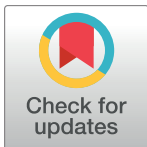RESEARCH ARTICLE

Alterations in cellular pharmacokinetics and pharmacodynamics of elvitegravir in response to ethanol exposure in HIV-1 infected monocytic (U1) cells

Narasimha M. Midde, Namita Sinha, Pradeep B. Lukka, Bernd Meibohm, Santosh Kumar*

Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis TN, United States of America

* ksantosh@uthsc.edu

Abstract

Ethanol consumption is negatively associated with antiretroviral therapy (ART) adherence and general health in HIV positive individuals. Previously, we demonstrated ethanol-mediated alterations to metabolism of elvitegravir (EVG) in human liver microsomes. In the current study, we investigated ethanol influence on the pharmacokinetic and pharmacodynamic interactions of EVG in HIV infected monocytic (U1) cells. U1 cells were treated with 5 µM EVG, 2 µM Cobicistat (COBI), a booster drug, and 20 mM ethanol for up to 24 hours. EVG, HIV p24 levels, alterations in cytochrome P450 (CYP) 3A4, MRP1, and MDR1 protein expressions were measured. Presence of ethanol demonstrated a significant effect on the total exposures of both EVG and EVG in combination with COBI. Ethanol also increased the HIV replication despite the presence of drugs and this elevated HIV replication was reduced in the presence of MRP1 and MDR1 inhibitors. Consequently, a slight increase in EVG concentration was observed in the presence of MRP1 inhibitor but not with MDR1 inhibitor. Furthermore, CYP3A4, MRP1 and MDR1 protein levels were significantly induced in treatment groups which included ethanol compared to those with no treatment. In summary, these findings suggest that Ethanol reduces intra cellular EVG exposure by modifying drug metabolism and transporter protein expression. This study provides valuable evidence for further investigation of ethanol effects on the intracellular concentration of EVG in ex vivo or in vivo studies.

Introduction

Highly active antiretroviral therapy (HAART) regimen consisting of integrase strand transfer inhibitors (INSTI), nucleoside (or nucleotide) reverse transcriptase inhibitors (NRTI), nonnucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitors (PI) dramatically transformed HIV infection from fatal to a chronic but manageable disease. Among different classes of drugs, INSTIs such as elvitegravir (EVG), dolutegravir or raltegravir have become 'standard' drugs in the recommended regimens owing their superior efficacy and safety in clinical trials and retrospective evaluations[1, 2]. While highly effective, a major limitation of

HAART therapy is its failure to eradicate the virus from cells even after years of therapy. Intracellular presence of EVG is a requisite for its action on its pharmacological target[3], while HIV therapeutics are optimized based on plasma pharmacokinetics[4]. Owing to most HAART therapeutic intracellular targets, it is of utmost importance to understand the intracellular pharmacokinetics of these essential drugs. This was further substantiated by several clinical studies which demonstrated a weak or no correlation of plasma INSTI, NRTIs or PIs concentration with antiviral efficacy in the treatment of HIV infected patients[5–8].

Intracellular EVG concentration is influenced by several factors including physicochemical properties of the drug, pharmacokinetic properties such as protein binding, hepatic metabolism, drug transporters and drug-drug interactions. Since EVG is predominantly metabolized by cytochrome P450 (CYP) 3A4[9] a strong CYP3A4 inhibitor such as cobicistat (COBI) is co-administered as a booster to increase EVG bioavailability. It was reported that EVG is a weak inducer of CYP3A4 and this effect can be countered by the presence of COBI in the liver[10]. But how EVG-drug interactions change the CYP3A4 induction at the cellular level has not been established. The intracellular antiretroviral drug concentrations are also dictated by the activity of efflux transporters[11, 12]. Several recent studies revealed that antiretroviral drugs can act as substrates and inducers for membrane transporters especially for efflux transporters [13, 14], which explain, at least in part, the reported high intracellular drug variability in HIV positive patients[15, 16]. Most of the recommended HAART therapy consists of multiple drugs and many of these drugs are known to modulate transporters. Moreover, concurrent use of other prescribed medication or drugs of abuse increases the complexity of the problem.

Ethanol, the most commonly consumed legal drugs in the world, is at low levels primarily metabolized by alcohol dehydrogenase (ADH), but at higher alcohol concentration both ADH and CYP2E1 are requires for metabolism[17]. Previously, we reported that exposure of ethanol increases the expression of both multidrug resistance protein 1 (MRP1) and CYP enzymes in U937 macrophages[18, 19]. Additionally, we reported altered CYP2E1 and CYP3A4 mRNA expression levels in chronic treatment of both ethanol and HIV protease inhibitors darunavir and ritonavir in macrophages[20]. At the systemic level, ethanol consumption (0.7 g/kg) with 600 mg of abacavir, an NRTI used in the treatment of HIV, increases abacavir total exposure by 41%, maximum concentration by 15% and half-life by 26% in 25 male HIV-infected individuals[21]. However, the effect of ethanol on the intracellular HIV drug concentration or efficacy in the clinical setting is not documented to date.

Since the intracellular concentrations of an active drug moiety determines the virologic suppression achieved in the body, understanding the drug interactions that lead to fluctuations in the intracellular drug concentrations are critical to improve treatment outcomes. However, there is a substantial knowledge gap on the impact of ethanol and HIV drug interactions at the cellular level, especially in monocytic cells. Monocytes are important targets of HIV-1 infection, and ART concentrations, especially PIs, is suboptimal in monocytes[22]. Further, HIV-infected monocytes infiltrate into the brain and spread the virus in perivascular macrophages and microglia that eventually form sanctuary sites for HIV-1 in the brain[23, 24]. In this study we examined the effect of acute exposure of ethanol and potential mechanism on the intracellular pharmacokinetics of EVG and its effectiveness on the viral suppression in HIV infected monocytic (U1) cells.

Materials and methods

Cell culture and treatments

HIV-infected U937 cell line—the U1 cells were obtained through the NIH AIDS Reagent Program (Germantown, MD). U1 cells were cultured in Roswell Park Memorial Institute (RPMI)

1640 media (Corning Inc, Tewksbury, MA) supplemented with 10% fetal bovine serum (Atlanta biologicals, Atlanta, GA), 1% L-glutamine, nonessential amino acid solution, sodium bicarbonate, and penicillin-streptomycin solution. Cells were plated at 0.9×10^6 cells/well of 6-well plates and were differentiated into macrophages with 100 nM Phorbol 12-myristate 13-acetate (PMA) for 72 hours prior to treatment. On the day of experiment, medium was removed and cells were washed twice with PBS and treated with media containing clinically relevant concentrations of (a) EVG (5 μM), (b) EVG+COBI (2 μM), (c) EVG+ethanol (20 mM), (d) EVG+COBI+ethanol. Ethanol-treated plates were placed in an incubator that humidified with 20 mM ethanol to prevent any loss of ethanol due to evaporation.

At predetermined time points (0.5, 1, 3, 6, 12, and 24 hours), cells were collected for concentration-time profile experiments and medium was collected for HIV p24 measurement. At each time point, cells were washed twice with PBS and incubated with 400 μL of cold Radioimmunoprecipitation assay (RIPA) buffer for 10 minutes and the cell lysates were collected. For evaluation of the influence of efflux transporters, cells were incubated with either EVG+COBI +ethanol and MK-571 (MRP1 inhibitor) at 50 μM or PSC-833 (multidrug resistance1 (MDR1)) inhibitor at 5 μM for 24 hours before collecting cell lysate and media samples.

LC-MS/MS analysis of elvitegravir

A previously reported LC-MS/MS method was used to quantitate EVG in cell lysates, culture medium, and plasma samples used in this study[25]. Calibrants (0.003, 0.006, 0.013, 0.025, 0.05, 0.1, 0.15, 0.25, 0.5, and 1 μM) were prepared by serial dilution approach with the corresponding working solutions. EVG extraction from the calibrants and study samples was performed by adding 3-volumes of cold acetonitrile which contained a 138 nM ritonavir (RTV) as internal standard (IS). All samples were centrifuged at 8000 rpm for 10 minutes at 4°C and clear supernatants were transferred into vails with low-volume inserts for LC-MS/MS analysis. Samples (5 μL) were injected onto a Waters Xterra® MS C18 Column (125Å, 3.5 μ, 4.6 X 50 mm) (Waters Corporation, Milford, MA) attached to a Shimadzu Nexera HPLC system coupled with a tandem mass spectrometer (Triple Quad 5500 from AB SCIEX, Framingham, MA) with electron spray ionization in positive mode. Data was acquired and analyzed with the Analyst software package (ver.1.6.2, AB SCIEX, Framingham, MA). Concentrations were quantified by the Quantitative Analysis module (Analyst ver 1.6.2, AB SCIEX, Framingham, MA) from a standard curve fitted with a linear-regression model with a weighting factor of $1/x^2$.

HIV p24 ELISA

Influence of the combination of drugs on HIV replication was determined by measurement of HIV p24 levels in the cell culture medium. After initial screening of p24 levels at different time points, we decided to use the 24 hour samples from all the experiments for p24 quantitation which provided consistent optical density (OD) values. p24 antigen production was detected per manufacturer's protocol (ZeptoMetrix Corp, Buffalo, NY). The concentration of p24 in the samples was quantitated by comparing with standards' absorbance values and reported in percentage of control. The vehicle-treated samples were used as control and set as 100%.

In-Cell Western analysis

Detecting low levels of protein expression in native cells using traditional Western blotting can be difficult and therefore some studies successfully used In-Cell Western blotting (ICW) to quantify minor alterations in protein expression[26, 27]. We used manufacturer recommended (LI-COR Biosciences, NE) protocol in this study to perform ICW. U1 cells (0.03×10^6) were plated in sterile 96-well plates and differentiated with 100 nM PMA for 72 hours at 37°C

in a humidified incubator while maintaining 5% CO_2 air flow. On the day of treatment, each well was flushed with sterile PBS (250 μl) followed by addition of a 250 μl RPMI media containing various combinations of drugs previously mentioned. These plates were incubated for 24 hours prior to fixation. Medium from each well was removed by careful aspiration and cells were immediately fixed by addition of cold (-20°C) methanol (200 μl) followed by incubation of the plate at ambient temperature for 10 minutes with gentle shaking. Following incubation, methanol was carefully removed and wells were gently rinsed thrice with PBS (200 μl). After the last rinse, blocking was done by adding a 150 μl of Li-Cor blocking buffer and incubating the plate at room temperature with gentle shaking for 1.5 hours. Meanwhile, the primary antibodies were diluted in Li-Cor Odyssey blocking buffer using following dilutions: 3A4 (Rabbit Pab, Abcam # ab3572) 1: 200; MRP1 (mouse mab, Millipore # MAB4100) 1:50; MDR1(mouse mab, Millipore # MAB4334) 1:50; β-Actin (Mouse mAb Cell Signaling # 3700) 1:2000; β-Actin (Rabbit mAb Cell Signaling # 4970) 1:200. After blocking, 50 μl of desired antibody added to test wells and incubated overnight at 4°C with gentle shaking. On the following day, wells were washed five times with 200 μl PBS containing Tween-20 (0.1%) for 5 minutes at room temperature with gentle shaking. Secondary antibodies, goat anti-Mouse Mab (Li-Cor # 926–68170) and goat anti-Rabbit Mab (Li-Cor # 827–08365) were diluted in 1:1000 ratio in blocking buffer (0.2% -Tween-20, 0.01% SDS). After the final wash, wells were incubated with 50 μl of secondary antibody mixture for 1 hour in the dark at room temperature. After five washes, plates were scanned using Li-Cor Odyssey® SA and analyzed by using Image Studio V4.0 software (LI-COR Biosciences, NE). Background was subtracted from control wells and target protein signal intensities from triplicate wells were normalized to their respective β-actin intensities. Signal intensities were expressed as percent of relative responses (mean ± standard errors of mean) compared to vehicle controls.

Pharmacokinetic and statistical analysis

The concentration-time profiles were analyzed by non-compartmental modeling using Phoenix WinNonlin 6.4 (Certara L.P., Princeton, NJ). Area under the curve (AUC_{tot}) was calculated by the log trapezoidal rule by log-linear regression. The maximum concentration achieved (C_{max}) and the time to maximum concentration (T_{max}) were determined from the concentration-time profiles. Effect of ethanol on EVG AUC_{tot} and C_{max} were compared using unpaired t test with Welch's correction. T_{max} values were compared using Wilcoxon rank-sum test. All other data involving more than two groups were compared by one way ANOVA and multiple comparisons were performed by Tukey's post hoc test. ANOVA data was presented along with multiplicity adjusted p values for each comparison. All the statistical analyses were done by Prism 6 (GraphPad Software, La Jolla, CA). A p-value $\leq$ 0.05 among treatment groups was considered significant.

Results

Intracellular ethanol-drug interaction

The mean intracellular concentration-time curve for EVG, EVG co-exposed with COBI or ethanol, and EVG co-exposed with both COBI and ethanol are shown in Fig 1 and Table 1. The mean exposure of (AUC_{tot}, μM*h/mg) of EVG alone (1733 ± 122) and EVG+COBI (40207 ± 3031) were decreased to 1733 ± 122 and 40207 ± 3031, respectively, when exposed along with ethanol. The difference is statistically significant (Table 1) and shows 39% (EVG vs EVG+EtOH t(4) = 2.794, p = 0.0491, student t test) and 26% (EVG+COBI vs EVG+COBI+EtOH t(7) = 3.413, p = 0.0112) decrease due to ethanol exposure. The maximal concentrations (C_{max}) after treatment with EVG alone and EVG+COBI were 82 ± 4 μM and 1896 ± 162 μM, respectively. When cells
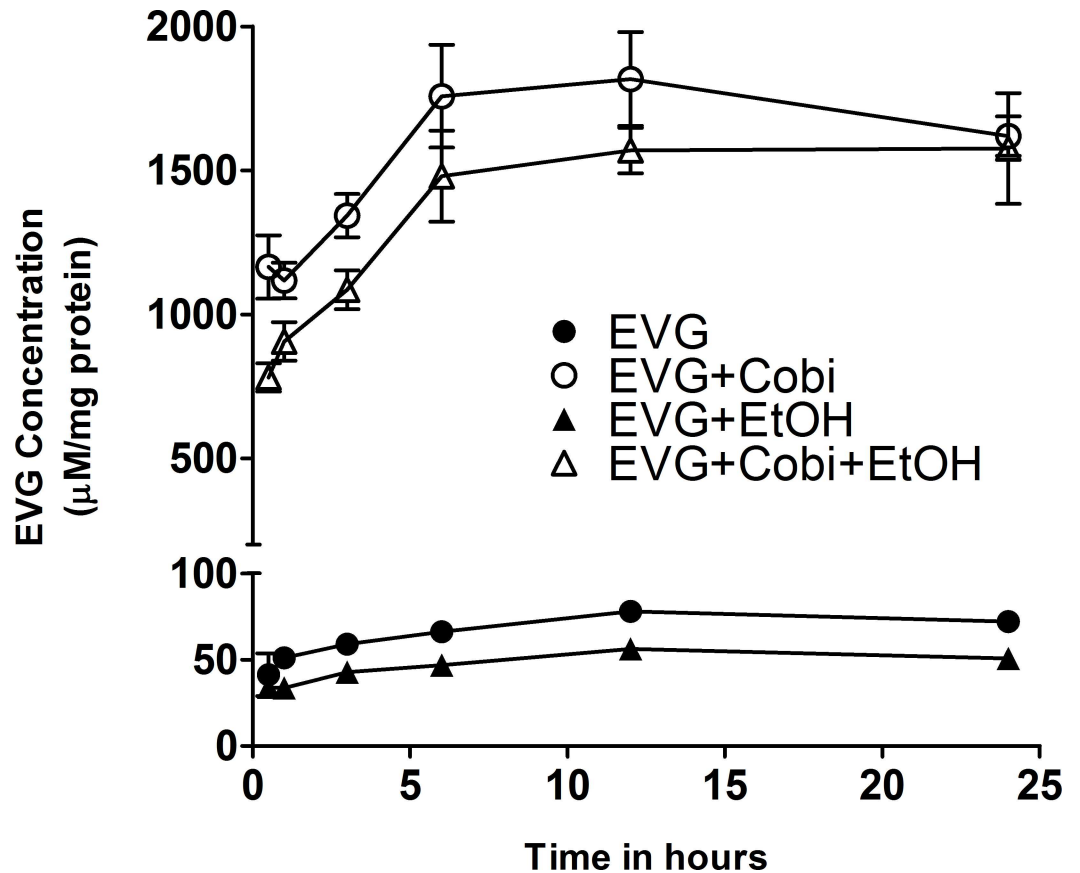
Fig 1. Concentration-time profiles for the accumulation of elvitegravir in U1 cells with or without cobicistat and ethanol. Results expressed as means ± SEM of the cellular concentrations measured (µM/mg of protein). EVG, Elvitegravir; COBI, Cobicistat; EtOH, ethanol.

doi:10.1371/journal.pone.0172628.g001

were co-treated with EVG and EVG+COBI combinations along with ethanol, C_{max} was found to be 61 ± 5 µM and 1756 ± 53 µM, respectively. While ethanol decreased the maximal EVG concentrations (C_{max}) by 26% (EVG vs EVG+EtOH $t(5) = 3.587$, $p = 0.0158$) after treatment with EVG alone, it had a lesser impact (7%) on EVG C_{max} when co-administered with COBI. The median T_{max} (hours; h) values for EVG and EVG along with COBI were found to be 12 h, and ethanol exposure demonstrated 6 h for EVG and 18 h for EVG+COBI exhibiting no significant influence of ethanol co-treatment on T_{max} of EVG.

Ethanol and elvitegravir interaction on HIV-1 replication

To understand the pharmacodynamic influence of ethanol and drug interaction, HIV-1 replication was indirectly assessed by measuring HIV p24 protein levels after treatment (24 hours).

Table 1. Pharmacokinetic parameters of elvitegravir in U1 cells. AUC_{tot}, area under the plasma concentration-time curve from time 0 to 24 hours; C_{max}, measured maximum plasma concentration; T_{max}, time to reach peak concentration. *$p < 0.05$ compared to EVG, #$p<0.05$ compared to EVG+COBI. Data expressed as mean ± SEM from 4–5 independent experiments for AUC_{tot} and C_{max}, and as median (range) for T_{max}. EVG, Elvitegravir; COBI, Cobicistat; EtOH, ethanol.

Pharmacokinetic parameters	EVG	EVG+COBI	EVG+EtOH	EVG+COBI+EtOH
AUC_{tot} (µMXh/mg)	1733 ± 122	40207 ± 1031	1055 ± 210*	34734 ± 1229#
C_{max} (µM)	82 ± 4	1896 ± 162	61 ± 5*	1756 ± 53
T_{max} (h)	12 (11.5)	12 (18)	18 (18)	6 (6)

doi:10.1371/journal.pone.0172628.t001
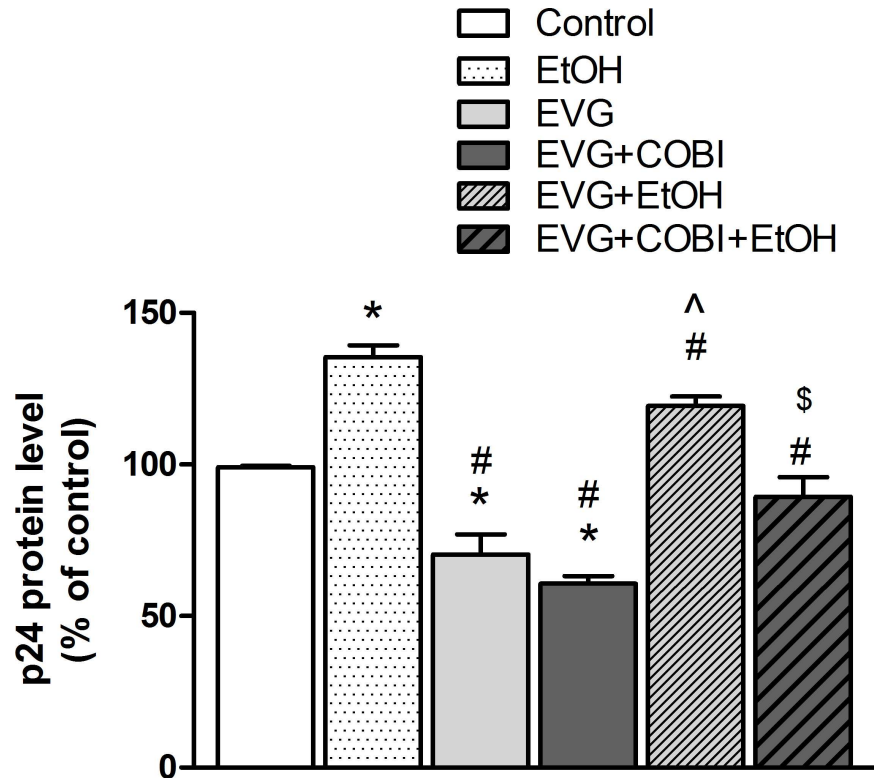
Fig 2. Alterations in HIV replication of U1 cells after drug exposure. PMA-differentiated U1 cells were treated with indicated drugs for 24 hours and HIV replication was determined using an ELISA for p24 levels in culture media. *$p < 0.05$ compared to control, #$p < 0.05$ compared to EtOH. ^$p < 0.05$ compared to EVG, and $$p < 0.05$ compared to EVG+COBI. Values were mean ± SEM from 3 individual experiments. EVG, Elvitegravir; COBI, Cobicistat; EtOH, ethanol.

doi:10.1371/journal.pone.0172628.g002

Data are presented in Fig 2. Ethanol exposure alone had a 35% increase compared to vehicle-treatment ($F_{(5,16)} = 30.20$, $p = 0.0005$, one-way ANOVA). Both EVG and EVG+COBI reduced the p24 levels by 30% and 40%, respectively, which is statistically significant compared to both vehicle ($p = 0.0054$ and $p = 0.0003$ respectively) and ethanol treatment groups ($p < 0.0001$ and $p < 0.0001$ respectively). Similarly, co-exposing cells to EVG and EVG+COBI along with ethanol enhanced p24 levels by 31% and 19%, respectively. This increase is statistically significant when compared to EVG ($p < 0.0001$) and EVG+COBI ($p = 0.0055$) groups but not with the vehicle treatment.

Ethanol and EVG effects on CYP3A4, MRP1, and MDR1 protein expression

In-Cell Western blot analysis was performed to understand the mechanism for alterations in intracellular PK parameters of EVG in response to ethanol exposure. CYP3A4, MRP1, and MDR1 proteins expression were detected, and the analyzed results are depicted in Fig 3, respectively. EVG alone or EVG treatment along with COBI showed no change in expression of CYP3A4; however, ethanol alone or in combination with EVG and EVG+COBI exhibited a significant induction of CYP3A4 compared to control ($F_{(5,51)} = 26.73$, $p < 0.0001$, one-way ANOVA, Fig 3A). No significant difference was seen in ethanol exposed EVG or EVG+COBI combination from ethanol alone treatment.
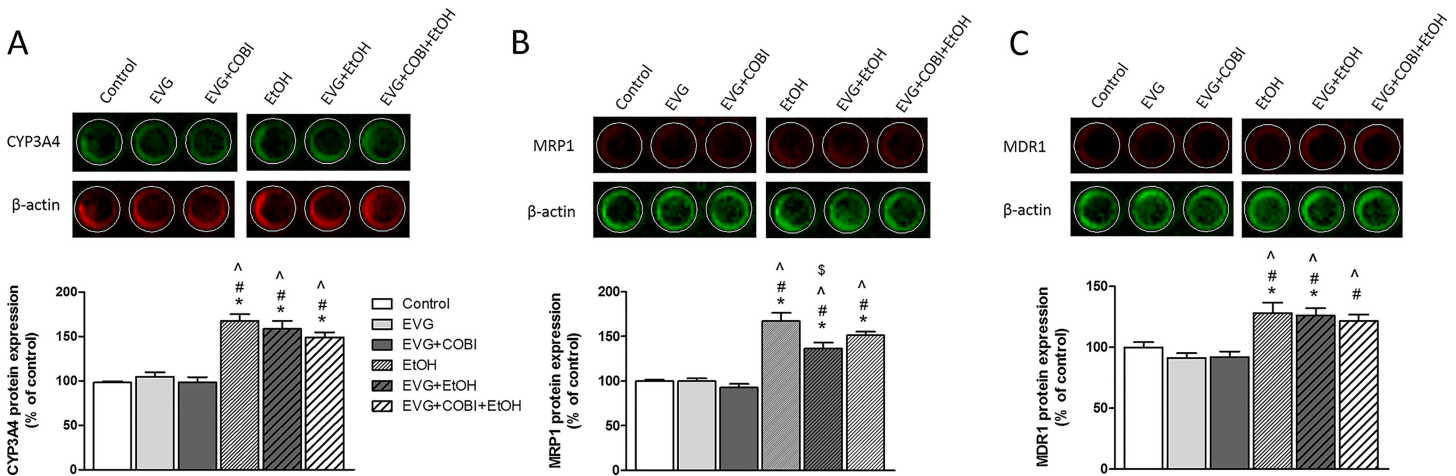
Fig 3. Induction of CYP3A4, MRP1, and MDR1 protein expression was analyzed by In-Cell Western (ICW) assay. Top section in each panel is a respective representative immunoblot of CYP3A4, MRP-1 or MDR1. Bottom plot in each panel demonstrates respective mean CYP3A4, MRP-1 or MDR1 fluorescence signal intensities of triplicates and error bars represent SEM values. *p< 0.05 compared to control, #p<0.05 compared to EVG, and ^p<0.05 compared to EVG+COBI, $p<0.05 compared to EtOH. Values were mean ± SEM from 3 individual experiments. EVG, Elvitegravir; COBI, Cobicistat; EtOH, ethanol.

Similarly, EVG or EVG+COBI treatments showed no effect on MRP1 expression. Ethanol upregulated MRP1 protein levels when treated alone or in combination with EVG or EVG+ COBI compared to control ($F_{(5,50)}$ = 28.85, p<0.001, one-way ANOVA, Fig 3B). EVG when exposed along with ethanol demonstrated an attenuation of ethanol-induced increase of MRP1 levels (p = 0.0043). Moreover, EVG exposure along with COBI and ethanol showed a decreasing trend in of ethanol-induced MRP1 expression but this was not statistically significant.

While drugs alone (EVG or EVG+COBI) showed no influence on the expression of MDR1, ethanol treatment both in the absence and presence of EVG exhibited a significant induction of MDR1 compared to control ($F_{(5,30)}$ = 9.411, p<0.028, one-way ANOVA, Fig 3C). The impact of ethanol co-exposure with EVG and COBI was not statistically significant from control. No significant change was observed in EVG and EVG+COBI combinations that are co-treated with ethanol compared to ethanol alone exposure.

Effect of MRP1 and MDR1 inhibition on intracellular EVG concentration

Since the induction of CYP3A4, MRP1, and MDR1 induction in response to ethanol treatment (Fig 3), we tested the possibility of MRP1 and MDR1 transporter effects on EVG availability. For this experiment, ethanol co-treatment with EVG and COBI as control was chosen, which represents the influence of both ethanol and COBI. The cells that are exposed to the MRP1 inhibitor, MK-571 demonstrated a 12% increase in the EVG concentration compared to control and is statistically significant ($F_{(2,26)}$ = 5.461, p = 0.031, one-way ANOVA, Fig 4). In contrary, the MDR1 inhibitor, PSC-833 showed no significant effect on intracellular EVG concentration.

Effect of MRP1 and MDR1 inhibition in HIV replication

We determined HIV p24 levels in the presence of MK-571 or PSC-833 while keeping ethanol co-exposure with EVG and COBI as constant to observe the influence of these inhibitors on the HIV replication. As shown in Fig 5, ethanol increased the p24 levels by 36% compared to control ($F_{(6,20)}$ = 18.89, p<0.0001, one-way ANOVA) but MK-571 or PSC-833 did not show
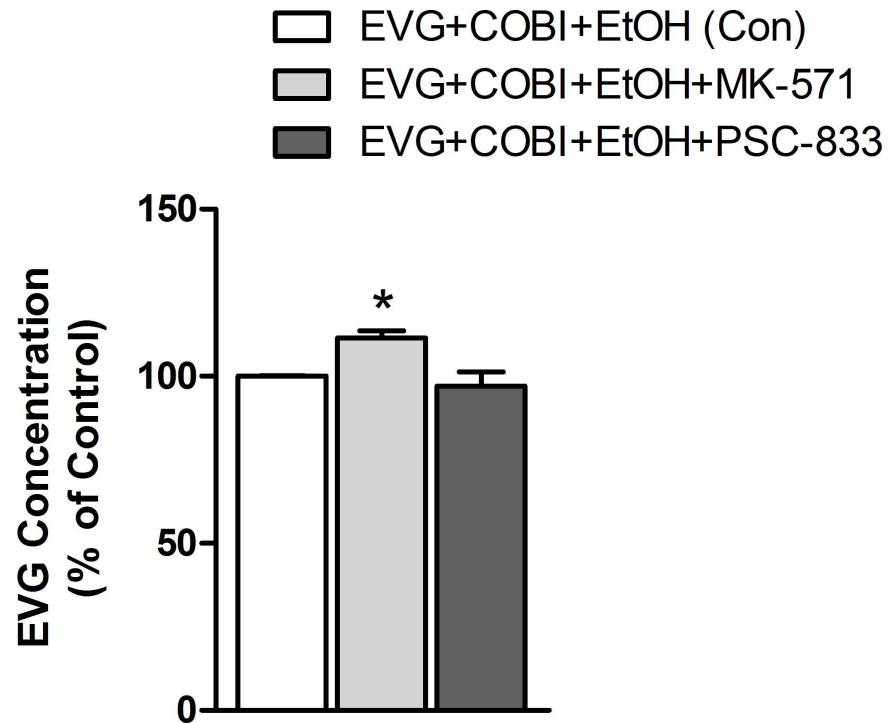
Fig 4. Intracellular elvitegravir concentration in the presence of MRP1 or MDR1 inhibitors and cobicistat + ethanol. EVG quantified from cell lysates using LC MS/MS after 24 hours treatment with indicated drugs. Data are expressed as mean ± SEM from 3 independent experiments. *$p < 0.05$ compared to EVG+COBI+EtOH (Control). EVG, Elvitegravir; COBI, Cobicistat; EtOH, ethanol.

doi:10.1371/journal.pone.0172628.g004

any effect on the p24 levels. PSC-833 in combination with EVG, COBI, and ethanol showed a decrease in p24 levels compared to vehicle or PSC-833 alone control ($p<0.05$). However, both MK-571 and PSC-833 in the presence of EVG, COBI, and ethanol failed to show significant change when compared to EVG, COBI, and ethanol combination.

Discussion

Optimum intracellular concentration is critical to the success of ART in HIV treatment and is affected by several external or internal dynamics. However, only a few clinical studies investigated the effects of ethanol on ART by measuring the plasma pharmacokinetics[21, 28, 29]. In general, it is conceived that small molecules that interact with ethanol dehydrogenase or CYP2E1 and are likely to be influenced by ethanol exposure. This interaction could lead to clinically significant changes in the pharmacokinetic properties of those drugs[30]. However, the influence of ethanol drinking on the intracellular concentration of ART or its effects on the pharmacodynamics is relatively unclear. Therefore, the current study investigated the intracellular drug-drug interactions between EVG and ethanol in combination with or without COBI in HIV-infected monocytic (U1) cells. Herein, we report a decreased EVG AUC and C_{max} values and increased HIV replication in the presence of ethanol. In addition, mechanistic studies revealed that ethanol induced alterations in EVG cellular pharmacokinetics and associated effect on HIV replication is, at least partially, due to induction of CYP3A4, MRP1, and MDR1 proteins.

EVG is required to penetrate the HIV-infected cell to exert its antiviral activity. Usually, ART plasma concentration is used to assess the intracellular drug level and to understand
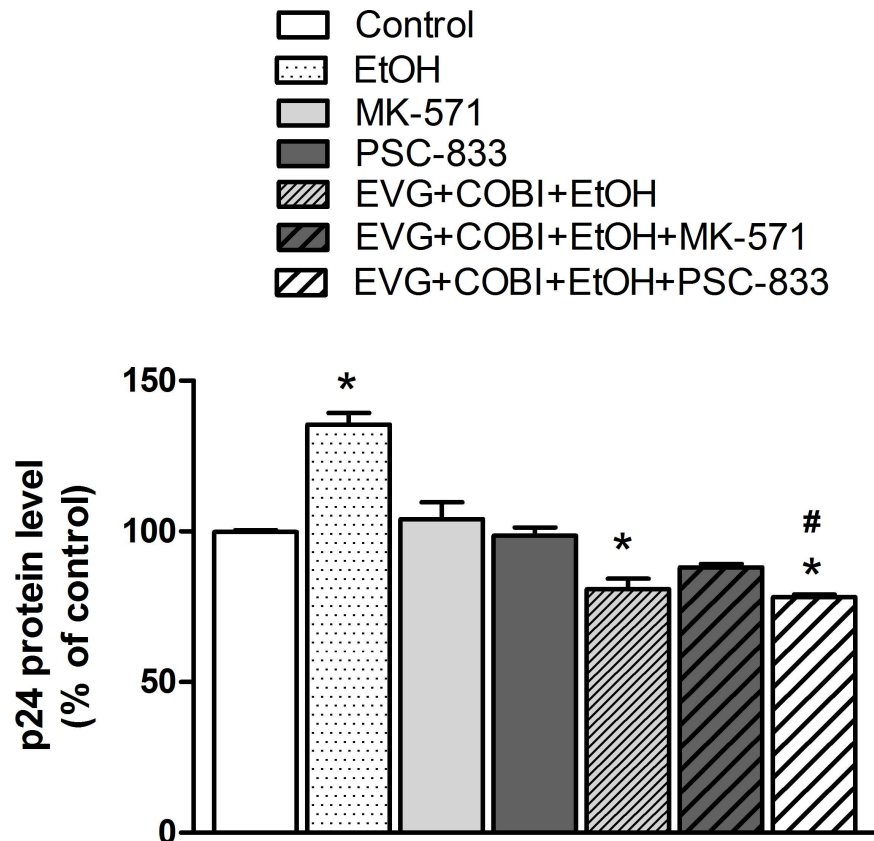
Fig 5. Changes in HIV replication of U1 cells after drug exposure. PMA-differentiated U1 cells were treated with indicated drugs for 24 hours and HIV replication was determined using an ELISA for p24 levels in culture media. *$p < 0.05$ compared to control, #$p < 0.05$ compared to PSC-833. Values were means ± SEM from 3 individual experiments. EVG, Elvitegravir; COBI, Cobicistat; EtOH, ethanol.

overall intracellular drug disposition[31] Currently, there is no information on EVG uptake into cells and the relationship between its plasma and cellular concentrations. Nonetheless, evaluation of therapeutic levels in the cells based on plasma concentrations may not be accurate especially for integrase inhibitors. Like nucleoside/nucleotide reverse transcriptase inhibitors, intracellular half-life of integrase inhibitors is critical to achieve antiviral effect[32]. For instance, an initial Phase III study of Isentress® (raltegravir), an integrase inhibitor show that 400 mg twice-daily is more effective than 800 mg once daily[8]. Moreover, considering high protein binding and poor cell penetration capability (~5% of plasma concentration) of integrase inhibitors[33, 34], small changes as a result of co-exposure of ethanol may greatly influence the intracellular concentration of EVG. Therefore, it is worth noting that the observed considerable influence of ethanol on the extent of EVG overall exposure irrespective of COBI presence may have impact on clinical outcomes in HIV treatment.

Our findings that ethanol causes an increase in HIV replication is in agreement with previously reported observations[35–37]. EVG or COBI-boosted EVG displayed a significant viricidal activity. However, this activity is not as pronounced as EVG effects in HIV-infected patients or on freshly infected cells[38, 39]. The inherent constitutive expression of HIV in the U1 cells could be the likely reason for this reduced effect of EVG in these experiments[40]. However, ethanol exposure increases HIV p24 levels despite the presence of EVG or EVG and COBI. This increase is attributable to either reduced intracellular drug exposure as described

above or by stimulating HIV replication through other mechanisms. For example, a recent study showed that ethanol enhances HIV infection in cord blood monocyte-derived macrophages by inhibiting anti-HIV microRNA and interferon alpha expression[35]. This important finding will require further investigation using HIV-infected primary macrophages or clinical samples under both acute and chronic alcohol consumption conditions.

The intracellular concentration of drugs is majorly controlled by passive transport, active uptake, and efflux from the cells[31]. EVG is a substrate for CYP3A4, and this drug metabolizing enzyme is present in the monocytic cells[18, 41]. Ethanol alone or when co-administered with EVG and COBI upregulated CYP3A4 protein levels. This observation is consistent with the reported induction of CYP3A4 mRNA and protein in monocytes[18]. Similarly, recently we reported decreased microsomal CYP3A efficiency by ethanol in metabolizing EVG[25]. Of interest, other ART drugs such as PIs are also metabolized by CYP3A4. When purified CYP3A4 enzyme exposed to 20 mM ethanol and PIs at different concentrations, ethanol alters spectral binding as well as inhibitory properties of PIs with the enzyme[42]. In addition to modifying above properties ethanol also decreases the catalytic efficiency of the CYP3A4 to metabolize nelfinavir[43]. These effects of ethanol are in corroboration with other studies that used animal models and in vitro cell lines to demonstrate that in addition to CYP2E1 ethanol markedly influences CYP3A4 [44–46]. Taken together, these results suggest that ethanol can alter CYP3A4 mediated EVG metabolism by modulating CYP3A4 at different points including mRNA, protein or functional level.

Reported evidence indicates that ART intracellular levels are also dependent on MRP1 and MDR1 or other efflux transporters expressed in monocytes[47, 48]. Therefore, we tested the hypothesis that ethanol co-administration with EVG modulates these molecular targets, which in turn, influences intracellular level of EVG. Similarly, we have previously reported upregulation of MRP1 mRNA as a result of ethanol exposure to U937 monocytic cells[18]. Our current observations of induction of MRP1 by ethanol are in agreement with our earlier studies. Besides, a published study has demonstrated induction of CYP3A4 and MDR1 mRNA in human adenocarcinoma LS180 cells by EVG[49]. In contrary, we observed induction of these proteins by EVG only with the combination of ethanol but not by EVG alone. This discrepancy may arise due to comparison of mRNA expression with protein or use of different cell model systems in these studies. For instance, phenotypically different subsets of macrophages M1 and M2 exhibited significant variations in intracellular concentrations of lopinavir with elevated levels of MDR1 expression in M2 type[50]. Regardless, when cells were exposed to MRP1 and MDR1 inhibitors along with EVG, COBI, and ethanol combination, EVG intracellular concentration was slightly increased but no noticeable impact was observed on HIV replication. These results suggest that these efflux transporters may not be solely responsible for ethanol-mediated effects on intracellular EVG concentration.

In conclusion, our findings clearly suggest modulation of intracellular concentration of EVG and HIV-1 replication in HIV-infected U1 monocytic cells, perhaps through drug efflux transporter MRP1 and metabolic enzyme CYP3A4. The findings of this study could be strengthened by providing further evidence of the role of MRP1 and CYP3A4 using functional assays in HIV-infected primary macrophage. Antiretroviral drug-drug interactions can also be led by other CYPs and transporters and ethanol can induce other molecular targets[14] that may indirectly influence EVG concentration in the cells. Overall, the findings from this study suggest that ethanol-drug interactions may have clinically relevant effects on EVG containing ART regimens for HIV treatment. These important in vitro findings should guide the future in vivo or ex vivo drug-drug interaction studies to understand the effects of ethanol on intracellular concentration of this integrase inhibitor.

Supporting information

S1 File. Supporting information that contains original data and/or statistical analysis for Figs 1 to 5.
(XLSX)

Author Contributions

Conceptualization: NNM SK.

Data curation: NNM SK.

Formal analysis: NNM PBL SK.

Funding acquisition: SK.

Investigation: NNM NS PBL.

Methodology: NNM NS PBL.

Project administration: SK BM.

Resources: SK BM.

Supervision: SK BM.

Validation: NNM SK BM.

Visualization: NNM PBL SK BM.

Writing – original draft: NNM.

Writing – review & editing: NNM PBL SK BM.

References

1. Schafer JJ, Squires KE. Integrase inhibitors: a novel class of antiretroviral agents. The Annals of Pharmacotherapy. 2010; 44(1):145–56. doi: 10.1345/aph.1M309 PMID: 20040702

2. Guidelines A. What to Start | Adult and Adolescent ARV Guidelines. AIDSinfo. 2016.

3. Shimura K, Kodama EN. Elvitegravir: a new HIV integrase inhibitor. Antivir Chem Chemother. 2009; 20 (2):79–85. doi: 10.3851/IMP1397 PMID: 19843978

4. Zha W, Zha BS, Zhou F, Zhou H, Wang G. The cellular pharmacokinetics of HIV protease inhibitors: current knowledge and future perspectives. Curr Drug Metab. 2012; 13(8):1174–83. PMID: 22746305

5. Lötsch J, Harder S, Stürmer M, Doerr H-W, Geisslinger G, Staszewski S, et al. Association of saquinavir plasma concentrations with side effects but not with antiretroviral outcome in patients infected with protease inhibitor-susceptible human immunodeficiency virus type 1. Antimicrob Agents Chemother. 2007; 51(9):3264–72. doi: 10.1128/AAC.00036-07 PMID: 17576836

6. Josephson F. Drug-drug interactions in the treatment of HIV infection: focus on pharmacokinetic enhancement through CYP3A inhibition. J Intern Med. 2010; 268(6):530–9. doi: 10.1111/j.1365-2796.2010.02301.x PMID: 21073558

7. Bazzoli C, Jullien V, Le Tiec C, Rey E, Mentre F, Taburet AM. Intracellular Pharmacokinetics of Antiretroviral Drugs in HIV-Infected Patients, and their Correlation with Drug Action. Clin Pharmacokinet. 2010; 49(1):17–45. doi: 10.2165/11318110-000000000-00000 PMID: 20000887

8. Rizk ML, Hang Y, Luo WL, Su J, Zhao J, Campbell H, et al. Pharmacokinetics and pharmacodynamics of once-daily versus twice-daily raltegravir in treatment-naive HIV-infected patients. Antimicrob Agents

Chemother. 2012; 56(6):3101–6. PubMed Central PMCID: PMCPMC3370742. doi: 10.1128/AAC. 06417-11 PMID: 22430964

9. Klibanov OM. Elvitegravir, an oral HIV integrase inhibitor, for the potential treatment of HIV infection. Current Opinion in Investigational Drugs (London, England: 2000). 2009; 10(2):190–200.

10. DeJesus E, Rockstroh JK, Henry K, Molina J-M, Gathe J, Ramanathan S, et al. Co-formulated elvitegravir, cobicistat, emtricitabine, and tenofovir disoproxil fumarate versus ritonavir-boosted atazanavir plus co-formulated emtricitabine and tenofovir disoproxil fumarate for initial treatment of HIV-1 infection: a randomised, double-blind, phase 3, non-inferiority trial. Lancet. 2012; 379(9835):2429–38. doi: 10. 1016/S0140-6736(12)60918-0 PMID: 22748590

11. Minuesa G, Purcet S, Erkizia I, Molina-Arcas M, Bofill M, Izquierdo-Useros N, et al. Expression and functionality of anti-human immunodeficiency virus and anticancer drug uptake transporters in immune cells. J Pharmacol Exp Ther. 2008; 324(2):558–67. doi: 10.1124/jpet.107.131482 PMID: 18042828

12. Köck K, Grube M, Jedlitschky G, Oevermann L, Siegmund W, Ritter CA, et al. Expression of adenosine triphosphate-binding cassette (ABC) drug transporters in peripheral blood cells: relevance for physiology and pharmacotherapy. Clin Pharmacokinet. 2007; 46(6):449–70. doi: 10.2165/00003088-200746060-00001 PMID: 17518506

13. Griffin L, Annaert P, Brouwer KLR. Influence of drug transport proteins on the pharmacokinetics and drug interactions of HIV protease inhibitors. J Pharm Sci. 2011; 100(9):3636–54. doi: 10.1002/jps. 22655 PMID: 21698598

14. 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. doi: 10.1016/j.lfs.2010.09.012 PMID: 20932495

15. Fayet Mello A, Buclin T, Franc C, Colombo S, Cruchon S, Guignard N, et al. Cell disposition of raltegravir and newer antiretrovirals in HIV-infected patients: high inter-individual variability in raltegravir cellular penetration. The Journal of Antimicrobial Chemotherapy. 2011; 66(7):1573–81. doi: 10.1093/jac/dkr151 PMID: 21508009

16. Fletcher CV, Staskus K, Wietgrefe SW, Rothenberger M, Reilly C, Chipman JG, et al. Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues. Proc Natl Acad Sci U S A. 2014; 111(6):2307–12. doi: 10.1073/pnas.1318249111 PMID: 24469825

17. Cederbaum AI. Alcohol metabolism. Clin Liver Dis. 2012; 16(4):667–85. doi: 10.1016/j.cld.2012.08.002 PMID: 23101976

18. Jin M, Arya P, Patel K, Singh B, Silverstein PS, Bhat HK, et al. Effect of alcohol on drug efflux protein and drug metabolic enzymes in U937 macrophages. Alcohol Clin Exp Res. 2011; 35(1):132–9. doi: 10. 1111/j.1530-0277.2010.01330.x PMID: 21039635

19. Jin M, Ande A, Kumar A, Kumar S. Regulation of cytochrome P450 2e1 expression by ethanol: role of oxidative stress-mediated pkc/jnk/sp1 pathway. Cell Death Dis. 2013; 4.

20. Rao PS, Kumar S. Chronic Effects of Ethanol and/or Darunavir/Ritonavir on U937 Monocytic Cells: Regulation of Cytochrome P450 and Antioxidant Enzymes, Oxidative Stress, and Cytotoxicity. Alcohol Clin Exp Res. 2016; 40(1):73–82. PubMed Central PMCID: PMCPMC4700550. doi: 10.1111/acer. 12938 PMID: 26727525

21. McDowell JA, Chittick GE, Stevens CP, Edwards KD, Stein DS. Pharmacokinetic interaction of abacavir (1592U89) and ethanol in human immunodeficiency virus-infected adults. Antimicrob Agents Chemother. 2000; 44(6):1686–90. PMID: 10817729

22. Perno CF, Newcomb FM, Davis DA, Aquaro S, Humphrey RW, Calio R, et al. Relative potency of protease inhibitors in monocytes/macrophages acutely and chronically infected with human immunodeficiency virus. J Infect Dis. 1998; 178(2):413–22. PMID: 9697721

23. Alexaki A, Liu Y, Wigdahl B. Cellular reservoirs of HIV-1 and their role in viral persistence. Curr HIV Res. 2008; 6(5):388–400. PubMed Central PMCID: PMC2683678. PMID: 18855649

24. Kedzierska K, Crowe SM. The role of monocytes and macrophages in the pathogenesis of HIV-1 infection. Curr Med Chem. 2002; 9(21):1893–903. PMID: 12369874

25. Midde NM, Rahman MA, Rathi C, Li J, Meibohm B, Li W, et al. Effect of Ethanol on the Metabolic Characteristics of HIV-1 Integrase Inhibitor Elvitegravir and Elvitegravir/Cobicistat with CYP3A: An Analysis Using a Newly Developed LC-MS/MS Method. PLoS One. 2016; 11(2):e0149225. PubMed Central PMCID: PMCPMC4752462. doi: 10.1371/journal.pone.0149225 PMID: 26872388

26. Egorina EM, Sovershaev MA, Osterud B. In-cell Western assay: a new approach to visualize tissue factor in human monocytes. J Thromb Haemost. 2006; 4(3):614–20. doi: 10.1111/j.1538-7836.2005. 01781.x PMID: 16371116

27. Hughes AJ, Spelke DP, Xu Z, Kang CC, Schaffer DV, Herr AE. Single-cell western blotting. Nat Methods. 2014; 11(7):749–55. PubMed Central PMCID: PMCPMC4077215. doi: 10.1038/nmeth.2992 PMID: 24880876

28. McCance-Katz EF, Gruber VA, Beatty G, Lum PJ, Rainey PM. Interactions between alcohol and the antiretroviral medications ritonavir or efavirenz. J Addict Med. 2013; 7(4):264–70. doi: 10.1097/ADM. 0b013e318293655a PMID: 23666322

29. Gruber VA, Rainey PM, Lum PJ, Beatty GW, Aweeka FT, McCance-Katz EF. Interactions between alcohol and the HIV entry inhibitor Maraviroc. Journal of the International Association of Providers of AIDS Care. 2013; 12(6):375–7. doi: 10.1177/2325957413495567 PMID: 23881910

30. Chan L-N, Anderson GD. Pharmacokinetic and pharmacodynamic drug interactions with ethanol (alcohol). Clin Pharmacokinet. 2014; 53(12):1115–36. doi: 10.1007/s40262-014-0190-x PMID: 25267448

31. Zhou F, Zhang J, Li P, Niu F, Wu X, Wang G, et al. Toward a new age of cellular pharmacokinetics in drug discovery. Drug Metab Rev. 2011; 43(3):335–45. doi: 10.3109/03602532.2011.560607 PMID: 21395404

32. Koh Y, Haim H, Engelman A. Identification and characterization of persistent intracellular human immunodeficiency virus type 1 integrase strand transfer inhibitor activity. Antimicrob Agents Chemother. 2011; 55(1):42–9. PubMed Central PMCID: PMCPMC3019619. doi: 10.1128/AAC.01064-10 PMID: 21060108

33. Massud I, Martin A, Dinh C, Mitchell J, Jenkins L, Heneine W, et al. Pharmacokinetic profile of raltegravir, elvitegravir and dolutegravir in plasma and mucosal secretions in rhesus macaques. J Antimicrob Chemother. 2015; 70(5):1473–81. PubMed Central PMCID: PMCPMC4398473. doi: 10.1093/jac/dku556 PMID: 25630643

34. Avery LB, Zarr MA, Bakshi RP, Siliciano RF, Hendrix CW. Increasing extracellular protein concentration reduces intracellular antiretroviral drug concentration and antiviral effect. AIDS Res Hum Retroviruses. 2013; 29(11):1434–42. PubMed Central PMCID: PMCPMC3809607. doi: 10.1089/AID.2013.0031 PMID: 23601085

35. Mastrogiannis DS, Wang X, Dai M, Li J, Wang Y, Zhou Y, et al. Alcohol enhances HIV infection of cord blood monocyte-derived macrophages. Curr HIV Res. 2014; 12(4):301–8. PubMed Central PMCID: PMCPMC4153785. PMID: 25053361

36. Agudelo M, Figueroa G, Yndart A, Casteleiro G, Munoz K, Samikkannu T, et al. Alcohol and Cannabinoids Differentially Affect HIV Infection and Function of Human Monocyte-Derived Dendritic Cells (MDDC). Front Microbiol. 2015; 6:1452. PubMed Central PMCID: PMCPMC4686798. doi: 10.3389/fmicb.2015.01452 PMID: 26733986

37. Bagasra O, Kajdacsy-Balla A, Lischner HW, Pomerantz RJ. Alcohol intake increases human immunodeficiency virus type 1 replication in human peripheral blood mononuclear cells. J Infect Dis. 1993; 167 (4):789–97. PMID: 8450242

38. DeJesus E, Berger D, Markowitz M, Cohen C, Hawkins T, Ruane P, et al. Antiviral activity, pharmacokinetics, and dose response of the HIV-1 integrase inhibitor GS-9137 (JTK-303) in treatment-naive and treatment-experienced patients. J Acquir Immune Defic Syndr. 2006; 43(1):1–5. doi: 10.1097/01.qai. 0000233308.82860.2f PMID: 16936557

39. Canducci F, Ceresola ER, Saita D, Castagna A, Gianotti N, Underwood M, et al. In vitro phenotypes to elvitegravir and dolutegravir in primary macrophages and lymphocytes of clonal recombinant viral variants selected in patients failing raltegravir. J Antimicrob Chemother. 2013; 68(11):2525–32. doi: 10. 1093/jac/dkt220 PMID: 23798668

40. Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science (New York, NY). 1987; 238(4828):800–2.

41. Nagai F, Hiyoshi Y, Sugimachi K, Tamura HO. Cytochrome P450 (CYP) expression in human myeloblastic and lymphoid cell lines. Biol Pharm Bull. 2002; 25(3):383–5. PMID: 11913539

42. Kumar S, Kumar A. Differential effects of ethanol on spectral binding and inhibition of cytochrome P450 3A4 with eight protease inhibitors antiretroviral drugs. Alcohol Clin Exp Res. 2011; 35(12):2121–7. doi: 10.1111/j.1530-0277.2011.01575.x PMID: 21682753

43. 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. doi: 10.1016/j.bbrc.2010.10.014 PMID: 20937259

44. Feierman DE, Melinkov Z, Nanji AA. Induction of CYP3A by ethanol in multiple in vitro and in vivo models. Alcohol Clin Exp Res. 2003; 27(6):981–8. doi: 10.1097/01.ALC.0000071738.53337.F4 PMID: 12824820

45. Kostrubsky VE, Strom SC, Wood SG, Wrighton SA, Sinclair PR, Sinclair JF. Ethanol and isopentanol increase CYP3A and CYP2E in primary cultures of human hepatocytes. Arch Biochem Biophys. 1995; 322(2):516–20. doi: 10.1006/abbi.1995.1495 PMID: 7574728

46. Roberts BJ, Shoaf SE, Song BJ. Rapid changes in cytochrome P4502E1 (CYP2E1) activity and other P450 isozymes following ethanol withdrawal in rats. Biochem Pharmacol. 1995; 49(11):1665–73. PMID: 7786308

47. Giraud C, Manceau S, Decleves X, Goffinet F, Morini JP, Chappuy H, et al. Influence of development, HIV infection, and antiretroviral therapies on the gene expression profiles of ABC transporters in human lymphocytes. J Clin Pharmacol. 2010; 50(2):226–30. doi: 10.1177/0091270009343696 PMID: 19837908

48. Turriziani O, Gianotti N, Falasca F, Boni A, Vestri AR, Zoccoli A, et al. Expression levels of MDR1, MRP1, MRP4, and MRP5 in peripheral blood mononuclear cells from HIV infected patients failing anti-retroviral therapy. J Med Virol. 2008; 80(5):766–71. doi: 10.1002/jmv.21152 PMID: 18360888

49. Zembruski NCL, Büchel G, Jödicke L, Herzog M, Haefeli WE, Weiss J. Potential of novel antiretrovirals to modulate expression and function of drug transporters in vitro. J Antimicrob Chemother. 2011: dkq501.

50. Cory TJ, He H, Winchester LC, Kumar S, Fletcher CV. Alterations in P-Glycoprotein Expression and Function Between Macrophage Subsets. Pharm Res. 2016.