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Unmasking efavirenz neurotoxicity: Time matters to the underlying mechanisms

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ABSTRACT

Efavirenz is an anti-HIV drug that presents relevant short- and long-term central nervous system adverse reactions. Its main metabolite (8-hydroxy-efavirenz) was demonstrated to be a more potent neurotoxin than efavirenz itself. This work was aimed to understand how efavirenz biotransformation to 8-hydroxy-efavirenz is related to its short- and long-term neuro-adverse reactions. To access those mechanisms, the expression and activity of Cyp2b enzymes as well as the *thiolomic* signature (low molecular weight thiols plus *S*-thiolated proteins) were longitudinally evaluated in the hepatic and brain tissues of rats exposed to efavirenz during 10 and 36 days. Efavirenz and 8-hydroxy-efavirenz plasma concentrations were monitored at the same time points. Cyp2b induction had a delayed onset in liver ($p < 0.001$), translating into increases in Cyp2b activity in liver and 8-hydroxy-efavirenz plasma concentration ($p < 0.001$). Moreover, an increase in *S*-cysteinyl-glycinylated proteins ($p < 0.001$) and in free low molecular weight thiols was also observed in liver. A distinct scenario was observed in hippocampus, which showed an underexpression of Cyp2b as well as a decrease in *S*-cysteinylated and *S*-glutathionylated proteins. Additionally, the observed changes in tissues were associated with a marked increase of *S*-glutathionylation in plasma. Our data suggest that the time course of efavirenz biotransformation results from different mechanisms for its short- and long-term neurotoxicity. The difference in the redox profile between liver and hippocampus might explain why, despite being mostly metabolized by the liver, this drug is neurotoxic. If translated to clinical practice, this evidence will have important implications in efavirenz short- and long-term neurotoxicity prevention and management.

1. Introduction

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor (NNRTI), one of the most commonly prescribed classes of drugs for the treatment of human immunodeficiency virus (HIV) type 1-infection. At the recommended 600 mg oral daily intake throughout the patient's life, EFV has been associated with central nervous system (CNS) deleterious effects (Fumaz et al., 2005). Most of the neuro-adverse EFV reactions occur at a snapshot in time, during the first weeks of treatment, and rapidly disappear (Fumaz et al., 2005). However they can persist for a long time in a significant proportion of patients, which may negatively impact a patient's quality of life (Leutscher et al., 2013).

Nearly 90% of EFV biotransformation occurs *via* CYP2B6 into 8-OH-EFV (Klein et al., 2005; Ward et al., 2003). The link between the generation of this metabolite and neuro-adverse EFV reactions has been strongly supported by non-clinical (Brandmann et al., 2013; Harjivan et al., 2014; Tovar-y-Romo et al., 2012) and clinical studies (Aouri et al., 2016; Grilo et al., 2016). This metabolite can undergo subsequent metabolic pathways, including phase II conjugations (Aouri et al., 2016). EFV is an inducer of CYP2B6, *i.e.* of its own metabolism (Ward et al., 2003; Desta et al., 2007; U.S. Food and Drug Administration, 2014) and at high concentrations EFV may inhibit 8-OH-EFV detoxification by glucuronidation (Grilo et al., 2016). Recently, 8OHEFV plasma concentrations were related to mood changes in HIV-infected

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patients on long-term EFV treatment (Grilo et al., 2016). Studies using human liver microsomes have revealed that the rate of formation of 8-OH-EFV displays considerable variability between samples (Ward et al., 2003), which suggested that systemic EFV exposure is likely to be affected by inter-individual variability in CYP2B6 activity and by drug-drug interactions involving this isoform. This assumption has been subsequently corroborated by several pharmacogenetics studies focusing on CY2B6 polymorphisms in HIV-infected patients taking EFV (Burger et al., 2006; Lamba et al., 2003). *In vitro* studies showed that 8-OH-EFV is one order of magnitude more toxic to neurons and astrocytes (Brandmann et al., 2013) than EFV, in a dose-dependent manner. Furthermore, bioactivation of 8OHEFV upon oxidation seems to be responsible for the generation of highly reactive electrophilic quinoid species, such as a quinoneimine and 7,8-EFV-quinone (Harjivan et al., 2014). These electrophiles are mainly detoxified by the free sulfhydryl (thiol) group in cysteines. Thiols can redox-cycle through three main fractions, protein (P)-bound (*S*-thiolated, RSSP) and non-protein-bound, which includes the reduced and oxidized fractions. The latter are usually assessed in combination under the common LMWT designation. RSSPs result from oxidative formation of disulfide bonds between the thiol groups of cysteine residues in proteins and LMWTs, such as cysteine (CysSH) or glutathione (GSH) (Eaton, 2006). Notably, brain function can be disrupted by many electrophilic neurotoxicants that irreversibly modify proteins (Lopachin and Decaprio, 2005). In addition, the toxicological activity of endogenous and exogenous quinoid metabolites can also stem from their potential pro-oxidant activity, which can yield reactive oxygen species (ROS) (Bolton et al., 2000; Monks and Jones, 2002). *S*-thiolation of protein cysteines represents a protective mechanism that blocks nucleophilic thiolate residues, thereby preventing irreversible reactions with electrophiles (Dalle-Donne et al., 2005a; Dalle-Donne et al., 2006; Dalle-Donne et al., 2005b). Together with LMWTs, the RSSPs contribute to define the unique “redox code” of tissues (Jones and Sies, 2015), that controls the delicate balance between injury and adaptive response after an insult.

Taking these observations into account we describe herein the use of a rat model to investigate correlations between EFV biotransformation to 8-hydroxy-EFV, Cyp2b auto-induction, and changes in the *thiolomic* signature, as well as their role in short- and long-term neurotoxicity.

2. Materials and methods

2.1. Drugs and chemicals

EFV was kindly provided by Dr. Frederick A. Beland (*National Center for Toxicological Research*, Arkansas, USA). High-performance liquid chromatography (HPLC)-grade solvents were purchased from *VWR* (Belgium). β-Glucuronidase (Type VII-A from *E. coli*, E.C. 3.2.1.31, 1000 U/mL), Arylsulfatase (Type H-1, from *E. coli*, E.C. 3.1.6.1., 1000 U/mL) and reagents used for *thiolomic* profile determination were purchased from *Sigma-Aldrich* (USA), with the exception of trichloroacetic acid (TCA), which was purchased from *Roth* (Germany). A kit from *NZYTech* (Portugal) was used for the cDNA synthesis. Primers and PowerUp™ SYBR® Green Master Mix used for quantitative real time PCR (qPCR) were purchased from *Applied Biosystems, Thermo Fisher Scientific* (USA).

2.2. Animals

Experiments were performed with male *Wistar* rats (*Rattus norvegicus* L.), aged 13 weeks, with a mean body weight of 293 ± 60 g, obtained from the *NOVA Medical School* animal facility. Animals were housed two *per* cage in polycarbonate cages with wire lids (*Tecniplast*, Italy), under 12 h light/dark cycles (8 am–8 pm), at room temperature (22 ± 2.0 °C) and a relative humidity of 60 ± 10%. Rats were maintained on a standard laboratory diet (SDS RM1, *Special Diets Services*, UK) and *ad libitum* reverse osmosis water.

Rats were randomly assigned into four groups: 10-day control (CTL), 10-day EFV-exposed, 36-day CTL, and 36-day EFV-exposed. In order to reduce the number of animals used in the experiments (*3Rs approach)*, the same rat was used for at least two experiments (*i.e.*, one hippocampus was used to determine ethoxycoumarin *O*-deethylase (ECOD) activity and the other for Cyp2b expression). A number of 8 animals *per* group was used for *thiolomic* profile (RSSP + LMWT) analysis. For Cyp2b expression and activity analysis a number of 6 animals *per* group were used.

NIH Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985), the European guidelines for the protection of animals used for scientific purposes (European Union Directive 2010/63/EU) and the Portuguese Law n° 113/2013 concerning ethical use of animals were followed. The experimental procedures (protocol n° 14/2016/CEFCM) received prior approval by the *Institutional Ethics Committee* of the *NOVA Medical School* for animal care and use in research.

2.3. Experimental protocol

Animals from the EFV groups were administered 9 mg/kg/day of EFV by oral gavage, suspended in 2 mL of reverse osmosis water, and CTL groups were administered the same volume of reverse osmosis water. The administrations were performed using a sterile polypropylene feeding tube (15 gauge; tip diameter: 3 mm; length: 78 mm; *Instech Laboratories*, Inc., USA) to reduce the risk of trauma, perforation and cross contamination (Morton et al., 2001). All animals underwent a 7-day period of acclimatization, handled daily by the same individual for a period of 2 min each, and accustomed to the gavage position, in a different animal facility room.

The animals were weighed at baseline and twice a week during the entire study. The amounts of EFV were adjusted accordingly, in order to ensure a constant daily dose. EFV or vehicle was administered daily in the morning, at approximately the same schedule.

At day 10 or 36, approximately 1–2 h after EFV administration, rats were anesthetized by intraperitoneal injection with medetomidine (0.5 mg/kg body weight; Domitor®, *Pfizer Animal Health*) and ketamine (75 mg/kg body weight; Imalgene 1000®, *Mérial*, Lyon, France). Cardiac puncture was performed for blood sampling and the plasma samples were stored at − 80 °C until use. The animals were then decapitated under deep anesthesia, brains were removed from skull, and hippocampus and prefrontal cortex were dissected. The liver was also rapidly removed.

2.4. Cyp2b1 and Cyp2b2 gene expression

Liver, hippocampus and prefrontal cortex were collected and homogenized in Trizol® (*Life Technologies*) using a tissue homogenizer (*Heidolph* DIAX 900). Total RNA extraction was performed according to the Trizol® manufacturer's instructions. The RNA concentration was determined prior to cDNA synthesis by measuring the absorbance at 260 nm on a SPECTROstar Omega spectrometer (*BMG Labtech*, Ortengerg, Germany) operating in the LVis Plate mode. cDNA was synthesized from 1 μg RNA according to the manufacturer's instructions. Quantitative real time PCR (qPCR) was carried out in a final volume of 15 μL with 2 × PowerUp™ SYBR® Green Master Mix and 0.3 μM of each primer, plus 2 μL of cDNA was added as template.

Rat specific primers were used for the housekeeping gene *β-actin* (Forward 5′-AAGTCCCTCACCCTCCCAAAAG-3′; Reverse 5′-AAGCAATGCTGTCACCTTCCC-3′) (Peinnequin et al., 2004) and for the target genes *Cyp2b1* (Forward 5′-GCTCAAGTACCCCCATGTCG-3′; Reverse 5′-ATCAGTGTATGGCATTTTACTGCGG-3′) and *Cyp2b2* (Forward 5′-CTTTGCTGGCACTGAGACCG-3′; Reverse 5′-ATCAGTGTATGGCATTTTGGTACGA-3′) (Schilter et al., 2000). The efficiency of each reaction was estimated with a calibration curve built using serial cDNA dilutions (1, 10^{-1}, and 10^{-2}) in order to construct a standard curve for each gene and tissue. The reaction was performed on an *Applied*