

## Short Communication

# Glucuronidation of the Antiretroviral Drug Efavirenz by UGT2B7 and an in Vitro Investigation of Drug-Drug Interaction with Zidovudine<sup>S</sup>

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### ABSTRACT:

The non-nucleoside reverse transcriptase inhibitor efavirenz (EFV) is directly conjugated by the UDP-glucuronosyltransferase (UGT) pathway to form EFV-*N*-glucuronide (EFV-G), but the enzyme(s) involved has not yet been identified. The glucuronidation of EFV was screened with UGT1A and UGT2B enzymes expressed in a heterologous system, and UGT2B7 was shown to be the only reactive enzyme. The apparent  $K_m$  value of UGT2B7 (21  $\mu\text{M}$ ) is similar to the value observed for human liver microsomes (24  $\mu\text{M}$ ), whereas the variant allozyme UGT2B7\*2 (Tyr<sup>268</sup>) displayed similar kinetic parameters. Because 3'-azido-3'-deoxythymidine (AZT), one of the most current nucleoside reverse transcriptase inhibitors prescribed in combination with EFV, is also conjugated by UGT2B7, the potential metabolic interaction between EFV and AZT

has been studied using human liver microsomes. Glucuronidation of both drugs was inhibited by one another, in a concentration-dependent manner. At  $K_m$  values (25 and 1000  $\mu\text{M}$  for EFV and AZT, respectively), EFV inhibited AZT glucuronidation by 47%, whereas AZT inhibited EFV glucuronidation by 23%. With a  $K_i$  value of 17  $\mu\text{M}$  for AZT-glucuronide formation, EFV appears to be one of the most selective and potent competitive inhibitor of AZT glucuronidation in vitro. Moreover, assuming that concentrations of EFV achieved in plasma ( $C_{\text{max}} = 12.9 \mu\text{M}$ ) are in a range similar to its  $K_i$  value, it was estimated that EFV could produce a theoretical 43% inhibition of AZT glucuronidation in vivo. We conclude that UGT2B7 has a major role in EFV glucuronidation and that EFV could potentially interfere with the hepatic glucuronidation of AZT.

Efavirenz [(EFV) (Sustiva); Bristol-Myers Squibb Co., Princeton, NJ] is the preferred non-nucleoside reverse transcriptase inhibitor (NNRTI) agent for initial therapy for HIV infection. A typical antiretroviral treatment regimen consists of two nucleoside reverse transcriptase inhibitors (NRTIs) plus a protease inhibitor or an NNRTI. The 2008 IAS-USA guidelines recommend either of two basic three-drug regimens for treatment of antiretroviral-naïve patients: 1) EFV plus two NRTIs; or 2) a ritonavir-boosted protease inhibitor (lopinavir, atazanavir, fosamprenavir, darunavir, or saquinavir) plus two NRTIs [tenofovir and emtricitabine; abacavir and lamivudine; or 3'-azido-3'-deoxythymidine (zidovudine; AZT) and lamivudine] (Hammer et al., 2008). A recent study on HIV-infected adults of Southern Africa further revealed that among patients who began nevirapine-based or EFV-based antiretroviral therapy between January 1998 and September 2004, 1321 of 1822 EFV-treated patients

(72.5%) received it in combination with the NRTI pair AZT plus lamivudine (Nachegea et al., 2008). Thus, a significant proportion of HIV-infected, highly active antiretroviral therapy-naïve adults receive EFV and AZT in combination.

A large interpatient variability was found to affect EFV bioavailability (coefficient of variation up to 118%) (Marzolini et al., 2001), which might be explained in part by its extensive metabolism. Primary hydroxylation of EFV by cytochromes CYP2B6 and CYP3A4 at position 8-OH and 7-OH, respectively, leads to the formation of inactive oxidized metabolites, which subsequently undergo conjugation by the UDP-glucuronosyltransferase (UGT) pathway (Mutlib et al., 1999; Ward et al., 2003). EFV is also directly conjugated to form EFV-*N*-glucuronide (EFV-G), which was present in urine of several species, including humans (Mutlib et al., 1999). EFV-G is a significant metabolite in urine of humans and animals given the first dose, and in bile duct-cannulated and noncannulated rats, the EFV-G was clearly seen as the major metabolite in urine and bile at early time points of collection (day 1, 0–4 h). After multiple dosing, the 8-OH-EFV-G became the predominant metabolite. No data clearly specify the contribution of glucuronidation to EFV metabolism, and the implication of variations in the glucuronidation pathway remains unknown. Furthermore, 8-OH-EFV represents >90% of the EFV-hydroxylated metabolites, and CYP2B6 alleles explain to a large extent the observed interindividual variability in EFV exposure (Ward et al., 2003). Moreover, CYP2B6 loss-of-function alleles were associated with a decrease in EFV-hydroxylated metabolites, but also with an increased levels of EFV-G, likely indicating the redirection of EFV

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**ABBREVIATIONS:** EFV, efavirenz; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; AZT, zidovudine; UGT, UDP-glucuronosyltransferase; EFV-G, EFV-*N*-glucuronide; AZT-G, AZT-glucuronide; HLM, human liver microsomes.

metabolism through the glucuronidation pathway (di Iulio et al., 2009). The enzyme(s) involved in *N*-glucuronidation pathway of EFV has not yet been characterized.

AZT, one of the most common NRTI prescribed in combination with EFV, is also predominantly metabolized by UGTs, to an inactive 5'-*O*-glucuronide metabolite (AZT-glucuronide; AZT-G). In humans, this enzymatic reaction is catalyzed exclusively by UGT2B7 (Barbier et al., 2000). The assessment of potential drug interactions at the metabolism level thus becomes essential considering that EFV and AZT are frequently used in combination and that they both undergo glucuronidation.

The initial objective of our study was to identify the UGT enzyme(s) involved in the metabolism of the active parent drug EFV. Data indicate that UGT2B7 is the only UGT isoform tested producing EFV-G in vitro, displaying glucuronidation activity similar to that observed with human liver microsomes (HLM). Accordingly, we then sought to investigate potential interaction with AZT, primarily in HLM. Results indicate that EFV could potentially interfere with hepatic glucuronidation of AZT.

### Materials and Methods

**Chemicals and Reagents.** AZT (3'-azido-3'-deoxythymidine) and  $\beta$ -glucuronidase from *Escherichia coli* Type VII were obtained from Sigma-Aldrich (St. Louis, MO), and EFV [(*S*)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2*H*-3,1-benzoxazin-2-one] was obtained from NIH AIDS Research and Reference Reagent Program (Germantown, MD). AZT-G, AZT-d4-G, and EFV-d4 were purchased from Toronto Research Chemical Inc. (Toronto, Canada). EFV-G and EFV-d4-G (racemic) were produced from in vitro enzymatic assays, purified on Stata X cartridges (60 mg; Phenomenex, Torrance, CA), and quantified on an EFV calibration curve following  $\beta$ -glucuronidase (25,000 units/ml) treatment.

**High-Performance Liquid Chromatography Tandem Mass Spectrometry.** The separation of EFV and AZT was performed onto a Gemini C18 column 3  $\mu$ M packing material, 100  $\times$  4.6 mm (Phenomenex). Isocratic condition with 90% methanol/10% water/0.5 mM ammonium formate with a flow rate of 0.9 ml/min was used to elute AZT-G, and for EFV-G the isocratic condition was 80% methanol/20% water/0.4 mM ammonium formate with a flow rate of 0.9 ml/min. The glucuronides were quantified by tandem mass spectrometry (model API 3200; Biosystems-Sciex, Concord, ON, Canada). Negative-product multiple reaction monitoring ions pairs were *m/z* 442  $\rightarrow$  124.9 for AZT-G, *m/z* 445  $\rightarrow$  127.9 for AZT-d3-G, *m/z* 489.7  $\rightarrow$  314.1 for EFV-G, and *m/z* 493.7  $\rightarrow$  318.1 for EFV-rac-d4-G. The ion source temperature was set at 550°C. The ion spray voltage, declustering potential, and entrance potential were set at -4200, -50, and -10 V. The collision energy for AZT-G and EFV-G were -30 and -25 V, respectively.

**Enzymatic and Competition Assays.** Human pooled liver microsomes (BD Biosciences, Woburn, MA) and recombinant UGT1A- and UGT2B-HEK293 microsomal proteins were used in enzymatic assays using a standard procedure (Villeneuve et al., 2003). Initial screening consisted of 16-h incubation at 37°C with 0.2 mM EFV or AZT. Kinetic parameters were then assessed for HLM as well as UGT2B7\*1 and \*2 allelic variants in the presence of increasing concentrations of EFV (varying from 1 to 75  $\mu$ M) or AZT (ranging from 100 to 5000  $\mu$ M) for 1-h incubation at 37°C. Absolute glucuronidation activities were corrected by UGT relative protein content and expressed as relative glucuronidation activities (pmol/min/mg). Eadie-Hofstee plots (*V* as a function of *V*/*S*) confirmed by visual inspection of fitted functions (*V* as a function of [*S*]) were used to select the best-fit enzyme kinetic model. Kinetic parameter calculations were performed with Sigma Plot 8.0 software assisted by Enzyme Kinetics 1.1 software (SPSS Inc., Chicago, IL). Inhibition constants ( $K_i$ ) were assessed using AZT and EFV concentrations ranging from 100 to 5000  $\mu$ M and 5 to 50  $\mu$ M, respectively.  $K_i$  values were estimated using Dixon plots and by fitting the kinetic data into a competitive inhibition model using nonlinear regression analysis with the GraphPad software version 4.01 (GraphPad Software, Inc., San Diego, CA):  $v = V_{\max} [S]/([S] + K_m/(1 + 1/K_i))$ . Values are expressed as the mean of at least two experiments performed in triplicate. Results were expressed as mean  $\pm$  S.D.

TABLE 1

Kinetic parameters for the glucuronidation of EFV and AZT by UGT2B7 and human liver microsomes

Relative  $V_{\max}$  means that velocities were normalized for UGT protein content. Results are expressed as mean  $\pm$  S.D. of two independent experiments done in triplicate.

Microsomes		Apparent $K_m$	Relative $V_{\max}$	$V_{\max}/K_m$
		$\mu$ M	pmol/min/mg	$\mu$ l/min/mg
EFV	UGT2B7			
	*1 (His <sup>268</sup> )	20.7 $\pm$ 2.3	1.6 $\pm$ 0.1	0.08 $\pm$ 0.01
	*2 (Tyr <sup>268</sup> )	16.1 $\pm$ 5.8	1.5 $\pm$ 0.4	0.09 $\pm$ 0.03
	Human liver	24.0 $\pm$ 3.3	7.8 $\pm$ 0.8	0.32 $\pm$ 0.04
AZT	UGT2B7			
	*1 (His <sup>268</sup> )	502 $\pm$ 65	221 $\pm$ 20	0.44 $\pm$ 0.06
	*2 (Tyr <sup>268</sup> )	719 $\pm$ 90	135 $\pm$ 24*	0.19 $\pm$ 0.01
	Human liver	1018 $\pm$ 70	1541 $\pm$ 130	1.52 $\pm$ 0.17

\* *P* values <0.05 vs. UGT2B7\*1.

Statistical significance was calculated by paired Student's *t* test. All tests were two sided.

**Prediction of the AZT-EFV Interaction.** The evaluation of the theoretical percent inhibition achievable in vivo at pharmacological concentrations (peak plasma levels) of EFV and AZT was predicted using the equation:  $i = 100 [I]/(K_i (1 + [S]/K_i) + [I])$  (Resetar et al., 1991). In this equation, *i* = percent inhibition, [*I*] = EFV and AZT at pharmacological steady-state concentration achieved in plasma, respectively. The extent of inhibition of AZT hepatic clearance by EFV was predicted based on the increase in the AUC ratio of AZT (AUC<sub>(+EFV)</sub>/AUC<sub>(control)</sub>) caused by the presence of EFV, using the equation: AUC<sub>(+EFV)</sub>/AUC<sub>(control)</sub> = 1/[( $f_m/(1 + [I]/K_i) + 1 - f_m$ ), where [*I*] is the steady-state concentration of EFV in vivo,  $f_m$  is the fraction of AZT metabolism via glucuronidation in the liver, and  $K_i$  is the inhibition constant for EFV generated in vitro (Ito et al., 1998).

### Results and Discussion

**Glucuronidation of EFV and AZT by Heterologous UGTs: A Main Role for UGT2B7.** Assays with HLM demonstrated the formation of a single glucuronic acid conjugate for both drugs, EFV-G and AZT-G. Incubations with recombinant human UGT1A and UGT2B enzymes revealed that UGT2B7 is the only heterologous enzyme capable of producing EFV-G and AZT-G (Supplemental Fig. S1). The recombinant human UGT1A3, UGT1A8, UGT1A9, and UGT1A10 are also able to form EFV-G but with an apparent velocity at least 48-fold lower than UGT2B7. Hence, these data provide the first experimental evidence for a major role of UGT2B7 in EFV glucuronidation.

Kinetic analyses were then performed to compare human liver and UGT2B7 microsomes for the glucuronidation of EFV and AZT, and all kinetics were well described by the Michaelis-Menten equation (Table 1; Supplemental Fig. S2). Apparent  $K_m$  values for UGT2B7 and HLM are similar for EFV-G formation (24 and 21  $\mu$ M for HLM and UGT2B7, respectively), which emphasizes the importance of UGT2B7 in EFV glucuronidation. The variants UGT2B7\*1 (His<sup>268</sup>) and UGT2B7\*2 (Tyr<sup>268</sup>) also displayed similar  $K_m$  values, and we observed no statistically different apparent velocities when normalized by UGT protein content. The codon 268 polymorphism was already shown not to have a functional significance for other drugs such as morphine and epirubicin (Coffman et al., 1998; Innocenti et al., 2001). Whether other coding polymorphisms result in impaired catalytic activity toward EFV is still unknown, whereas several promoter variants were reported to alter expression of UGT2B7 (Duguay et al., 2004). Kinetics for AZT were comparable to previous reports (Barbier et al., 2000; Uchaipichat et al., 2008), and a 2.3-fold reduced apparent velocity of AZT-G formation was observed for UGT2B7\*2 compared with UGT2B7\*1 (Supplemental Fig. S2), in accordance with the previous study of Barbier et al. (2000).

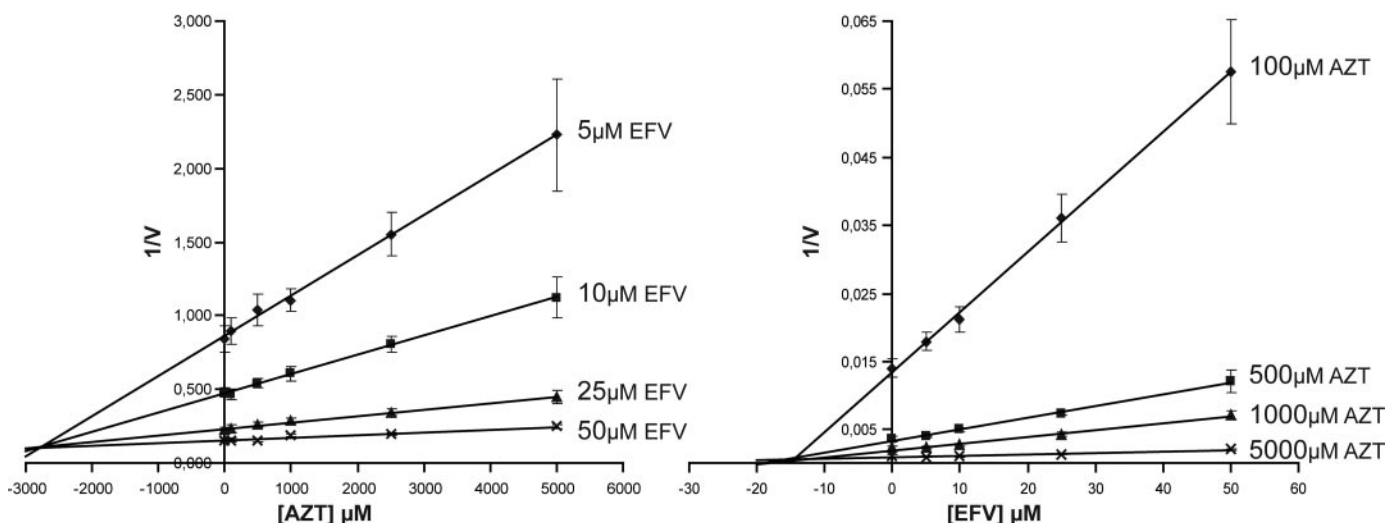
**A Inhibition of EFV glucuronidation by AZT****B Inhibition of AZT glucuronidation by EFV**

FIG. 1. Representative Dixon plots for the inhibition of efavirenz glucuronidation (A) and zidovudine glucuronidation (B) by the alternate substrate catalyzed by human liver microsomes.

**Evidence of a Potential EFV-AZT Interaction.** Because UGT2B7 is primarily involved in conjugation of EFV and AZT, frequently coadministered in HIV patients, we investigated whether these drugs may interfere with each other's conjugation in HLM. A marked decrease in the AZT-G formation by EFV was shown to be dose dependent. At 5000  $\mu\text{M}$  AZT, the mean of AZT glucuronidation was reduced to 21% of the control rate by 25  $\mu\text{M}$  EFV, and this reduction reached 57% for 100  $\mu\text{M}$  AZT. At  $K_m$  values of both drugs (25 and 1000  $\mu\text{M}$  for EFV and AZT, respectively), EFV inhibited AZT glucuronidation by 47%, whereas AZT inhibited EFV glucuronidation by 23%. The apparent  $K_i$  values estimated from Dixon plots were 2700  $\mu\text{M}$  for inhibition of EFV glucuronidation by AZT and 17  $\mu\text{M}$  for inhibition of AZT glucuronidation by EFV (Fig. 1). Using the simultaneous nonlinear regression method, a  $K_i$  of  $3902 \pm 211$   $\mu\text{M}$  was derived for AZT on EFV glucuronidation and of  $16.8 \pm 1.2$   $\mu\text{M}$  for EFV on AZT glucuronidation, which is consistent with the  $K_m$  values independently determined from the competition study. For the inhibition of AZT upon EFV-G,  $K_i > K_m$  indicate that the affinity of UGT2B7 for EFV is greater than for AZT, in accordance with the kinetic described above. It is noteworthy that previous  $K_i$  values for AZT glucuronidation inhibition ranged from 38  $\mu\text{M}$  [ethinylestradiol (Herber et al., 1992)] to 47 000  $\mu\text{M}$  [sulfisoxazole (Resetar et al., 1991)]. Moreover, kinetic experiments in the absence of bovine serum albumin as performed herein, overestimate  $K_m$  and underestimate true  $K_i$ , particularly for substrates of UGT2B7 (Rowland et al., 2007). Based on these observations, EFV would then appear to be in the lowest range of competitive inhibitors for AZT glucuronidation in vitro.

At steady-state, after an AZT oral dose of 200 mg twice daily, the maximum concentration achieved in plasma is approximately 1.2  $\mu\text{g}/\text{ml}$  (4.5  $\mu\text{M}$ ) with a relatively short half-life (0.5–3 h) (Hoetelmans, 1999). For EFV, oral administration of 600 mg daily has been reported to produce mean steady-state  $C_{\text{max}}$  of 12.9  $\mu\text{M}$ , drug concentrations ranging from 125 to 15,230  $\mu\text{g}/\text{l}$  (median 2188  $\mu\text{g}/\text{l}$ , 0.4–48  $\mu\text{M}$ ), and a half-life of >40 h (Marzolini et al., 2001). Because AZT plasma concentration is far less than its  $K_i$  value of 3902  $\mu\text{M}$ , an interaction on EFV glucuronidation in the presence of AZT is unlikely. Conversely, considering that the pharmacological drug concentration of EFV and its apparent  $K_i$  (16.8  $\mu\text{M}$ ; assessed

herein) are in the same range (ratio  $[I]/K_i$  value of 0.77) (Tucker et al., 2001), an inhibition of EFV on UGT2B7-mediated AZT glucuronidation is possible, but it remains to be demonstrated in vivo. According to in vitro data, we estimated that the competitive inhibitor EFV could reduce AZT glucuronidation by approximately 43% at steady-state maximum concentrations (see *Materials and Methods*). The magnitude of the potential EFV inhibition of AZT hepatic clearance was predicted based on the increase in the AUC ratio at the maximum inhibitor concentration in plasma ( $[\text{EFV}]_{\text{max}} = 12.9$   $\mu\text{M}$ ) and average inhibitor concentration in plasma ( $[\text{EFV}]_{\text{min}} = 5.6$   $\mu\text{M}$  and  $[\text{EFV}]_{\text{max}} = 12.9$   $\mu\text{M}$ , so  $[\text{EFV}]_{\text{ave}} = (5.6 + 12.9)/2 = 9.25$   $\mu\text{M}$ ) (EFV). Because glucuronidation is mainly responsible for hepatic clearance of AZT,  $f_m$  (the fraction of the metabolic process subject to inhibition) was taken as 1. The predicted in vivo AUC ratio of AZT is 1.75 at  $[\text{EFV}]_{\text{max}}$  and 1.54 at  $[\text{EFV}]_{\text{ave}}$ , meaning that EFV could perhaps increase AZT AUC upon concurrent use. The maximum unbound EFV (inhibitor) concentration in plasma was also used to predict the magnitude of the interaction between EFV and AZT in vivo. However, because EFV is highly bound (approximately 99.5–99.75%) to human plasma proteins, only calculations that incorporated the total EFV concentration predicted a clinically significant interaction. This prediction is only a rough estimate of the potential drug interactions in vivo given that several factors might influence drug interaction in this context, such as the presence of compensatory pathways, extrahepatic metabolism, or extensive binding to plasma proteins. Drugs that are at the greatest risk of having a high (>5)  $\text{AUC}_i/\text{AUC}$  ratio are high extraction drugs metabolized by a single enzyme, simultaneously presented to the liver along with an inhibitor in which the ratio of liver concentration ( $[I]$ ) to potency of inhibition ( $K_i$ ) is high (Williams et al., 2004). AZT is a good example, with a high hepatic extraction and a metabolism catalyzed specifically by UGT2B7. Therefore, the potential for a high  $\text{AUC}_i/\text{AUC}$  ratio would be elevated, given a high  $I/K_i$  ratio of a coadministered inhibitor. We showed that EFV has a strong in vitro inhibitory effect upon UGT2B7-mediated AZT glucuronidation, making the EFV-AZT interaction possible. Moreover, in spite of the high glucuronidation capacity of UGT2B7, there is literature evidence that shows that the glucuronidation activity of this enzyme can be limited by drug-drug interaction. An example is the observed 2-fold increase in AUC of AZT with concomitant valproic



acid administration (Lertora et al., 1994). Moreover, an increase of 225% in the AZT drug plasma  $C_{\min}$  was reported with the coadministration of EFV (U.S. prescribing information, [http://packageinserts.bms.com/pi/pi\\_sustiva.pdf](http://packageinserts.bms.com/pi/pi_sustiva.pdf)).

To the best of our knowledge, the clinical significance of variation in the EFV glucuronidation pathway has not yet been studied. Conversely, the importance of polymorphisms in the gene that encodes the hepatic enzyme primarily responsible for EFV oxidation (CYP2B6) has been the subject of intense research (for a review, see Rodriguez-Novoa et al., 2006). As an example, a study observed that carriers of a CYP2B6 polymorphism (the variant G516T) have greater plasma EFV exposure during the first 24 weeks of antiretroviral therapy, and they experienced frequent central nervous system-related side effects during the first week of treatment (Rodriguez-Novoa et al., 2005). Moreover, a more recent investigation also concludes that dosage adjustment in accordance with the type of polymorphism (CYP2B6, CYP2A6, or CYP3A4) is required to maintain EFV within the therapeutic target levels (Arab-Alameddine et al., 2009). In addition to cytochrome P450s, other polymorphic enzymes such as UGT2B7 may contribute to interindividual variability of EFV plasma levels.

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