

RESEARCH ARTICLE

Effect of Methamphetamine on Spectral Binding, Ligand Docking and Metabolism of Anti-HIV Drugs with CYP3A4

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Abstract

Cytochrome P450 3A4 (CYP3A4) is the major drug metabolic enzyme, and is involved in the metabolism of antiretroviral drugs, especially protease inhibitors (PIs). This study was undertaken to examine the effect of methamphetamine on the binding and metabolism of PIs with CYP3A4. We showed that methamphetamine exhibits a type I spectral change upon binding to CYP3A4 with δA_{\max} and K_D of 0.016 ± 0.001 and $204 \pm 18 \mu\text{M}$, respectively. Methamphetamine-CYP3A4 docking showed that methamphetamine binds to the heme of CYP3A4 in two modes, both leading to N-demethylation. We then studied the effect of methamphetamine binding on PIs with CYP3A4. Our results showed that methamphetamine alters spectral binding of nelfinavir but not the other type I PIs (lopinavir, atazanavir, tipranavir). The change in spectral binding for nelfinavir was observed at both δA_{\max} (0.004 ± 0.0003 vs. 0.0068 ± 0.0001) and K_D (1.42 ± 0.36 vs. $2.93 \pm 0.08 \mu\text{M}$) levels. We further tested effect of methamphetamine on binding of 2 type II PIs; ritonavir and indinavir. Our results showed that methamphetamine alters the ritonavir binding to CYP3A4 by decreasing both the δA_{\max} (0.0038 ± 0.0003 vs. 0.0055 ± 0.0003) and K_D (0.043 ± 0.0001 vs. $0.065 \pm 0.001 \text{ nM}$), while indinavir showed only reduced K_D in presence of methamphetamine (0.086 ± 0.01 vs. $0.174 \pm 0.03 \text{ nM}$). Furthermore, LC-MS/MS studies in high CYP3A4 human liver microsomes showed a decrease in the formation of hydroxy ritonavir in the presence of methamphetamine. Finally, CYP3A4 docking with lopinavir and ritonavir in the absence and presence of methamphetamine showed that methamphetamine alters the docking of ritonavir, which is consistent with the results obtained from spectral binding and metabolism studies. Overall, our results demonstrated differential effects of methamphetamine on the binding and metabolism of PIs with CYP3A4. These findings have clinical implication in terms of drug dose adjustment of antiretroviral medication, especially

with ritonavir-boosted antiretroviral therapy, in HIV-1-infected individuals who abuse methamphetamine.

Introduction

Cytochrome P450 (CYP) belongs to the family of heme proteins that are involved in the biotransformation of xenobiotics. Among the various CYP isozymes, CYP3A4 metabolizes approximately 50% of the currently marketed drugs including several antiretroviral drugs [1]. Most antiretroviral drugs including protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) are substrates, inducers and/or inhibitors of CYP3A4 [2]. The highly active antiretroviral therapy (HAART) regimens contain multiple drugs including PIs and NNRTIs, which also causes potential drug-drug interactions through CYP3A4 [3, 4]. Among all the PIs, ritonavir is known to be the strongest inhibitor of CYP3A4 and is, therefore, used as a booster in the HAART regimen that contains NNRTIs and PIs. Based on the physicochemical properties of the PIs, several studies have shown that PIs exhibit two types of spectral changes upon binding with CYP3A4 known as type I and type II [5, 6].

Methamphetamine is a commonly used substance of abuse among HIV-1 infected population. Studies have shown that the risk of acquiring HIV-1 infection is higher among men who have sex with men (MSM) and abuse amphetamines compared to those MSM who don't abuse drugs [7, 8]. Furthermore, several *in vitro* and *in vivo* studies have shown the role of methamphetamine in altering HIV-1 pathogenesis by various mechanisms, including suppression of innate restriction factors in macrophages and increased viral loads in the brain [9, 10]. Few reports have shown the occurrence of fatal interactions in patients abusing methamphetamine while on ritonavir therapy resulting from inhibition of CYP2D6 mediated methamphetamine metabolism [11]. However, it's unclear whether methamphetamine also reduces the response to HAART by altering the bioavailability of HAART and increasing HAART-mediated toxicity that would ultimately result in increased HIV-1 pathogenesis in methamphetamine users. Since, methamphetamine is also partly metabolized by CYP3A4, which is known to metabolize NNRTIs and PIs, we propose that methamphetamine interacts with NNRTIs/PIs through CYP3A4. Therefore, in this study we determined the interaction of methamphetamine with CYP3A4 followed by the effect of methamphetamine on the interaction of PIs with CYP3A4 using spectral binding and docking studies.

Materials and Methods

Materials

Plasmid encoding CYP3A4 was generously provided by Dr. James Halpert (Skaggs School of pharmacy and pharmaceutical sciences, UCSD). Ni-NTA agarose column was obtained from Qiagen (Valencia, CA). Methamphetamine was purchased from Sigma chemicals (St. Louis, MO, USA). All the protease inhibitors (PIs) were obtained from NIH AIDS reagent center. XTerra[®] MS C18 column (4.6X50mm, i.d, 3.5 μm) was purchased from Waters (Milford, MA, USA). The other reagents used in the study were obtained from standard commercial sources.

Enzyme preparation

Histidine tagged CYP3A4 enzyme was expressed in *E. coli* and purified on Ni-NTA agarose column as described previously [12]. Briefly, CYP3A4 cDNA was introduced into TOPP3

strain of *E. coli* by transformation and the cells were plated on LB ampicillin plate with tetracycline. The next day, a viable and isolated colony was grown in TB media for 48 h to induce CYP3A4. The cultures were harvested by centrifugation and CYP3A4 was extracted from the membrane using detergent followed by ultracentrifugation. The supernatant was loaded on a Ni-NTA agarose column for further purification. The purified enzyme was dialyzed and final preparation was stored at -80°C until use.

Spectral binding assay

The spectral change was recorded by using a UV/Vis double beam spectrophotometer (6800 UV/VIS JENWAY spectrophotometer) as described earlier [6]. Briefly, $0.5\ \mu\text{M}$ of purified CYP3A4 enzyme was incubated with 2 ml of 0.1 M HEPES buffer for 3 min and distributed equally between the sample and reference cuvettes. Depending upon the solvent used to solubilize methamphetamine or PIs, equal quantities of either methanol or water was added into the reference cuvette with final volume of the external solvent $<1\%$. Increasing concentrations of methamphetamine was added to the sample cuvette while the same volume of solvent was added into the reference cuvette and the spectral change was recorded. For experiments involving PIs, different concentrations of PIs were added into the sample cuvette and the spectral change was recorded with a 2 min interval between each addition. PIs binding to CYP3A4 exhibit two different types of spectral change based on the presence of different kinds of functional groups. Type I ligands displace the heme water ligand, thus shifting the iron spin equilibrium towards the high-spin form resulting in a spectrum, which is characterized by an increase in the absorbance at 390 nm (high spin) and a decrease at 420 nm (low spin). Type II ligands containing nitrogen heterocycles replace the water ligand to stabilize the low-spin form resulting in a spectrum that is characterized by an increase in the spectrum at $\sim 430\ \text{nm}$ and a decrease at $\sim 400\ \text{nm}$. The effect of methamphetamine on the spectral binding of PIs was determined by incubating the CYP3A4 enzyme with $100\ \mu\text{M}$ of methamphetamine and titrating with increasing concentrations of PIs.

PIs Metabolism Assay

The effect of Methamphetamine on CYP3A4 mediated metabolism of PIs was assessed using human liver microsomes (HLMs) (Invitrogen, MA, USA), which possess high CYP3A4 activity. Briefly, the metabolite formations were assessed in incubation mixtures (0.5 mL) containing 100 mM phosphate buffer (pH 7.4), HLMs (0.1 mg/mL), and 5 mM MgCl_2 [13, 14]. The incubation mixtures were pre-incubated with six different concentrations of Meth ranging from $3\ \mu\text{M}$ to $1000\ \mu\text{M}$ for 10 min, followed by the addition of $5\ \mu\text{l}$ stock solution of PI. The entire mixture was incubated in water bath at 37°C for 15 min. The substrate concentrations of the PIs used were near their K_m values; ritonavir ($2\ \mu\text{M}$), lopinavir ($10\ \mu\text{M}$), and nelfinavir M-8 ($1\ \mu\text{M}$). Later, the reactions were initiated upon the addition of $100\ \mu\text{l}$ of 25 mM NADPH (final concentration of 5 mM) solution to each of the tube and incubated in water bath at 37°C for 60 min. After the incubation period, $500\ \mu\text{l}$ of Stop solution (Acetonitrile: water: FA in 47:50:3 ratio) containing $0.7\ \mu\text{M}$ of internal standard (IS), Atazanavir-d5, was added in each tube. Then, the tubes were vortexed and centrifuged at 500 g for 10 min and supernatant was transferred into vials for analysis by LC-MS/MS.

Analytical instrumentation

Ritonavir, lopinavir, lopinavir M1, nelfinavir, nelfinavir M8, and atazanvir d5 stock solutions were prepared in methanol where as hydroxy ritonavir was prepared in DMSO. Atazanavir d5 was used as an IS for all the analytes. Standard curves for each analyte with IS were generated

in blank HLMs incubation mixture as described above. The carry over test was performed by injecting an extra blank following the injection of upper limit of quantitation (ULOQ) of the standard curve with an IS. The LC-MS/MS methods were developed for each analyte along with its metabolite using 3200 QTRAP LC-MS/MS system, AB Sciex. The most suitable multiple reaction monitoring (MRM) transitions produced through electron spray ionization $[M+H]^+$ for precursor ions (Q1) and product ions (Q3) were found to be 721.2→296.2 for ritonavir, 737.5→312.2 for hydroxy ritonavir, 629.4→183.4 for lopinavir, 643.7→250.5 for lopinavir M1, 568.5→330.4 for nelfinavir, 584.5→330.3 for nelfinavir M8, and 710.3→340.5 for atazanavir d5. (Fig 1, Table 1). A source temperature of 500°C, ion spray voltage of 5500 V, curtain gas of 20, GS 1 & 2 of 50, and dwell time of 200 msec were used for the determination of all analytes. The MRM method parameters such as declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP), were listed in the Table 1 along with the MRM transitions.

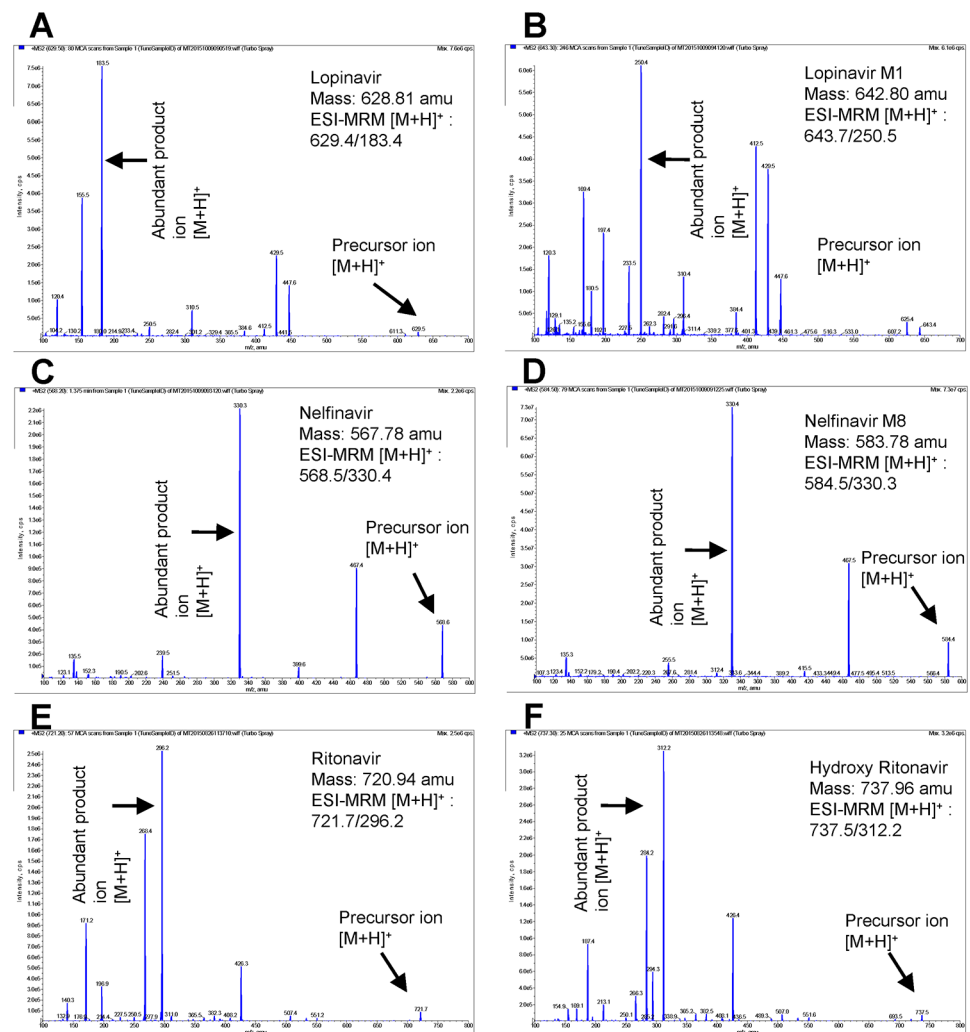


Fig 1. Development of LC-MS/MS methods to quantitate lopinavir, lopinavir M1, nelfinavir, nelfinavir M8, ritonavir, and hydroxy ritonavir in human liver microsomes. MS/MS spectra of (A) lopinavir, (B) lopinavir M1, (C) nelfinavir, (D) nelfinavir M8, (E) ritonavir, (F) hydroxy ritonavir with ESI proton adducts $[M+H]^+$ in positive mode. The y-axis shows intensity (CPS, count per second) and the x-axis shows the mass to charge ratio (m/z, amu).

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Table 1. Summary of optimized parameters and characteristics for positive mode LC—MS/MS detection of Protease inhibitors.

Name	MRM Parameters						t_r (min)
	Q1	Q3	DP (V)	EP (V)	CE (V)	CXP (V)	
Lopinavir	629.3	183.3	32	6	30	6	6.64
Lopinavir M1	643.3	250.3	32	6	37	6	6.36
Nelfinavir	568.6	330.4	70	7	50	5	5.42
Nelfinavir M8	584.6	330.4	70	7	50	5	4.49
Ritonavir	721.2	312.2	58	7	25	5	6.41
Hydroxy ritonavir	737.2	340.5	58	7	25	5	5.26

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All the analytes were separated on reverse phase XTerra[®] MS C18 column (4.6X50mm, i.d, 3.5 μ m) using a Shimadzu LC-20AD HPLC system (CA, USA). A binary gradient elution method comprising of mobile phases (acetonitrile with 0.1% formic acid and water with 0.1% formic acid) with a flow rate of 0.5 mL/min was employed. For separation of ritonavir, hydroxy ritonavir, lopinavir, and lopinavir M1, a gradient program starting with 35% acetonitrile with 0.1% formic acid for the first minute, followed by a linear gradient to 100% acetonitrile with 0.1% formic acid in 5 min, maintained for 1 min and then to 35% acetonitrile with 0.1% formic in 4 min with a total run time of 11 min was developed. Under the above conditions, ritonavir, hydroxy ritonavir, lopinavir, lopinavir M1, and IS exhibited retention times of 6.41, 5.26, 6.64, 6.36, and 4.33 min, respectively. For separation of nelfinavir and nelfinavir M8, the same gradient profile was used except for starting with 35% of 70% acetonitrile with 0.1% formic acid. Upon using the above method, nelfinavir, nelfinavir M8, and IS were eluted in 5.42, 4.49, and 6.33 min, respectively. Data acquisition and analyses were performed using the Analyst 1.4.2 software package (Applied Biosystems, Foster City, CA, USA).

Docking studies

The study was performed as described previously with modifications [15]. The initial CYP3A4 model was taken from Protein Data Bank (PDB, www.pdb.org). Currently, there are 17 crystal structures of human CYP3A4 available in PDB. On the basis of crystal resolution, residues completeness, and ligand size, 3NXU (at 2.00 Å resolution) was chosen. There are 2 asymmetric molecules in 3NXU. Chain A was used for docking simulations. The missing residues were reconstructed using 1TQN and 3V0M as the templates. Docking of PIs into the CYP3A4 active site in the absence and presence of methamphetamine was accomplished by Gold 5.2 software [16]. The center of the grid was placed on the centroid of ritonavir, a co-crystallized ligand in 3NXU. The residues within 20 Å of the ligand were defined as the binding pocket. Chemscore was used for scoring the interactions between the ligands and CYP3A4. The output solution was set 30 for each run. The first 10 poses were analyzed in detailed.

Statistical analysis

The K_D and δA_{max} were determined by fitting the curve (absorbance vs. concentration) with hyperbolic equation using non-regression analysis in sigma plot 11 (Systat Software, San Jose, CA). The p-values were determined using One way ANOVA with Dunnet's post-hoc test and values <0.05 were considered statistically significant.

Results and Discussion

Methamphetamine-CYP3A4 spectral binding

The current study was undertaken to examine the role of methamphetamine on the spectral binding of PIs with CYP3A4. In order to determine this, first we examined the spectral binding of methamphetamine with CYP3A4. The results showed an increase in absorbance at 390 nm and a decrease at 418 nm, suggesting that methamphetamine shows a type I spectral change with CYP3A4. The result is consistent with its physicochemical properties as it does not contain any reactive group with a lone pair, e.g. imidazole, which usually shows type II spectral change through covalent binding. The titration of methamphetamine with CYP3A4 showed a hyperbolic spectral binding (Fig 2A). Therefore, we fitted the methamphetamine-CYP3A4 binding using hyperbolic equation, which yielded a K_D of methamphetamine as $204 \pm 18 \mu\text{M}$ and δA_{max} at 0.016 ± 0.001 . The result from δA_{max} suggests that methamphetamine causes approximately 20% spectral transition (low to high spin) with CYP3A4 at saturating concentration of methamphetamine. This spectral transition with CYP3A4 is similar to spectral transition obtained from other type I ligands such as ethanol [17]. To determine and validate the nature of methamphetamine binding with CYP3A4, we performed methamphetamine docking with CYP3A4. The results show that methamphetamine interact with CYP3A4 active site in two different binding modes, both of which can undergo N-demethylation (Fig 2B).

The involvement of various CYP enzymes including CYP3A4 in the metabolism of methamphetamine has been shown in previous studies, including ours [18–22]. Our docking data is consistent with methamphetamine CYP3A4 interaction that prefers N-demethylation route of metabolism. Further, spectral binding of methamphetamine with CYP3A4 suggests that methamphetamine-CYP3A4 interaction can be studied using type I spectral change. It is important to mention that all the ligands, which are either substrates, inhibitors, or activators of CYP3A4, do not necessarily show spectral change. If a ligand does not show spectral change with CYP3A4, then it is very difficult to study ligand-CYP3A4 interaction and its effect on the metabolism, inhibition, or activation of other CYP3A4 ligands. Although the K_D obtained from spectral binding is higher than the plasma concentration of methamphetamine (2–10 μM) [23], it is likely that its concentration in the liver is higher than in plasma because methamphetamine is rapidly metabolized in the liver. Further, the methamphetamine level observed in the post-mortem brains of chronic abusers ranges between 0.8 mM to 1mM [24]. CYP3A4 is known to be present in different brain cells [25], and in some cells, the local CYP3A4 concentration is as high as the liver CYP3A4. Thus, CYP3A4-methamphetamine interaction at such concentration is of significance in the brain. Recent study has shown that methamphetamine mediates blood brain barrier (BBB) dysfunction leading to internalization of occludin [26]. A leaky BBB may also increase the transport of other compounds including its own, as well as, NNRTIs and PIs in the brain. Thus, in addition to liver, potential drug-drug interactions between methamphetamine and NNRTIs/PIs through CYP3A4 may also occur in the brain.

Since CYP3A4 is involved in the metabolism of both PIs and NNRTIs, it is important to study drug-drug interactions in the presence of methamphetamine [27]. We and others have earlier characterized the binding of CYP3A4 with PIs and classified PIs-CYP3A4 interactions into type I PIs, type II PIs, and unbound PIs (PIs that do not show spectral change) [5, 6]. Furthermore, we have shown that ethanol differentially alters the binding of both type I and type II PIs with CYP3A4, suggesting a three-way ethanol-CYP3A4-PI interaction [6, 17]. In this study, we have studied the effect of methamphetamine on the spectral binding of both type I and type II PIs.

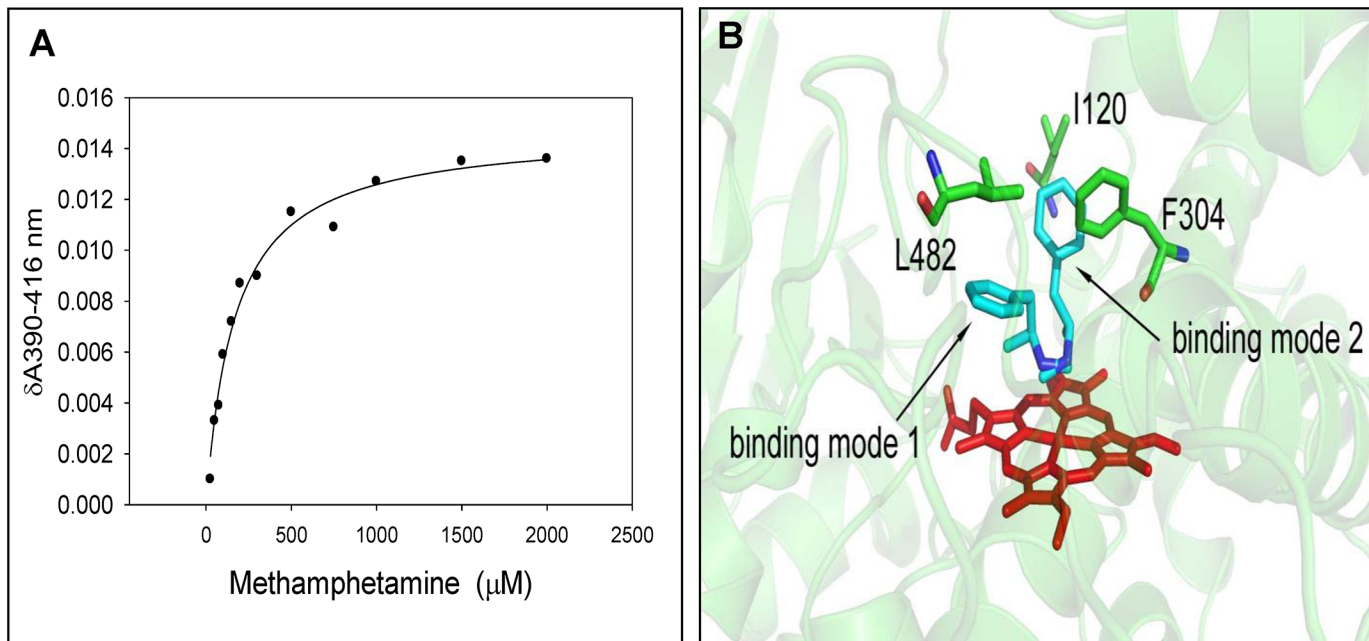


Fig 2. Spectral binding of methamphetamine with CYP3A4. (A) The spectral binding was performed at varying concentrations of methamphetamine from 1 μM to 2 mM. The graph was plotted using difference in absorbance at 390 and 416 nm vs. concentration of methamphetamine. The graph was fitted with hyperbolic equation using non-linear regression analysis in sigma plot 11. (B) Docking simulation of methamphetamine binding with CYP3A4 in two different binding modes. The heme, methamphetamine, and interacting amino acid residues of CYP3A4 are represented in red, green, and blue, respectively.

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Effect of methamphetamine on interaction of Type I PIs with CYP3A4

We examined the role of methamphetamine on spectral binding of four type I PIs (atazanavir, nelfinavir, tipranavir and lopinavir) with CYP3A4. Our results showed that methamphetamine causes a significant decrease in the δA_{max} of nelfinavir (0.004 ± 0.0003 vs. 0.0068 ± 0.0001) and a slight increase in δA_{max} with tipranavir (0.0061 ± 0.0002 vs. 0.0050 ± 0.0005). However, it did not show any change in δA_{max} with atazanavir and lopinavir (Fig 3, Table 2). Further, methamphetamine slightly increased the K_D values of lopinavir, atazanavir, and tipranavir, while it significantly decreased the K_D of nelfinavir (1.42 ± 0.36 vs. 2.93 ± 0.08 μM) (Table 2). To determine the effect of methamphetamine on CYP3A4-mediated metabolism of these PIs (whose metabolites are commercially available), we performed inhibition studies using HLMs. Inhibition studies were performed by analyzing the rate of metabolite formation of lopinavir M1 from lopinavir and decrease in the substrate concentration of nelfinavir M8 in the presence of varying concentrations of methamphetamine. Our studies showed that methamphetamine did not affect the metabolism of either lopinavir or nelfinavir M8 as shown in the Fig 4A and 4B.

The results are more or less similar to the results obtained by CYP3A4-PI binding experiments. In general, methamphetamine did not significantly alter the binding of Type I PIs with CYP3A4, except for nelfinavir. In consistence with the spectral findings, our inhibition studies in microsomal system did not show any significant effect of methamphetamine on type I PIs metabolism.

To corroborate these findings, we performed docking studies with Type I PIs in the absence and presence of methamphetamine. Docking of PIs in the active site of CYP3A4 in the absence of methamphetamine showed binding modes that could be classified into 2–3 clusters in the top ten poses for each PI. The predicted binding a region with the modes of the 4 PIs occupied boundaries being closer to I helix, B-C loop, K-L loop, and C-terminal loop (Fig 5A). Average

Type I Protease Inhibitors

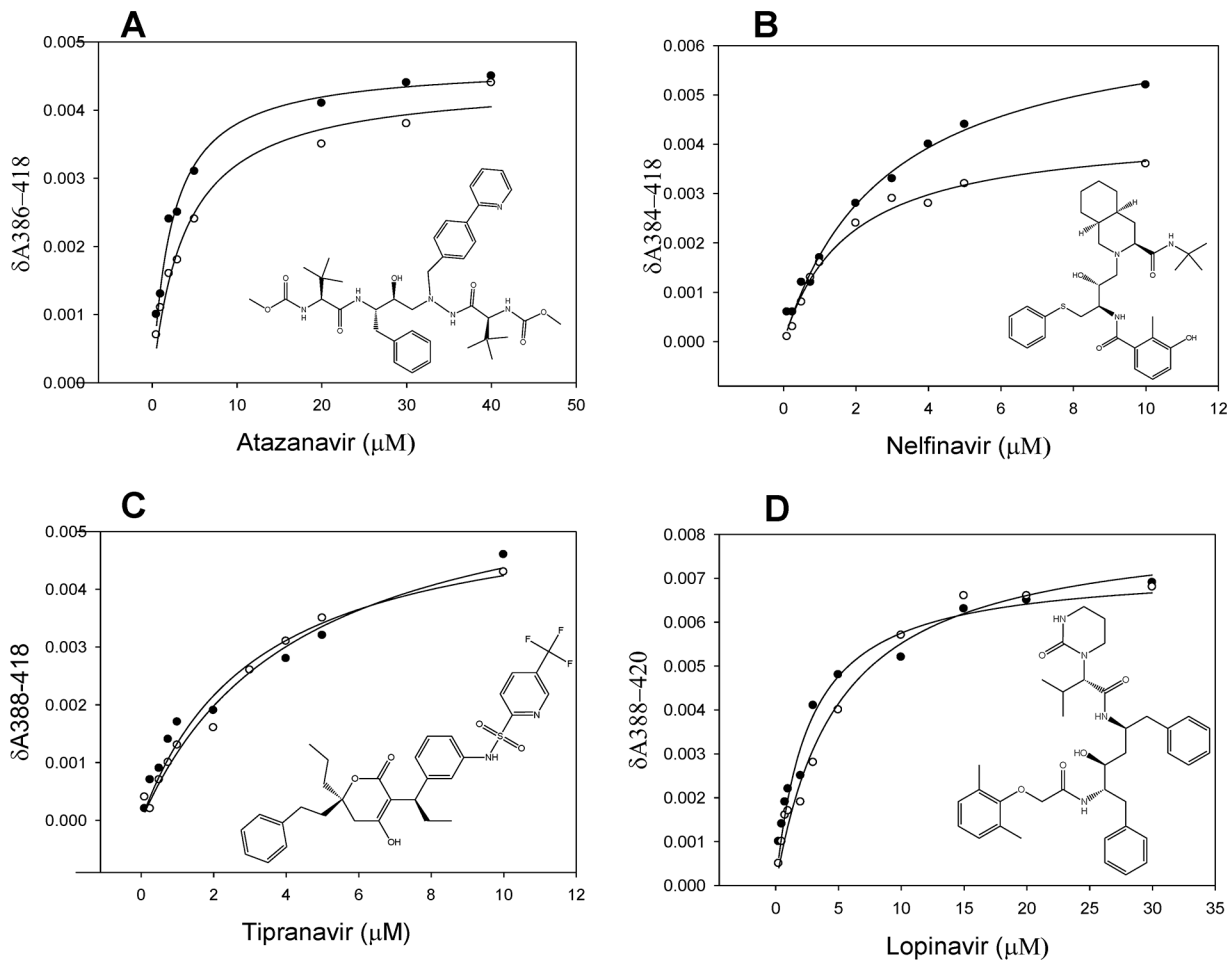


Fig 3. Spectral binding of type I PIs with CYP3A4 in the absence (filled circles) and presence (open circles) of methamphetamine. (A-D) The spectral binding was performed at varying concentrations of atazanavir, nelfinavir, tipranavir and lopinavir. The K_D and δA_{max} for each PI with and without methamphetamine are presented in Table 2. One way ANOVA with Dunnet's post-hoc test was employed to calculate the statistical significance. A p-value <0.05 is indicated by * and <0.1 is indicated by #.

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Table 2. The K_D and δA_{max} of all the PIs with and without methamphetamine.

	No Methamphetamine		100 μ M Methamphetamine		
	PIs	δA_{max}	KD (μ M)	δA_{max}	KD (μ M)
Type I	Lopinavir	0.0070 \pm 0.0002	2.5 \pm 0.1	0.0078 \pm 0.0005	4.1 \pm 0.8
	Atazanavir	0.0045 \pm 0.0002	3.2 \pm 0.8	0.0046 \pm 0.0002	4.7 \pm 0.7
	Tipranavir	0.0050 \pm 0.0005	3.1 \pm 0.1	0.0061 \pm 0.0002	4.7 \pm 0.4
	Nelfinavir	0.0068 \pm 0.0001	2.9 \pm 0.1	0.0040 \pm 0.0003*	1.4 \pm 0.3 [#]
Type II	Ritonavir	0.0055 \pm 0.0003	0.065 \pm 0.001	0.0038 \pm 0.0003*	0.043 \pm 0.001*
	Indinavir	0.0044 \pm 0.0002	0.174 \pm 0.030	0.0039 \pm 0.0002	0.086 \pm 0.010*

*-indicates a p-value <0.05;

#-Indicates p-value <0.1

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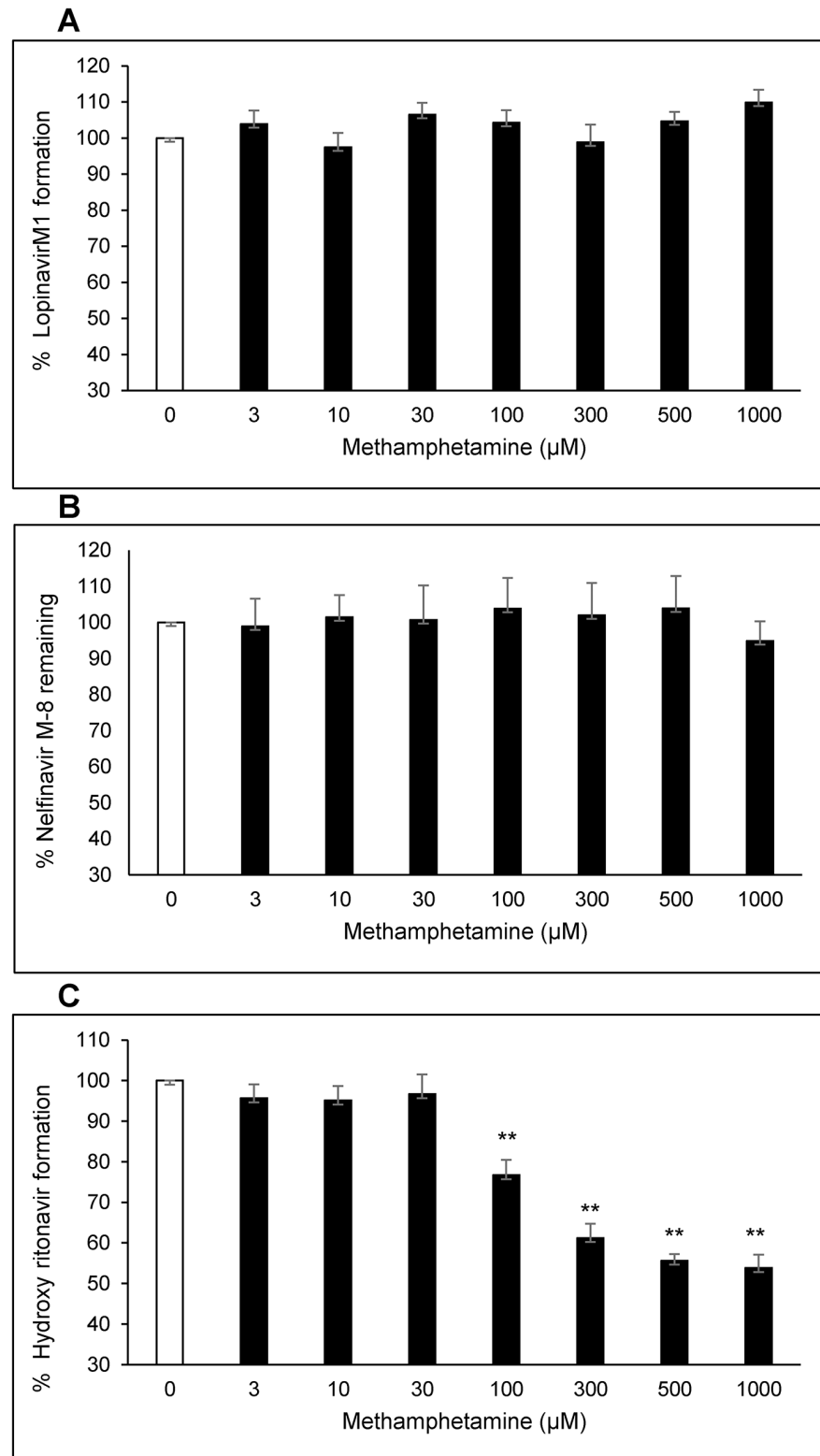


Fig 4. Effect of methamphetamine on metabolism of PIs in CYP3A4 human liver microsomes. Effect of methamphetamine on (A) lopinavir M1 formation from lopinavir, (B) nelfinavir M8 degradation and, (C) hydroxy ritonavir formation from ritonavir. One way ANOVA with Dunnet's post-hoc test was employed to calculate the statistical significance and ** denotes p-value <0.01.

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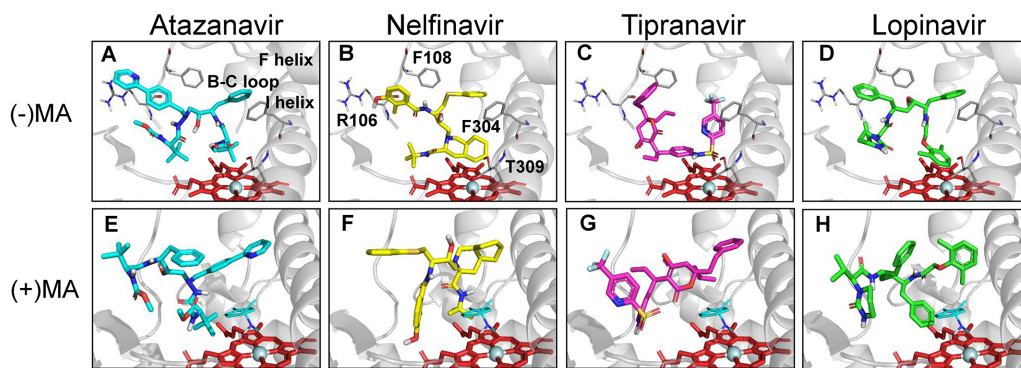


Fig 5. Docking simulations of PIs in the absence and presence of methamphetamine. Structures of (A) Atazanavir, (B) Nelfinavir, (C) Tipranavir and (D) Lopinavir with their preferred binding sites pointing towards the heme moiety of CYP3A4in the absence of methamphetamine. Docking simulations of (E) Atazanavir, (F) Nelfinavir, (G) Tipranavir and (H) Lopinavir with heme moiety of CYP3A4 in the presence of methamphetamine. R-methamphetamine in binding mode 1 was shown in cyan sticks. Docking was performed as described in Materials and Methods.

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docking scores in the major cluster (cluster with highest average docking score) for type I PIs ranged from 44.62 to 51.60 (Table 3). The docking scores of different PIs were in general consistent with the relative ranking of the experimental K_D values. Since the crystal structure of CYP3A4 complexed with MA is not available, we first predicted the possible binding modes of MA in the active site of CYP3A4. Our docking studies suggested two possible binding modes for both R-methamphetamine and S-methamphetamine (Fig 2B). The docking scores of Type I PIs in the presence of R-methamphetamine in relatively more preferred binding mode 1 did not show significant differences in average scores compared to that in the absence of methamphetamine (Table 3). These results are in agreement with binding and metabolism studies in which we saw no significant effect of methamphetamine. Even though there is slight difference in the average scores with S-methamphetamine in both binding modes, we do not think these values make a significant difference in terms of metabolism (data not shown).

Using molecular modeling it is difficult to predict which binding mode or which molecule is more stable, because CYPs exhibit considerable structural flexibility [28, 29]. This has been manifested by our docking simulations that no significant differences between the docking scores have been observed.

Overall, our data suggests that methamphetamine does not alter the spectral change (δA_{max} and K_D), inhibition of CYP3A4, and substrate docking of type I PIs with CYP3A4s. Together

Table 3. The statistical results of PIs docking into CYP3A4 active site in the presence of methamphetamine.

	R-MA mode1	Average score ^[a]	Average score ^[b]	Δ Score	Average distance (Å) ^[c]	Average distance (Å) ^[d]	Δ Distance (Å)
Type I	Lopinavir	51.6	47.84	3.76	3.16	5.45	-2.29
	Atazanavir	44.62	41.88	2.74	3.61	6.42	-2.81
	Tipranavir	46.09	46.9	-0.81	3.75	6.33	-2.58
	Nelfinavir	49.16	47.57	1.59	3.6	5.31	-1.71
Type II	Ritonavir	57.56	46.75	10.81	3.16	5.7	-2.54
	Indinavir	55.88	52.37	3.51	3.37	5.74	-2.37

^a. Average score of major cluster in the absence of methamphetamine

^b. Average score of major cluster in the presence of methamphetamine

^c. The average distance between the atom of site closest to Fe and Fe for all top 10 conformers in the absence of methamphetamine.

^d. The average distance between the atom of site closest to Fe and Fe for all top 10 conformers in the presence of methamphetamine.

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these results suggest that there is no major conformational change in the active site of CYP3A4 when type I PIs bind with CYP3A4 in the presence of methamphetamine. Overall, the findings from type I PIs suggest no significant drug-drug interaction between methamphetamine and type I PIs. This is in contrast with our previous study with ethanol-CYP3A4 interaction, in which, ethanol showed altered metabolism of nelfinavir [17]. Therefore, a drug-dose adjustment with the type I PIs when given alone may not be needed. However, further *ex vivo* study is also needed to test whether the metabolism, bioavailability, and efficacy of type I PIs is altered in methamphetamine users.

Effect of methamphetamine on interaction of Type II PIs with CYP3A4

We also examined the effect of methamphetamine on spectral binding of two type II PIs (ritonavir and indinavir) with CYP3A4. Methamphetamine significantly decreased the δA_{\max} of ritonavir (0.0038 ± 0.0003 vs. 0.0055 ± 0.0003) but didn't affect indinavir (0.0039 ± 0.0002 vs. 0.0044 ± 0.0002) (Fig 6, Table 2). However, methamphetamine significantly decreased the K_D values of both ritonavir and indinavir by approximately 33% and 50%, respectively (0.043 ± 0.0001 vs. 0.065 ± 0.001 for ritonavir and 0.086 ± 0.01 vs. 0.174 ± 0.03 μM for indinavir) (Table 2). We also assessed the formation of hydroxy ritonavir, one of the major metabolites of ritonavir through CYP3A4 pathway, in the presence of varying concentrations of methamphetamine [30]. Our results demonstrated a significant decrease in the formation of hydroxy ritonavir in CYP3A4 microsomes with increasing concentrations of methamphetamine. As shown in Fig 4C, 100 μM of methamphetamine significantly decreased the formation of hydroxy ritonavir ($23.3 \pm 3.8\%$) with a further decrease in the metabolite level as the methamphetamine concentration increased to 1000 μM ($46.2 \pm 3.3\%$).

Further, we performed ritonavir docking to CYP3A4 to verify whether methamphetamine alters binding mode of ritonavir. The docking study showed that ritonavir has three sites that can interact with the heme of CYP3A4 (Fig 7A). However, the thiazole site of ritonavir is the most prominent because it exhibits 6 conformers with CYP3A4 with the highest score of 66.92, and the distance between ritonavir and heme is the shortest (2.75 Å). Interestingly, in the presence of methamphetamine, ritonavir exhibits altered binding with CYP3A4, in which thiazole group of ritonavir points away from the heme. Similarly, the docking scores of ritonavir binding are lower (50.33 vs. 66.92) and cluster are higher (10 vs. 3) in the presence of methamphetamine than in the absence of methamphetamine (S1 Table). Indinavir docking into CYP3A4 showed a slight decrease in the average score of major cluster in the presence of methamphetamine both binding modes (Table 3).

Ritonavir exhibited the lowest K_D among all the drugs studied, which is consistent with the previous finding [6], and with the finding that it is the most potent inhibitor of CYP3A4 [30]. Most importantly, methamphetamine further increased the binding affinity of both the type II PIs with CYP3A4. It is possible that methamphetamine causes a conformational change in CYP3A4 active site that enables the binding of type II PIs with CYP3A4 relatively more strongly. On the other hand, methamphetamine decreased the magnitudes of binding of ritonavir with CYP3A4 as determined by a decrease in δA_{\max} and a decrease in ritonavir metabolism. These findings suggest that methamphetamine acquires selective binding with certain pool of CYP3A4, which can't be replaced by ritonavir, a strongest CYP3A4 inhibitor known. Furthermore, docking studies suggest that thiazole group of ritonavir moves away from the heme site of CYP3A4 in the presence of methamphetamine suggesting a decrease in the magnitude of binding. Thus, the docking study is consistent with the finding that the δA_{\max} decreases in the presence of methamphetamine and also with decreased metabolite formation. Similarly, an increased binding affinity with ritonavir (relative to lopinavir) in the

Type II Protease Inhibitors

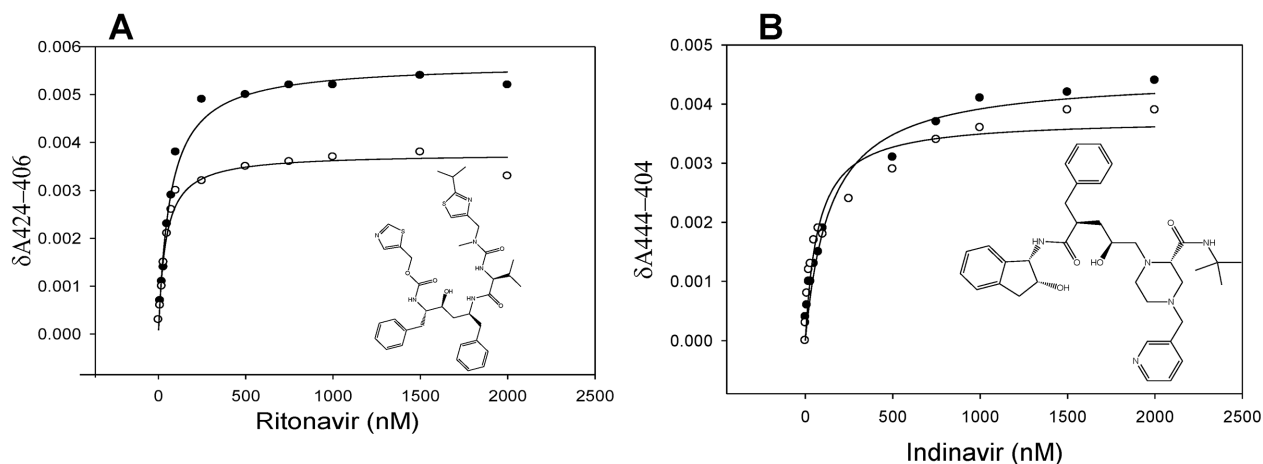


Fig 6. Spectral binding of type II PIs with CYP3A4 in the absence (filled circles) and presence (open circles) of methamphetamine. (A, B) The spectral binding was performed at varying concentrations of ritonavir and indinavir. The K_D and δA_{max} for each PI with and without methamphetamine are presented in Table 2. One way ANOVA with Dunnet's post-hoc test was employed to calculate the statistical significance. A p-value <0.05 is indicated by * and <0.1 is indicated by #.

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presence of methamphetamine can be explained by a higher magnitude of decreased binding energy and increased clusters with ritonavir compared to lopinavir (S1 Table). It is likely that methamphetamine binding causes a conformation change in the active site of CYP3A4 that leads to increased affinity with ritonavir and indinavir.

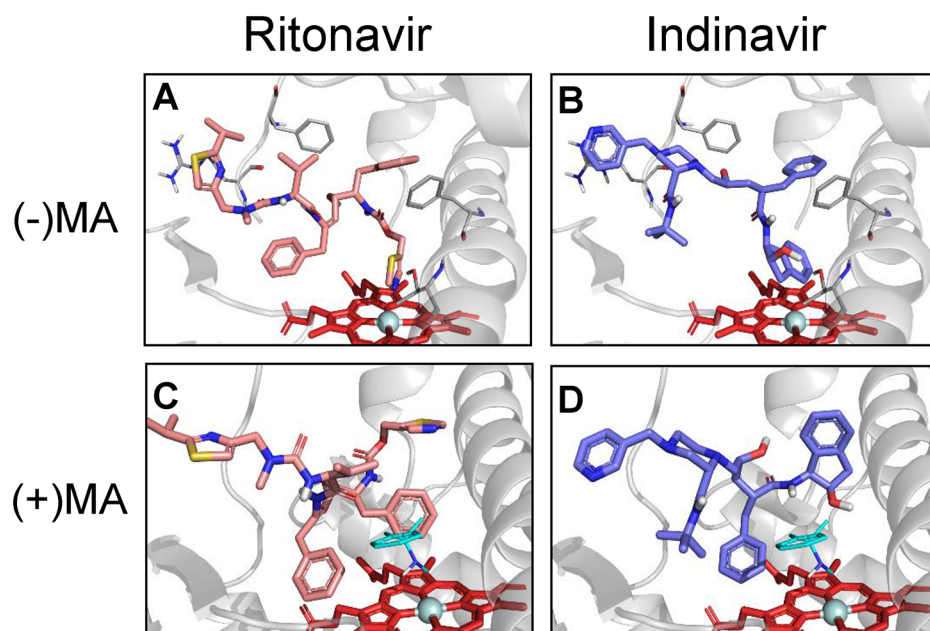


Fig 7. Docking simulations of Type II PIs in the absence and presence of methamphetamine. Structures of (A) Ritonavir and (B) Indinavir with their preferred binding sites pointing towards the heme moiety of CYP3A4 in the absence of methamphetamine. Docking simulations of (C) Ritonavir and (D) Indinavir with heme moiety of CYP3A4 in the presence of methamphetamine. R-methamphetamine in binding mode 1 was shown in cyan sticks. Docking was performed as described in Materials and Methods.

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Overall, our findings suggest that methamphetamine alters type II PIs-CYP3A4 interactions, which lead to decreased metabolism of these PIs, especially ritonavir. The results have clinical implications because an altered interaction of type II PIs, especially ritonavir, is expected to alter the inhibition characteristics of ritonavir and thus the metabolism of other PIs or NNRTIs. An increased metabolism of type II PIs is expected to decrease their bioavailability and therefore decrease the response of HAART. In addition, an increased metabolism may cause increased accumulation of their metabolites leading to increased toxicity. On the other hand since ritonavir is used as an inhibitor of CYP3A4 to increase the bioavailability of other PIs and NNRTIs, an increased inhibition may further increase the bioavailability of other drugs. Although this may cause an improved response to HAART, this may also cause increased drug-mediated toxicity. Therefore, there is a need for drug dose adjustment in methamphetamine users who are on ritonavir-boosted PIs or NNRTIs in their antiretroviral regimen. Further studies are needed to examine the metabolism, bioavailability, and efficacy of these drugs in the presence of methamphetamine in HIV-1-infected monocytes and lymphocytes, as well as, using ex-vivo study in HIV-infected methamphetamine users who are on HIV medication.

Supporting Information

S1 Table. The difference in docking scores and clusters of CYP3A4 bound with ligands between with and without the presence of methamphetamine (MA).
(DOC)

Author Contributions

Conceived and designed the experiments: AN NKV WL SK AK. Performed the experiments: AN JL AA LW. Analyzed the data: AN JL AA LW. Contributed reagents/materials/analysis tools: WL SK AK. Wrote the paper: AL WL SK AK.

References

1. Anzenbacher P, Anzenbacherova E. Cytochromes P450 and metabolism of xenobiotics. *Cell Mol Life Sci.* 2001; 58(5–6):737–47. Epub 2001/07/05. PMID: [11437235](#).
2. Fichtenbaum CJ, Gerber JG. Interactions between antiretroviral drugs and drugs used for the therapy of the metabolic complications encountered during HIV infection. *Clinical pharmacokinetics.* 2002; 41(14):1195–211. doi: [10.2165/00003088-200241140-00004](#) PMID: [12405866](#).
3. Walubo A. The role of cytochrome P450 in antiretroviral drug interactions. *Expert Opin Drug Metab Toxicol.* 2007; 3(4):583–98. Epub 2007/08/19. doi: [10.1517/17425225.3.4.583](#) PMID: [17696808](#).
4. Pal D, Mitra AK. MDR- and CYP3A4-mediated drug-drug interactions. *J Neuroimmune Pharmacol.* 2006; 1(3):323–39. Epub 2007/11/28. doi: [10.1007/s11481-006-9034-2](#) PMID: [18040809](#).
5. Chiba M, Jin L, Neway W, Vacca JP, Tata JR, Chapman K, et al. P450 interaction with HIV protease inhibitors: relationship between metabolic stability, inhibitory potency, and P450 binding spectra. *Drug metabolism and disposition: the biological fate of chemicals.* 2001; 29(1):1–3. PMID: [11124221](#).
6. Kumar S, Kumar A. Differential effects of ethanol on spectral binding and inhibition of cytochrome P450 3A4 with eight protease inhibitors antiretroviral drugs. *Alcoholism, clinical and experimental research.* 2011; 35(12):2121–7. doi: [10.1111/j.1530-0277.2011.01575.x](#) PMID: [21682753](#); PubMed Central PMCID: PMC4082664.
7. Plankey MW, Ostrow DG, Stall R, Cox C, Li X, Peck JA, et al. The relationship between methamphetamine and popper use and risk of HIV seroconversion in the multicenter AIDS cohort study. *Journal of acquired immune deficiency syndromes.* 2007; 45(1):85–92. doi: [10.1097/QAI.0b013e3180417c99](#) PMID: [17325605](#); PubMed Central PMCID: PMC3486782.
8. Buchacz K, McFarland W, Kellogg TA, Loeb L, Holmberg SD, Dille J, et al. Amphetamine use is associated with increased HIV incidence among men who have sex with men in San Francisco. *AIDS.* 2005; 19(13):1423–4. Epub 2005/08/17. 00002030-200509020-00011 [pii]. PMID: [16103774](#).

9. Wang X, Wang Y, Ye L, Li J, Zhou Y, Sakarcan S, et al. Modulation of intracellular restriction factors contributes to methamphetamine-mediated enhancement of acquired immune deficiency syndrome virus infection of macrophages. *Curr HIV Res.* 2012; 10(5):407–14. Epub 2012/05/18. CHIVR-EPUB-20120511-2 [pii]. PMID: [22591364](#); PubMed Central PMCID: PMC3934555.
10. Marcondes MC, Flynn C, Watry DD, Zandonatti M, Fox HS. Methamphetamine increases brain viral load and activates natural killer cells in simian immunodeficiency virus-infected monkeys. *Am J Pathol.* 2010; 177(1):355–61. Epub 2010/05/22. doi: [10.2353/ajpath.2010.090953](#) S0002-9440(10)60091-0 [pii]. PMID: [20489154](#); PubMed Central PMCID: PMC2893678.
11. Hales G, Roth N, Smith D. Possible fatal interaction between protease inhibitors and methamphetamine. *Antiviral therapy.* 2000; 5(1):19. PMID: [10846588](#).
12. Domanski TL, He YA, Khan KK, Roussel F, Wang Q, Halpert JR. Phenylalanine and tryptophan scanning mutagenesis of CYP3A4 substrate recognition site residues and effect on substrate oxidation and cooperativity. *Biochemistry.* 2001; 40(34):10150–60. Epub 2001/08/22. bi010758a [pii]. PMID: [11513592](#).
13. Hirani VN, Raucy JL, Lasker JM. Conversion of the HIV protease inhibitor nelfinavir to a bioactive metabolite by human liver CYP2C19. *Drug metabolism and disposition: the biological fate of chemicals.* 2004; 32(12):1462–7. doi: [10.1124/dmd.104.001743](#) PMID: [15448116](#).
14. Walsky RL, Obach RS. Validated assays for human cytochrome P450 activities. *Drug metabolism and disposition: the biological fate of chemicals.* 2004; 32(6):647–60. doi: [10.1124/dmd.32.6.647](#) PMID: [15155557](#).
15. Sheng Y, Chen Y, Wang L, Liu G, Li W, Tang Y. Effects of protein flexibility on the site of metabolism prediction for CYP2A6 substrates. *Journal of molecular graphics & modelling.* 2014; 54:90–9. doi: [10.1016/j.jmgl.2014.09.005](#) PMID: [25459760](#).
16. Jones G, Willett P, Glen RC, Leach AR, Taylor R. Development and validation of a genetic algorithm for flexible docking. *Journal of molecular biology.* 1997; 267(3):727–48. doi: [10.1006/jmbi.1996.0897](#) PMID: [9126849](#).
17. Kumar S, Earla R, Jin M, Mitra AK, Kumar A. Effect of ethanol on spectral binding, inhibition, and activity of CYP3A4 with an antiretroviral drug nelfinavir. *Biochem Biophys Res Commun.* 2010; 402(1):163–7. Epub 2010/10/13. doi: [10.1016/j.bbrc.2010.10.014](#) S0006-291X(10)01865-6 [pii]. PMID: [20937259](#).
18. Kuwayama K, Tsujikawa K, Miyaguchi H, Kanamori T, Iwata YT, Inoue H. Interaction of 3,4-methylenedioxyamphetamine and methamphetamine during metabolism by in vitro human metabolic enzymes and in rats. *J Forensic Sci.* 2012; 57(4):1008–13. Epub 2012/01/04. doi: [10.1111/j.1556-4029.2011.02039.x](#) PMID: [22211929](#).
19. Meyer MR, Peters FT, Maurer HH. The role of human hepatic cytochrome P450 isozymes in the metabolism of racemic 3,4-methylenedioxyethylamphetamine and its single enantiomers. *Drug metabolism and disposition: the biological fate of chemicals.* 2009; 37(6):1152–6. Epub 2009/03/21. [pii]. PMID: [19299523](#).
20. Kreth K, Kovar K, Schwab M, Zanger UM. Identification of the human cytochromes P450 involved in the oxidative metabolism of "Ecstasy"-related designer drugs. *Biochem Pharmacol.* 2000; 59(12):1563–71. Epub 2000/05/09. S0006295200002847 [pii]. PMID: [10799653](#).
21. Earla R, Kumar S, Wang L, Bosinger S, Li J, Shah A, et al. Enhanced methamphetamine metabolism in rhesus macaque as compared with human: an analysis using a novel method of liquid chromatography with tandem mass spectrometry, kinetic study, and substrate docking. *Drug metabolism and disposition: the biological fate of chemicals.* 2014; 42(12):2097–108. doi: [10.1124/dmd.114.059378](#) PMID: [25301936](#); PubMed Central PMCID: PMC4244873.
22. Welter J, Meyer MR, Wolf EU, Weinmann W, Kavanagh P, Maurer HH. 2-methiopropamine, a thiophene analogue of methamphetamine: studies on its metabolism and detectability in the rat and human using GC-MS and LC-(HR)-MS techniques. *Analytical and bioanalytical chemistry.* 2013; 405(10):3125–35. doi: [10.1007/s00216-013-6741-4](#) PMID: [23361230](#).
23. Melega WP, Cho AK, Harvey D, Lacan G. Methamphetamine blood concentrations in human abusers: application to pharmacokinetic modeling. *Synapse.* 2007; 61(4):216–20. Epub 2007/01/19. doi: [10.1002/syn.20365](#) PMID: [17230548](#).
24. Talloczy Z, Martinez J, Joset D, Ray Y, Gacser A, Toussi S, et al. Methamphetamine inhibits antigen processing, presentation, and phagocytosis. *PLoS Pathog.* 2008; 4(2):e28. Epub 2008/02/20. doi: [10.1371/journal.ppat.0040028](#) 07-PLPA-RA-0573 [pii]. PMID: [18282092](#); PubMed Central PMCID: PMC2242831.
25. Meyer RP, Gehlhaus M, Knoth R, Volk B. Expression and function of cytochrome p450 in brain drug metabolism. *Curr Drug Metab.* 2007; 8(4):297–306. Epub 2007/05/17. PMID: [17504219](#).

26. Park M, Kim HJ, Lim B, Wylegala A, Toborek M. Methamphetamine-induced occludin endocytosis is mediated by the Arp2/3 complex-regulated actin rearrangement. *J Biol Chem*. 2013; 288(46):33324–34. Epub 2013/10/02. [pii]. PMID: [24081143](#); PubMed Central PMCID: PMC3829179.
27. Pal D, Kwatra D, Minocha M, Paturi DK, Budda B, Mitra AK. Efflux transporters- and cytochrome P-450-mediated interactions between drugs of abuse and antiretrovirals. *Life Sci*. 2011; 88(21–22):959–71. Epub 2010/10/12. doi: [10.1016/j.lfs.2010.09.012](#) S0024-3205(10)00400-5 [pii]. PMID: [20932495](#); PubMed Central PMCID: PMC3100475.
28. Pochapsky TC, Kazanis S, Dang M. Conformational plasticity and structure/function relationships in cytochromes P450. *Antioxidants & redox signaling*. 2010; 13(8):1273–96. doi: [10.1089/ars.2010.3109](#) PMID: [20446763](#); PubMed Central PMCID: PMC2959183.
29. Negishi M, Uno T, Darden TA, Sueyoshi T, Pedersen LG. Structural flexibility and functional versatility of mammalian P450 enzymes. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 1996; 10(7):683–9. PMID: [8635685](#).
30. Kumar GN, Rodrigues AD, Buko AM, Denissen JF. Cytochrome P450-mediated metabolism of the HIV-1 protease inhibitor ritonavir (ABT-538) in human liver microsomes. *The Journal of pharmacology and experimental therapeutics*. 1996; 277(1):423–31. PMID: [8613951](#).