In Vivo Profiling and Distribution of Known and Novel Phase I and Phase II Metabolites of Efavirenz in Plasma, Urine, and Cerebrospinal Fluid

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ABSTRACT

Efavirenz (EFV) is principally metabolized by CYP2B6 to 8-hydroxy-efavirenz (8OH-EFV) and to a lesser extent by CYP2A6 to 7-hydroxy-efavirenz (7OH-EFV). So far, most metabolite profile analyses have been restricted to 8OH-EFV, 7OH-EFV, and EFV-glucuronide, even though some metabolites represent a minor percentage of EFV metabolites present in vivo. We have performed a quantitative phase I and II metabolite profile analysis by tandem mass spectrometry of plasma, cerebrospinal fluid (CSF), and urine samples in 71 human immunodeficiency virus patients taking efavirenz, prior to and after enzymatic (glucuronidase and sulfatase) hydrolysis. We have shown that phase II metabolites constitute the major part of the known circulating efavirenz species in humans. The 8OH-EFV-glucuronide (gln) and 8OH-EFV-sulfate (identified for the first time) in humans were found to be 64- and 7-fold higher than the parent 8OH-EFV, respectively. In individuals \( n = 67 \) genotyped for CYP2B6, 2A6, and CYP3A metabolic pathways, 8OH-EFV/EFV ratios in plasma were an index of CYP2B6 phenotypic activity \((P < 0.0001)\), which was also reflected by phase II metabolites 8OH-EFV-glucuronide/EFV and 8OH-EFV-sulfate/EFV ratios. Neither EFV nor 8OH-EFV, nor any other considered metabolites in plasma were associated with an increased risk of central nervous system (CNS) toxicity. In CSF, 8OH-EFV levels were not influenced by CYP2B6 genotypes and did not predict CNS toxicity. The phase II metabolites 8OH-EFV-gln, 8OH-EFV-sulfate, and 7OH-EFV-gln were present in CSF at 2- to 9-fold higher concentrations than 8OH-EFV. The potential contribution of known and previously unreported EFV metabolites in CSF to the neuropsychological effects of efavirenz needs to be further examined in larger cohort studies.

Introduction

Efavirenz (EFV) (Fig. 1A) presents a large interindividual pharmacokinetic variability that can be attributed to differences in its metabolism (Csajka et al., 2003). A significant percentage of individuals under the current standard EFV dosage (600 mg once daily) experience neuropsychological toxicity, whereas a reduced dosage of 400 mg once daily has been recently shown to be equally efficacious with a reportedly more favorable tolerance profile (Puls et al., 2014). EFV tolerability is a major concern and, in fact, represents the main reason for premature antiretroviral treatment discontinuation (Lubomirov et al., 2011). Several studies have investigated the relationships between elevated EFV concentrations, genetic background, and neuropsychological effects, which have yielded controversial results as some associations have been found in some but not all studies (Marzolini et al., 2001; Gutierrez et al., 2005; Read et al., 2009; van Luin et al., 2009). In view of the conflicting results regarding the mechanism of EFV toxicity, Tovar-y-Romo et al. (2012) investigated, in vitro, the impact of EFV and two primary oxidized EFV metabolites on the function of primary neurone cultures, and found that the metabolite 8-hydroxy-efavirenz (8OH-EFV) (Fig. 1B) is a potent neurotoxin in vitro at concentrations 1 order of magnitude lower than those for EFV and 7-hydroxy-efavirenz (7OH-EFV). However, since this finding has as yet

ABBREVIATIONS: CNS, central nervous system; CSF, cerebrospinal fluid; 8,14-di-OH-EFV, 8,14-dihydroxy-efavirenz; DOF, decrease of function; D-SL, D-saccaric acid 1,4-lactone; EFV, efavirenz; ESI, electrospray ionization; gln, glucuronide; HR, high resolution; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LOF, loss of function; Mo-6-gln, morphine-6-glucuronide; MS, mass spectrometer; MW, molecular weight; 7OH-EFV, 7-hydroxy-efavirenz; 8OH-EFV, 8-hydroxy-efavirenz; RT, retention time; SHCS, Swiss HIV Cohort Study; TDM, therapeutic drug monitoring.
not been followed by a formal pharmacokinetic/dynamic analysis in patients, it remains unclear whether 8-hydroxy-efavirenz only can explain in vivo the neuropsychological disorders experienced by some patients on EFV.

In fact, the EFV metabolic pathway is complex, with different enzymes involved. EFV has three primary metabolites. It is mainly metabolized by CYP2B6 to 8OH-EFV, to a lesser extent by CYP2A6 to 7OH-EFV (Fig. 1C), and directly conjugated by UGT2B7 (Belanger et al., 2009; Kwara et al., 2009; Cho et al., 2011) to efavirenz N-glucuronide (EFV-N-gln) (Fig. 1D). All primary metabolites are detected in humans, although the abundance of EFV-N-gln in plasma is negligible, and the contribution of the N-glucuronidation pathway to the overall clearance of EFV appears to be minimal (Mutlib et al., 1999b; Kwara et al., 2009).

EFV also has secondary metabolites. The 8OH-EFV and 7OH-EFV are subject to phase II metabolism via glucuronidation and/or sulfation. The secondary metabolites 8OH-EFV-gln (Fig. 1E), 7OH-EFV-gln (Fig. 1F), and 7OH-EFV-sulfate (Fig. 1I) have been identified in humans. However, the 8OH-EFV-sulfate (Fig. 1H) has only been detected in urine samples from rats and cynomolgus monkeys (Mutlib et al., 1999b). 8OH-EFV can also undergo subsequent metabolism to 8,14-dihydroxy-efavirenz (8,14-di-OH-EFV) (Fig. 1G). The 8,14-di-OH-EFV has been barely detected in plasma (Avery et al., 2013). Mixed sulfate-glutathione diconjugates of EFV have also been detected in humans (Mutlib et al., 1999a).

Genetic variability in the main (CYP2B6) and accessory EFV metabolic pathways (CYP2A6, CYP3A4, and UGT2B7) can explain the differences in the metabolite profiles (Rotger et al., 2007; di Iulio et al., 2009). Overall, the majority of the studies so far have been restricted to the phase I metabolites (Cho et al., 2011; Avery et al., 2013) even though, as we will show, these metabolites are present in vivo at much lower levels than phase II metabolites.

Our hypothesis is that some metabolites, instead of EFV itself, may be responsible for the neuropsychological toxicity associated with EFV treatment. Therefore, in our studies, we investigated not only the phase I but also the major phase II metabolites that may also represent relevant phenotypic markers of the possible mechanism of this toxicity. Herein, we performed a quantitative phase I and II metabolites profile analysis in plasma, cerebrospinal fluid (CSF), and urine samples from individuals under EFV therapy. We correlated the differences in the metabolite profiles with genetic variants in CYP2B6 and CYP2A6, and examined the association between the concentrations of phase I and phase II EFV metabolites with both EFV treatment discontinuation and neuropsychological toxicity.

Materials and Methods

Study Population. A total of 71 plasma samples, 10 CSF samples, and 8 urine samples from 71 participants were obtained from four study population subsets:

- Swiss HIV Cohort Study (SHCS) (Schoeni-Affolter F et al., 2010) participants under EFV included in our previous study to evaluate the association of pharmacogenetic markers with time to treatment discontinuation during the first year of antiretroviral therapy. A total of 39 plasma samples were selected according to their genotype (Lubomirov et al., 2011).
- SHCS participants who presented extremely high EFV plasma levels during routine therapeutic drug monitoring (TDM) (n = 13 plasma samples).
- SHCS participants included in a previous study to evaluate the activity of monotherapy in the central nervous system (Monotherapy Switzerland/Thailand study (MOST) study) (Gutmann et al., 2010) who were receiving EFV during the control period of the trial (n = 9 plasma and n = 9 CSF samples). Among the CSF and plasma samples collected, six paired CSF-plasma samples were analyzed.
- During the routine medical follow-up, eight SHCS patients, under stable EFV regimen (600 mg once a day), agreed to donate a urine spot at the same time a TDM blood sample was collected during their usual medical follow-up visit (n = 8 urine and n = 8 plasma samples). These patients had normal hepatic and renal functions. They had undetectable viremia at the time of the visit, and no toxicity was reported in these patients except for one individual, who stopped the treatment because of central nervous system (CNS) toxicity.
- One special case of a patient with extreme EFV plasma levels, for whom a paired CSF-plasma sample was also collected during the investigation of very severe neuropsychological toxicity (Anagnostopoulos et al., 2013).

Samples were retrieved from the SHCS repository and/or from individuals treated with EFV followed in the routine TDM program for antiretrovirals at Lausanne University Hospital (Lausanne, Switzerland).

Genotype Data. Genotype data were obtained from 67 participants who were genotyped in the context of previous studies (Hasse et al., 2005; Rotger et al., 2005; di Iulio et al., 2009; Lubomirov et al., 2011; Anagnostopoulos et al., 2013) or for the present study for six decrease or loss-of-function variants in CYP2B6 (n = 4), A2A6 (n = 1), and 3A4 (n = 1). All participants gave written informed consent for genetic testing.

In the present study, genotyping was performed by TaqMan allelic discrimination (Applied Biosystems, Foster City, CA): C_7817765_60 for rs3745274 (CYP2B6*6), C_33845811_20 for rs35303484 (CYP2B6*1), C_33845840_20 for rs35979566 (CYP2B6*15), C_30634332_10 for rs2839933 (CYP2A6*9), and C_32306227_10 for rs4646437 (CYP3A4). The rs2839949 (CYP2B6*18) was analyzed by polymerase chain reaction and direct sequencing. For the 13 participants with extremely high EFV plasma levels, CYP2B6 and CYP2A6 were fully resequenced (Rotger et al., 2007; di Iulio et al., 2009).
Participants were classified into six genetic scores (Table 1) according to their CYP2B6, 2A6, and 3A4 genotype status, as previously described (Lubomirov et al., 2011), and categorized into three groups—group 1: score 1, individuals homozygous for the reference alleles of CYP2B6 and all accessory pathways; group 2: scores 3 and 4, individuals heterozygous for a loss-of-function (LOF) CYP2B6 and/or at least one LOF allele in the accessory pathways; and group 3: score 5, individuals homozygous for an LOF in CYP2B6, and score 6, individuals homozygous for an LOF in CYP2B6 and at least one LOF allele in the accessory pathways (Rotger et al., 2007; di Iulio et al., 2009).

Chemicals and Reagents. Efavirenz, 7OH-EFV, 8OH-EFV, and 8,14-di-OH-EFV (Fig. 1, A, B, C, and G) as well as their stable isotopically labeled internal standards were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Sulfatase, β-glucuronidase, and o-saccaric acid 1,4-lactone (D-SL; a specific glucuronidase inhibitor) were obtained from Sigma-Aldrich (St. Louis, MO).

Enzymatic Hydrolysis of Plasma, Urine, and CSF. A 100-μl aliquot of a patient’s sample (plasma, urine, and CSF) was mixed with 300 μl of acetonitrile and centrifuged at +4°C for 10 minutes at 20,000g (14,000 rpm) on a Mikro 200R Benchtop centrifuge (Hettich, Bäch, Switzerland). The supernatants (150 μl) were carefully transferred to a propylene tube and evaporated to dryness under a stream of nitrogen at room temperature to yield a solid extract residue that was reconstituted in a suitable buffer (see below) prior to the enzymatic treatment. For each enzymatic reaction, a control was prepared in parallel in the same conditions (without enzyme).
**Sulfatase treatment:** Prior to incubation, solid sample extract residues were constituted with 70 μl of buffer (20 mM acetate ammonium) and 30 μl of sulfatase from *Aerobacter aerogenes* (3.1 mg protein/ml). Fifty microliters of a solution of D-SL (a specific glucuronidase inhibitor) at 4 mg/ml in H₂O was added to inhibit the small quantity of glucuronidase (<10 IU/ml) present in the sulfatase enzyme. After 18 hours of incubation in a water bath at 37°C, the enzymatic reaction was terminated by adding 80 μl of internal standard solution containing EFV-d₅, 7OH-EFV-d₄, and 8OH-EFV-d₄ at 300 ng/ml in MeOH. After centrifugation of the reaction mixture at 14,000 rpm, 150 μl of supernatant was transferred into a high-performance liquid chromatography vial, and 20 μl was injected for the analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

**Glucuronidase treatment:** 20 μl of β-glucuronidase from *Helix pomatia*, type HP-2, and 130 μl of 20 mM acetate (pH adjusted to 4.7) were added to the sample extract residue. The mixture was vortex mixed for 5 seconds and incubated at 37°C in a water bath for 18 hours. A volume of 100 μl of internal standard solution in MeOH was used to stop the glucuronidase reaction. After centrifugation (10 minutes at 14,000 rpm), the supernatant was transferred into a high-performance liquid chromatography vial, and 20 μl was subjected to LC-MS/MS analysis.

**Mass Spectrometry Analysis.** The liquid chromatography system consisted of Rheos 2200 quaternary pumps, equipped with an online degasser and an HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) controlled by Janeiro-CNS software (Flux Instruments, AG, Thermo Fisher Scientific Inc., Waltham, MA). Separations were done on a 2.1 × 50-mm Atlantis dC18 3-mm analytical column (Waters, Milford, MA). The chromatographic system was coupled to a triple-stage quadrupole Quantum Ion Max mass spectrometer (MS) from Thermo Fisher Scientific, Inc., equipped with an electrospray ionization (ESI) interface and operated with Xcalibur 2.0 software (Thermo Fisher Scientific Inc.).

The mobile phase used for chromatography was 2 mM ammonium acetate in ultrapure water containing 0.1% formic acid (pH 3.5). Solution B consisted of 0.1% formic acid in acetonitrile. The mobile phase was delivered using the following stepwise gradient elution program: 25% of B at 0 minute to 40% at 6 minutes followed by an isocratic mode at 40% of B until 10.5 minutes, then the percentage was gradually increased to 60% of B over 14 minutes, with a flow rate of 0.3 ml/min. The second part of the run includes 2 minutes of intensive rinsing.
and a re-equilibration step to the initial solvent up to 20 minutes. The autosampler was maintained at 10°C. The volume injection was 20 μl. ESI in negative mode was carried out at a capillary temperature of 350°C, the ESI spray voltage was set at 4 kV, and the source-induced dissociation was set at 10 V. The sheath and auxiliary gas (nitrogen) flow rate were set at 35 and 10 (arbitrary units), respectively.

The Phase II metabolites were identified by detailed mass fragmentation analysis: full scan, neutral loss, precursor ion scan, and product ion scan. Precursor ion (Q1) scan data were obtained under negative ESI conditions m/z 330 for 7OH-EFV and 8OH-EFV, m/z 314 for EFV, and m/z 346 8,14-di-OH-EFV. Product ion scan (Q3) spectral data of the known synthetic metabolite standards were compared with those metabolite peaks obtained in plasma and urine samples. In neutral loss analysis, Q1 was fixed at m/z 410, and the loss was fixed at m/z 80. The hydroxyled efavirenz metabolites were identified by mass spectra comparison with previously published data (Mutlib et al., 1999a,b) as well as by enzymatic conversion to the corresponding hydroxylated efavirenz metabolites.

![Fig. 4. EFV metabolite profiling of plasma sample before (A) and after (B) sulfatase treatment. The first and second trace to m/z 410 (hydroxy-EFV-sulfate) and m/z 330 →285 (monohydroxy-EFV), respectively. (Bottom) Chromatographic profile of pure standard of 7OH-EFV and 8OH-EFV. AA, Peak Area; NL, Normalized Level; TIC, Total Ion Current.](image)

(100%: B with 0.5 ml/min) and a re-equilibration step to the initial solvent up to 20 minutes. The autosampler was maintained at 10°C. The volume injection was 20 μl. ESI in negative mode was carried out at a capillary temperature of 350°C, the ESI spray voltage was set at 4 kV, and the source-induced dissociation was set at 10 V. The sheath and auxiliary gas (nitrogen) flow rate were set at 35 and 10 (arbitrary units), respectively.

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**TABLE 1**

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<th>Score 4</th>
<th>Score 5</th>
<th>Score 6</th>
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<tr>
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<tr>
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<tr>
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<td>7</td>
<td>15</td>
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**Number of genotyped patients**

22 0 5 7 15 18

Description of genetic risk scores for efavirenz (Lubomirov et al., 2011) and distribution of genotyped patients (n = 67) according to genetic scores.
Fig. 5. (A) Plasma concentration of EFV and its metabolites (median + IQR 25–75). (B) Ratios of metabolites/EFV in patients from all subset studies (median). (C) CSF from MOST study (n = 9) (Gutmann et al., 2010) and one CSF sample (closed squares) from neurologic investigations (Anagnostopoulos et al., 2013) (median + interquartile range (IQR) 25–75). (D) Ratio of paired samples CSF/plasma from MOST study (n = 6) and one CSF sample from neurologic investigations (closed squares). (E) Metabolite profile analysis of urine normalized with creatinine (CRT) levels in urine (median + IQR 25–75) from a subset of consenting patients under a stable efavirenz regimen, collected at their regular medical follow-up visit, which includes TDM.
The identity of the newly identified metabolite 8OH-EFV-sulfate in humans was further confirmed by high-resolution (HR) mass spectrometry analysis using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer in the positive and negative mode (Thermo Fischer Scientific, Waltham, MA). MS data processing was carried out using the Xcalibur software (Thermo Fischer Scientific, Waltham, MA).

**Results**

**Efavirenz Metabolite Profile Analysis.** Typical chromatographic analysis by LC-MS/MS using the selected reaction monitoring of EFV and its hydroxy metabolites in a plasma extract sample is shown in Fig. 2. The third chromatographic profile in Fig. 2 shows the presence of signals at m/z 314+16 (330), which is indicative of at least three major monohydroxy metabolites either directly present in plasma or possibly produced during the spray/ionization step of the MS analysis (i.e., ion-source metabolite dissociation). The metabolite 8OH-EFV was unambiguously identified based on comparison of its retention time (RT = 11.8 minutes), precursor ion scan data, and MS/MS fragmentation pattern with synthetic metabolite standard. However, the 7OH-EFV and 8OH-EFV metabolites were calculated as the difference between total (i.e., after enzymatic hydrolysis) and direct dehydrolysis. The concentrations of phase II metabolites were calculated as the available for quantification. The concentrations of the glucuronides and sulfates in urine were normalized with creatinine urinary levels (in micromole/L).

**Statistical Analysis.** Statistical analyses were performed using Prism 6 (version 6.04) GraphPad Software, Inc. (San Diego, CA). Association between EFV and its metabolite concentration and genetic score or treatment continuation groups was evaluated on log_{10}-transformed data using t test, and P values <0.05 were considered statistically significant.

**Identification of the Metabolite 8OH-EFV-Sulfate.** The mass spectrum of the hydroxy metabolite Y eluted at 6.1 minutes was identified as the EFV-O-glucuronide because of the in-source dissociation of the glucuronide. This peak identification was confirmed in a separate sample analysis by single-ion monitoring at [M-H]^– = 506, corresponding to the molecular weight of EFV-O-glucuronide (MW = 507).

The mass spectrum of the hydroxy metabolite X eluted at 9.3 minutes obtained by full-scan analysis between m/z 100–600 is shown in Fig. 3A. It had a molecular weight of 410, compatible with a sulfate of either 7OH-EFV-4OH-EFV. Full-scan mass analysis of compound X and its comparison with published mass spectra data (Mutlib et al., 1999a,b) allows the unambiguous assignment of the metabolite as being the metabolite 8OH-EFV-sulfate, identified for the first time in humans. This metabolite was differentiated from the known 7OH-EFV-sulfate by comparison of their mass spectral fragmentation pattern. While the ESI-MS/MS of X showed a signal at [M+H]^+ at m/z 410 for the molecular weight of sulfate conjugates of both 8OH-EFV and 7OH-EFV, we observed an important fragment ion for the aglycone at m/z 330, which is the characteristic signature for 8-hydroxylated analogs of EFV (Mutlib et al., 1999b). For comparison in Fig. 3B, this signal at m/z 330 is actually missing in the mass spectrum of the sulfate conjugate of 7OH-EFV that could also be detected at 7.5 minutes in the metabolite profile analysis monitored at m/z 410 (Fig. 4A). The enzymatic hydrolysis of plasma by sulfatase results in a corresponding increase in the respective minor 7OH-EFV and major 8OH-EFV metabolites and confirms the major 8-substituted hydroxy-sulfate conjugate metabolite identification.

Finally, the HR mass spectrum of 8OH-EFV-sulfate shows a signal at m/z [M-H]^– = 409.97216 in excellent correspondence to the calculated molecular mass of m/z [M-H]^– = 409.97075. The difference between observed and calculated masses was <5 ppm, providing high confidence in providing a definitive identity assessment.

**Quantification of Phase I and II Metabolites of Efavirenz in Plasma, CSF, and Urine.** Total plasma concentrations of EFV and the six metabolites determined in the various body fluids from 71 participants prior to and after enzymatic treatment with glucuronidase and sulfatase (in the presence of the glucuronidase inhibitor D-SL) are shown in Fig. 5A and reported in Table 2. Considering the entire population, the 8OH-EFV-glucuronide and the 7OH-EFV-glucuronide were the major metabolites circulating in plasma (Table 2), with median (range) concentrations of 5827 ng/ml (414–17,107) and 949 ng/m (0–11,797). 8OH-EFV-gln was 64-fold higher than the parent 8OH-EFV (Fig. 5A). The median plasma levels of the newly identified metabolite 8OH-EFV-sulfate was 771 ng/ml (0–12,611), 7-fold higher than the

### Table 2

Concentrations (median and range) of EFV and its metabolites in plasma, CSF, and urine in patients under EFV regimen

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th></th>
<th>CSF</th>
<th></th>
<th>Urine</th>
<th></th>
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<tr>
<td></td>
<td>Concentrations (n = 71)</td>
<td>Metabolites /EFV ratios (n = 71)</td>
<td>Concentrations (n = 10)*</td>
<td>CSF/Plasma ratios (n = 72)</td>
<td>Urine/CRT ratios (n = 8)</td>
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<tr>
<td></td>
<td>Median</td>
<td>Range</td>
<td>%</td>
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<td>Range</td>
<td>%</td>
</tr>
<tr>
<td>EFV</td>
<td>2100</td>
<td>326–46,232</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>8OH-EFV</td>
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<td>0–1086</td>
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<td>0–66</td>
<td>4</td>
<td>0–11</td>
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<tr>
<td>8OH-EFV-gln</td>
<td>5827</td>
<td>414–17,107</td>
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<td>0–547</td>
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*Only 7 out of 10 CSF samples were available as paired CSF/plasma samples.
parent 8OH-EFV metabolite, whereas the plasma concentration of 7OH-EFV-sulfate was 269 ng/ml (0–3019) (Fig. 5A). The metabolite-to-EFV ratios in plasma, expressed as percentages, are shown in Fig. 5B and reported in Table 2. Interestingly, the median ratio percentage of 8OH-EFV-Gln/EFV is greater than 200, with plasma concentrations of 8OH-EFV-gln exceeding the level of EFV in 70% of the cases. Finally, the 8,14-di-OH metabolite of EFV was essentially not detectable in plasma.

EFV concentrations obtained after the reanalysis of CSF samples were greater than 0.5 ng/ml [the suggested CSF 50% maximal inhibitory concentration (IC50) for wild-type human immunodeficiency virus (Best et al., 2011)] in all samples. The median concentration of EFV in CSF was 16 ng/ml (range 9–31), besides the exceptional value at 143 ng/ml measured in the individual with severe neuropsychological toxicity (Anagnostopoulos et al., 2013) (Fig. 5C, closed square). The median concentration of the phase I metabolite 8OH-EFV in CSF was 4 ng/ml (range 0–11). In 6 out of 10 samples, the 8OH-EFV concentrations in CSF were higher than the reported toxicity threshold of 3.3 ng/ml associated with dendritic damage, as suggested by Tovar-y-Roma et al. (2012) in their in vitro study. Both 7OH-EFV and 7OH-EFV-sulfate were below the limit of quantification of 2 ng/ml of our assay (Best et al., 2011). By contrast, 8OH-EFV-gln, 8OH-EFV-sulfate, and 7OH-EFV-gln were present in CSF at concentrations of 15–56, 0–29, and 0–13 ng/ml, respectively (Fig. 5C). Overall, these phase II metabolites were present in CSF at about 2- to 9-fold higher concentrations than 8OH-EFV. The median CSF/plasma ratios (n = 7) were 1.5, 2.5, 0.45, 1.25, and 0.44% for EFV, 8OH-EFV, 8OH-EFV-gln, 8OH-EFV-sulfate, and 7OH-EFV-gln, respectively (Fig. 5D).

The results of the analysis of urine samples are shown in Fig. 5E, with EFV and metabolite levels normalized with creatinine urinary levels. 7OH-EFV-sulfate was consistently the principal urinary sulfate conjugate (Fig. 5E). Finally, 8,14-di-OH-sulfate was found in significant amounts in urine (data not shown).

**Phase I and II Metabolite Profiles in Plasma and CSF from Genotyped Participants.** Among the 67 participants with genotype data, 33% were classified into group 1, 18% into group 2, and 49% into group 3.

The metabolite-to-EFV concentration ratios in plasma in these genotyped participants are shown in Fig. 6. The median log ratios (8OH-EFV/EFV) were 0.8, 1.1, and 0% in groups 1, 2, and 3, respectively. As expected, the 8OH-EFV/EFV ratio was significantly lower (P < 0.0001) in group 3 as compared with the other groups.

The log-transformed 8OH-EFV-gln/EFV and 8OH-EFV-sulfate/EFV ratios in the three groups parallel those determined for 8OH-EFV/EFV. In comparison with groups 1 and 2, group 3 individuals had significantly lower median log-transformed 8OH-EFV-gln/EFV ratios (2.7, 2.7, and 2%, respectively; P < 0.0001) and log-transformed 8OH-EFV-sulfate/EFV ratios (1.8, 1.8, and 1%, respectively; P < 0.0001).

To evaluate the contribution of the accessory pathways (CYP2A6 and CYP3A4) in overall 7OH-EFV metabolite abundance in plasma samples, log-transformed ratios from individuals with score 5 (reference for both CYP2A6 and CYP3A4) versus score 6 [decrease of function (DOF) for CYP2A6 and/or CYP3A4] were compared. The median log-transformed 7OH-EFV-gln/EFV and 7OH-EFV-sulfate/EFV ratios were 1.6% versus 1.5%, and 0.9% versus 0.9%, respectively.

Exploratory analyses were carried out to examine whether hydroxy metabolite levels in CSF are influenced by genetic status. Among 10 CSF samples, 6 were obtained from genotyped participants, who were classified into score 1 (n = 2), score 2 (n = 3), and score 6 (n = 1). Median CSF levels of 8OH-EFV, 8OH-EFV-gln, and 8OH-EFV-sulfate in score 1 and score 4+6 groups were similar: 7 versus 8, 30 versus 52, and 19 versus 16 ng/ml, respectively.

**Fig. 6.** Metabolite/EFV logarithmic ratios stratified according to functional status of CYP2B6 in 67 patients. Ref, reference.

Of note, the paired sample from one individual with severe neuropsychological toxicity because of LOF/DOF alleles of CYP2B6 (Anagnostopoulos et al., 2013) had extreme EFV concentrations of
46,232 and 143 ng/ml in plasma and CSF, respectively (shown in Fig. 5, A and B, closed squares). In this individual, plasma levels of 8OH-EFV, 8OH-EFV-gln, and 8OH-EFV-sulfate concentrations were 37, 6895, and 1399 ng/ml, respectively, whereas the concentrations of 7OH-EFV, 7OH-EFV-gln, and 7OH-EFV-sulfate were 11, 6932, and 1750 ng/ml, respectively (Fig. 5A, closed squares). In CSF, despite extremely high EFV levels, 8OH-EFV, 7OH-EFV, and 7OH-EFV-sulfate were not detected, whereas the concentrations of 8OH-EFV-gln, 8OH-EFV-sulfate, and 7OH-EFV-gln were 15, 11, and 12 ng/ml, respectively.

**Phase I and II Metabolite Profiles, Early Treatment Discontinuation, and CNS Effects.** Data on EFV treatment discontinuation or dose reduction were available from our previous study (Lubomirov et al., 2011) or were retrieved from the SHCS (n = 29). A total of 51 of 68 participants discontinued the EFV-based therapy. In the present analysis, neither EFV nor any of its primary or secondary metabolites were found to be associated with increased risks of EFV treatment discontinuation (Supplemental Fig. 1).

Among the 51 patients who discontinued efavirenz-based treatment, the reason for discontinuation was reported in 42 patients: 15 patients stopped the treatment because of neurologic (CNS) toxicity, and 27 patients discontinued the therapy for other reasons (non-neurologic reasons, i.e., gastrointestinal toxicity, treatment failure, patient’s own wish, etc.). As shown in Fig. 7, no difference was observed in the plasma levels of EFV, 8OH-EFV, or any secondary metabolites in individuals who developed CNS toxic effects or who stopped therapy for other reasons.

Among the 15 patients who stopped EFV treatment because of CNS toxicity, the majority (11/15, 73%) had a decreased (score 4, n = 1) or loss-of-function (scores 5 and 6, n = 10) genotype for the major metabolic pathway CYP2B6, in line with previously reported results by Rotger et al. (2005). Finally, we examined data from patients with CSF-plasma paired samples: among the three out of seven patients with available CSF who developed CNS disorders and stopped EFV therapy, no difference was observed in the CSF concentrations of EFV, 8OH-EFV, or any of its metabolites.

**Discussion**

We performed a comprehensive quantitative metabolite profile analysis of EFV comprising both phase I and phase II metabolites in plasma, CSF, and urine.

In plasma, phase I metabolites were present at rather low levels (up to 200 ng/ml) in accordance with previously published data (Cho et al., 2011; Avery et al., 2013), whereas the phase II metabolites (8OH-EFV-gln, 7OH-EFV-gln, and 7OH-EFV-sulfate) were found at comparable or even higher levels (up to 17,107 ng/ml) than EFV itself. Our study was also the first to identify in humans the 8OH-EFV-sulfate that was found at higher levels than the known 7OH-EFV-sulfate. Previous reports have reported 8OH-EFV-sulfate in urine samples from rats and cynomolgus monkeys (Mutlib et al., 1999b). The identification of the new metabolite in humans may have consequences if EFV is used as a probe to phenotype the CYP2B6 activity (Rakhmanina et al., 2012). In previous studies, only the glucuronide of 8OH-EFV was considered as the single monohydroxy-EFV conjugate metabolite (Mutlib et al., 1999b). In the future, not only 8OH-EFV-gln but also 8OH-EFV-sulfate will have to be considered and hydrolyzed accordingly to yield 8OH-EFV. The 8,14-di-OH-EFV metabolite was almost undetectable in plasma. Recent in vitro investigations have shown that the 8,14-di-OH-EFV metabolite was not produced when 8OH-EFV was directly incubated in human liver microsomes (Ogburn et al., 2010). This finding is also in agreement with the absence of 8,14-di-OH-EFV in patients’ plasma. However, the sulfate of 8,14-di-OH-EFV was found in significant amounts in urine, suggesting that 8OH-EFV may first undergo a phase II conjugation step (i.e., sulfation or glucuronidation) prior to the second hydroxylation at position 14 (Ogburn et al., 2010).

In CSF, 8OH-EFV levels found in our study were in close correspondence to those reported previously (Avery et al., 2013; Winston et al., 2014). 8OH-EFV, 8OH-EFV-gln, 7OH-EFV-gln, and 8OH-EFV-sulfate were detected in CSF, with 8OH-EFV-glucuronide and 8OH-EFV-sulfate being the major metabolites, the latter with a median CSF/plasma ratio of 1.25%, similar to that of EFV (1.5%). These observations were unexpected with regard to the enhanced...
polarity of the metabolites that, a priori, should reduce their passage across the lipophilic blood-brain barrier. These findings are reminiscent of what has been observed for the archetypal example of morphine and its metabolite morphine-6-glucuronide (Mo-6-gln) (De Gregori et al., 2012). This latter metabolite, in addition to its “high polarity,” has also been reported in some studies (Huwyler et al., 1996; Sattari et al., 2011), but not all studies (Bourasset et al., 2003), to be a substrate of P-glycoprotein that should further decrease Mo-6-gln blood-brain barrier permeability. Additional carrier-mediated processes have been found to determine Mo-6-gln (and morphine) neuropharmacokinetics and brain distribution (Bourasset and Scherrmann, 2006). The transporters implicated in the active transport of Mo-6-gln across the blood-brain barrier include GLU-1 and a digoxin-sensitive transporter (probably oatp2) (Bourasset et al., 2003). Interestingly, despite a lower uptake and reduced blood-brain barrier permeability as compared with morphine (Wu et al., 1997), Mo-6-gln has now been recognized to play a large, if not major, role in the analgesic effect after morphine administration to patients (Klimas and Mikus, 2014; Mikus and Klimas, 2015). By analogy with Mo-6-gln, further in vitro studies in cell systems are warranted to clarify the potential role of transporters for the unexpected blood-brain barrier passage observed for 8OH-EFV-gln and 7OH-EFV-gln, their overall brain distribution, and possibly neurotoxicity.

In urine, 7OH-EFV-sulfate was the most abundant metabolite showing the opposite relative proportion with the plasma samples wherein 8OH-EFV-sulfate was the principal sulfate conjugate. It is unclear whether this is due to a possible difference in renal clearance of sulfate conjugates, although results should be interpreted with caution with the limited number of urine samples available.

We examined the relationships with genetic variants in the main (CYP2B6) and accesssory (CYP2A6 and CYP3A4) EFV metabolic pathways and the different metabolites. Individuals homozygous for LOF alleles in CYP2B6 presented the lowest 8OH-EFV metabolite-to-EFV concentration ratios that can be considered as a phenotypic index of CYP2B6 functionality. The reduced capacity to hydroxylate EFV at position 8 was not only observed at the primary phase I reaction, but was also reflected in the subsequent glucuronidation and sulfation steps. For instance, in the patient with LOF/DOF alleles of CYP2B6 (Anagnostopoulos et al., 2013), the plasma concentration of 8OH-EFV was among the lowest encountered in our study. Consistent with this, the corresponding 8OH-EFV-sulfate plasma level was also very low, whereas the 8OH-EFV-gln levels were in the lower range. The phenotypic activity of CYP2A6 that catalyzes 7-hydroxylation-EFV (Ogburn et al., 2010) could not be directly ascertained using 7OH-EFV since in our study, this metabolite was found to be present in plasma at levels mostly under the limit of quantification. Indirect comparison of CYP2A6 activity may be made via the analysis of subsequent phase II metabolites of 7OH-EFV. The 7OH-EFV-gln/EFV and 7OH-EFV-sulfate/EFV ratios were, however, similar in the score 5 and score 6 groups, which challenges our current knowledge of the EFV 7-hydroxylation mediated by CYP2A6 (Ogburn et al., 2010). It may be argued that the variability of the secondary glucuronidation and sulfation steps would obscure the impact of the primary CYP2A6-mediated 7-hydroxylation of EFV in vivo. Nevertheless, the patient with LOF/DOF alleles of CYP2B6 and extreme neurotoxicity requiring admission in a psychiatric hospital (Anagnostopoulos et al., 2013) had a very low 7OH-EFV level in plasma (as most LOF study participants) but had the highest 7OH-EFV-gln levels, in plasma and CSF, and among the highest plasma concentration of 7OH-EFV-sulfate in our study. This is consistent with our proposed compensatory CYP2A6-mediated mechanism (di Iulio et al., 2009) occurring when the CYP2B6 pathway is impaired, assuming that any formed 7OH-EFV is readily subjected to subsequent conjugation with glucuronide and sulfate.

We did not observe a correlation between metabolite levels and EFV treatment discontinuation because of CNS toxicity. In particular, the metabolite 8OH-EFV, which was reported to be a potent neurotoxin in vitro, with an effect distinct from EFV, is exactly the metabolite that is minimally produced when the CYP2B6 pathway is impaired, and remains overall at a relatively constant, low level both in plasma and CSF, regardless of CYP2B6 genotypes. This is an intriguing finding, as patients’ CYP2B6 genotype per se was found to be a predictor of EFV neurotoxicity, in line with previous data (Rotger et al., 2005). Thus, one must at least assert that 8OH-EFV is certainly not the single likely candidate for CNS toxicity; therefore, other metabolites have to be considered.

In conclusion, we performed a comprehensive quantitative metabolite profile analysis of EFV in plasma, CSF, and urine, and examined their relationships with the presence of certain genetic variants in CYP2B6, CYP2A6, and CYP3A4 and with treatment discontinuation because of CNS toxicity. During these investigations, we identified the new metabolite 8OH-EFV-sulfate, which was present at high concentrations in all body compartments. More generally, our study was the first to demonstrate that the dominant circulating (as well as in CSF and urine) metabolites of EFV are not EFV primary oxidized products (e.g., 7-OH- and 8-OH-EFV), but rather the downstream phase II EFV metabolites (glucuronide and sulfate) of the product of EFV oxidation by CYP2B6 and CYP2A6, some of them attaining concentrations even higher than those for EFV itself. We observed that phase II EFV metabolites were the principal metabolites present in CSF, an unexpected finding given the higher polarity of glucuronide and sulfate as compared with the lipophilic parent compound EFV. The clinical importance of these previously unreported EFV metabolites in CSF and their potential contribution to the neuropsychological effects of efavirenz needs to be examined in larger cohort studies.

Acknowledgments


Authorship Contributions

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