) and alcohol further decreased its K_D by >5-fold (0.041 ± 0.007 μM). This suggests that alcohol facilitates the binding of nelfinavir with CYP3A4. Alcohol also decreased $\delta A_{386-420}$ of nelfinavir-CYP3A4 type I spectral change by 2-fold (0.0062 ± 0.0002 vs. 0.012 ± 0.0006), because pre-incubation of alcohol alone induced spectral change as shown in Fig. 1 and Table 1.

3.3. Effect of ethanol on CYP3A4 inhibition by nelfinavir

To further examine the effect of alcohol on nelfinavir-CYP3A4 affinity, we performed CYP3A4 inhibition by nelfinavir using 7-BFC debenzylation reaction. The results are presented in Fig. 2. As shown before [3,22], the IC_{50} of nelfinavir was low (2.8 ± 0.5 μM) and showed complete inhibition at saturating nelfinavir concentration. As expected, alcohol (5–20 mM) decreased the IC_{50} of nelfinavir (Fig. 2). For example, 20 mM alcohol decreased the IC_{50} of nelfinavir by >3-fold (2.6 ± 0.5 vs. 8.3 ± 2.1 μM) suggesting possible inferior efficacy in HIV-1-infected individuals who are chronic alcohol users.

Our results clearly indicate that alcohol facilitates binding of nelfinavir to CYP3A4. As discussed with other proteins [11], we suggest that ethanol accesses CYP3A4 active site, replaces water molecules, and stabilizes nelfinavir through additional H-bonds and hydrophobic interactions. This hypothesis can be tested using X-ray crystallography of alcohol-ART–CYP3A4 bound structure, NMR, and/or high pressure spectroscopy. High pressure spectroscopy determines the solvation of P450 enzymes in the active site [13]. Since most CYP3A4 drugs, especially NNRTIs and PIs, are hydrophobic in nature, it is likely that alcohol will facilitate the binding of other NNRTIs and PIs with CYP3A4.

3.4. Effect of ethanol on nelfinavir metabolism

We examined the effect of ethanol on nelfinavir metabolism using recombinant purified CYP3A4. The optimization of nelfinavir metabolism showed a linear range of activity at 10 pmol CYP3A4 for 10 min reaction time (data not shown). As shown before by CYP3A4 [23,24], it produced two metabolites, perhaps 2-methoxy-3-hydroxynelfinavir (M1) and 1-hydroxynelfinavir (M3). The identification of these metabolites is based on previous reports [23–26]. Fig. 3 shows a representative LCMS and MRM chromato-

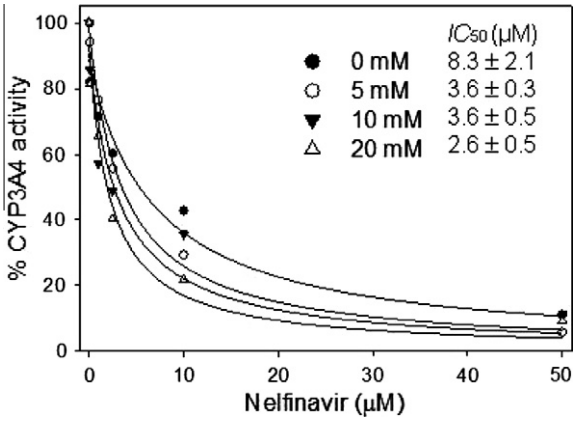

Fig. 2. Inhibition of CYP3A4 by nelfinavir at varying concentrations of ethanol (0, 5, 10, and 20 mM). The inhibition was performed at varying nelfinavir concentrations using 7-BFC substrate in the standard NADPH reaction. The IC_{50} was determined by fitting the data to a single site ligand binding equation using Sigma plot 11. Standard errors for fit to the equation are shown as ±. Results are the representative of at least two independent determinations. The variation between the experiments is <20%.

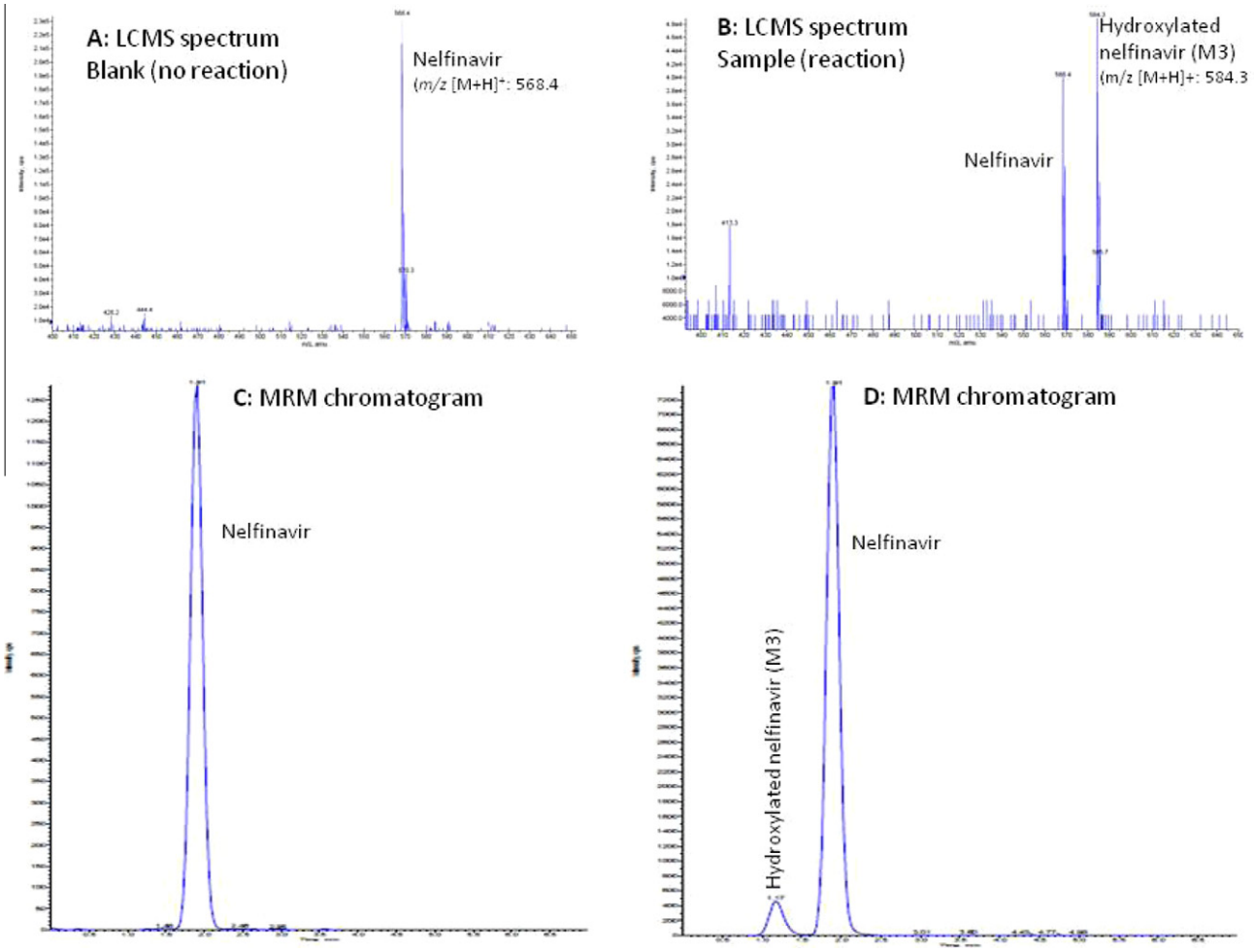

Fig. 3. LCMS and MRM chromatogram profiles of nelfinavir and its metabolite 1-hydroxynelfinavir (M3). (A) LCMS profile of nelfinavir in blank (no reaction), (B) LCMS profile of nelfinavir and M3 in sample (reaction by CYP3A4), (C) MRM chromatogram of nelfinavir in blank, and (D). MRM chromatogram of nelfinavir and M3 in sample. The molecular weight of nelfinavir and M3 are provided in (A) and (B).

gram profiles of nelfinavir and major metabolite M3. Since the amount of M3 was very low, especially at low nelfinavir concentrations, we used the amount of nelfinavir consumed to determine kinetic parameters. The k_{cat} and K_m of nelfinavir for CYP3A4 was found to be 1.4 ± 0.16 nmol/min/nmol P450 and 1.3 ± 0.06 μM, respectively (Fig. 4). Although alcohol did not show a significant change in k_{cat}, it decreased the K_m for nelfinavir by >3-fold, leading to a decrease in its catalytic efficiency (k_{cat}/K_m). However, it can be noted that although the k_{cat} was unchanged, alcohol decreased CYP3A4 activity at all the concentrations used for kinetic analysis (Fig. 4).

There is no report on kinetic parameters (k_{cat} and K_m) of nelfinavir in CYP3A4. A report of nelfinavir metabolism, by liver microsomes CYP2C19 for the formation of 3-hydroxynelfinavir (M8), shows very low k_{cat} (25 pmol/min/nmol P450) [25]. However, the k_{cat} was 40-fold higher with purified CYP2C19 (~1 nmol/min/nmol P450), which is similar to the k_{cat} obtained by recombinant purified CYP3A4 (1.4 nmol/min/nmol P450). The K_m of nelfinavir for CYP2C19 is much higher (22 μM) [25] than for CYP3A4 (1.3 μM). Nevertheless, this K_m is in the similar range to the K_D (0.23 μM) and IC_{50} (2.8 μM) of nelfinavir for CYP3A4. Furthermore, our results are similar to the k_{cat} and K_m of the recombinant and/or liver microsomes CYP3A4 for the metabolism of other PIs, such as indinavir [27,28], ritonavir [28,29], and saquinavir [30].

Although alcohol increased the binding affinity (K_D and IC_{50}) of nelfinavir for CYP3A4, it apparently decreased K_m for nelfinavir metabolism. It is not unusual because K_D and IC_{50} represent absolute affinity for substrate binding, while K_m represents substrate

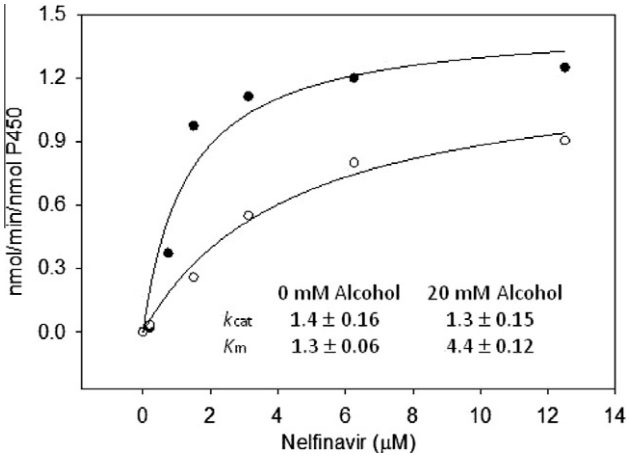

Fig. 4. Nelfinavir kinetics by CYP3A4 at 0 and 20 mM ethanol. The activity was performed using the standard NADPH method at varying concentrations of nelfinavir. The k_{cat} and K_m were determined by fitting the data to Michaelis–Menten equation using Sigma plot 11. Standard errors for fit to the equation are shown as ±. Results are the representative of three independent determinations. The variation between the experiments is <25%.

binding in an orientation that leads to fruitful collision followed by product formation and egress. Overall, it is evident from this study that ethanol increases nelfinavir-CYP3A4 binding and decreases its metabolism, which is likely to decrease nelfinavir clearance. Strong nelfinavir-CYP3A4 binding in the presence of alcohol may decrease bioavailability of nelfinavir at HIV-1 targets, such as macrophages and lymphocytes. Since macrophages also have alcohol-inducible CYP3A4 [9], the bioavailability of nelfinavir may further decrease at the target (HIV-1 proteases) in macrophages. This interpretation is consistent with the reports that alcohol-ART interaction decreases ART efficacy, especially of NNRTIs and PIs [31–33]. In addition, increased nelfinavir-CYP3A4 binding and decreased nelfinavir metabolism are likely to cause accumulation of nelfinavir that may lead to drug toxicity.

4. Conclusion

Alcohol compounds, especially ethanol, bind to the heme of CYP3A4 leading to type I spin transition. Furthermore, ethanol facilitates the binding of nelfinavir to CYP3A4 and decreases the catalytic efficiency of nelfinavir metabolism. Although alcohol is known to induce CYP3A4 leading to increased ART drug metabolism, this is the first report on alcohol-CYP3A4 physical interaction resulting in altered binding and metabolism of an ART. This is very important finding in context with the report that alcohol decreases the response to ART. The study has clinical relevance with regard to HIV-1 and its treatments, because the prevalence of mild-to-chronic alcohol consumption among HIV-1 individuals is three-times higher than the normal population [34,35].

Acknowledgments

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