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Profile of stress and toxicity gene expression in human hepatic cells treated with Efavirenz

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ABSTRACT

Hepatic toxicity and metabolic disorders are major adverse effects elicited during the pharmacological treatment of the human immunodeficiency virus (HIV) infection. Efavirenz (EFV), the most widely used non-nucleoside reverse transcriptase inhibitor (NNRTI), has been associated with these events, with recent studies implicating it in stress responses involving mitochondrial dysfunction and oxidative stress in human hepatic cells. To expand these findings, we analyzed the influence of EFV on the expression profile of selected stress and toxicity genes in these cells.

Significant up-regulation was observed with Cytochrome P450, family 1, subfamily A, polypeptide 1 (*CYP1A1*), which indicated metabolic stress. Several genes directly related to oxidative stress and damage exhibited increased expression, including Methalothionein 2A (*MT2A*), Heat shock 70 kDa protein 6 (*HSPA6*), Growth differentiation factor 15 (*GDF15*) and DNA-damage-inducible transcript 3 (DDIT3). In addition, Early growth response protein 1 (*EGR1*) was enhanced, whereas mRNA levels of the inflammatory genes Chemokine (C-X-C motif) ligand 10 (*CXCL10*) and Serpin peptidase inhibitor (nexin, plasminogen activator inhibitor type 1), member 1 (*SERPINE1*) decreased and increased, respectively. This profile of gene expression supports previous data demonstrating altered mitochondrial function and presence of oxidative stress/damage in EFV-treated hepatic cells, and may be of relevance in the search for molecular targets with therapeutic potential to be employed in the prevention, diagnosis and treatment of the hepatic toxicity associated with HIV therapy.

1. Introduction

Combined Antiretroviral Therapy (cART) has rendered human acquired immunodeficiency syndrome (AIDS) a chronic rather than mortal illness in the developed world. However, as the disease has come under control, there has been increasing concern about the adverse effects of this medication. Metabolic disturbances, particularly those related to lipid homeostasis, appear in nearly half of cART patients (Caron-Debarle et al., 2010) whereas significant drug-induced hepatotoxicity has been identified in up to a quarter of patients, a figure that probably belies actual rates, as 50% of subjects with increased liver enzymes are asymptomatic (Inductivo-Yu and Bonacini, 2008; Núñez, 2006, 2010). Mitochondrial toxicity is one of the main mechanisms responsible for cART-induced side effects. It has been attributed primarily to one component of this multi-drug therapy, namely nucleoside analogue reverse transcriptase inhibitors (NRTI), which are capable of inhibiting the enzyme responsible for mtDNA replication, mitochondrial DNA polymerase gamma (Pol-γ) (Walker et al., 2002). cART regimens usually comprise two NRTI plus either a boosted protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) (AIDSinfo, 2011). Efavirenz (EFV) is the most widely prescribed NNRTI. It is largely considered a safe drug, however its use has been related to psychiatric symptoms and to a lesser extent lipid and metabolic disorders and hepatotoxicity (Tashima et al., 2003; Gutierrez et al., 2005; Manfredi et al., 2005; Maggiolo, 2009). The mechanisms responsible for these toxic effects remain largely unknown; however, despite the fact that EFV does not inhibit Pol-γ, some of

Abbreviations: EFV, Efavirenz; HAART, highly active antiretroviral therapy; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial transmembrane potential; DNA, polymerase gamma (Pol-γ); ER, endoplasmatic reticulum; UPR, unfolded protein response.

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its actions display features of mitochondrial dysfunction. Recent studies report deleterious effects by EFV in human hepatic cells that point to specific mitochondrial alterations and metabolic disturbances (Apostolova et al., 2010; Blas-García et al., 2010). In particular, the stress response observed included major bioenergetic changes manifested as a reduction in mitochondrial oxygen consumption with specific inhibition at Complex I of the electron transport chain, a decrease in ATP production, a drop in the mitochondrial membrane potential ($\Delta\Psi_m$) and an augmentation in reactive oxygen species (ROS) generation. An enhancement of mitochondrial mass was also detected and associated with the induction of autophagy (in particular, mitophagy) and apoptosis (Apostolova et al., 2010, 2011). Importantly, the highest concentration of EFV employed in these studies (50 μM) compromised the viability and proliferation of Hep3B cells, leading to cell cycle arrest and induction of apoptotic cell death (Apostolova et al., 2010). In order to provide an initial assessment of potential stress-related pathways involved in the toxic effects of EFV, in the present work we have studied the expression of specific stress and toxicity-responsive genes in human hepatoma cells (Hep3B) treated with the same, clinically relevant, concentrations of the compound previously shown to modify mitochondrial function without exerting a major effect on cellular viability (10 and 25 μM). Several validating experiments have also been performed in primary human hepatocytes.

2. Materials and methods

2.1. Reagents and drugs

Efavirenz acquired from Sequoia Research Products (Pangbourne, UK), was dissolved in methanol (3 mg/ml) and the purity (98–100%) and stability of the solutions were evaluated by HPLC. The EFV concentrations employed (10 and 25 μM) are clinically relevant and were chosen taking into account the important inter-individual variability reported for the pharmacokinetics of this drug (Burger et al., 2006).

2.2. Cell culture

Hep3B cells (ATCC HB-8064) were employed due to their metabolic competence, as they possess an active cytochrome P450 system despite constituting a transformed human hepatoma cell line (Zhu et al., 2007). Specifically, EFV is primarily metabolized by CYP2B6, the activity of which has been reported to in Hep3B cells (Lin et al., 2012). These cells were cultured in MEM supplemented with 1 mM non-essential amino acids, 10% heat-inactivated foetal bovine serum, penicillin (50 units/mL) and streptomycin (50 μg/mL). Unless stated otherwise, all the reagents employed were purchased from GIBCO (Invitrogen, Eugene, OR). Primary human hepatocytes were obtained from liver biopsies (one man, two women), isolated following a two-step collagenase protocol and cultured as described elsewhere (Apostolova et al., 2010). The protocols employed complied with European Community guidelines for the use of human experimental models and were approved by the Ethics Committee of the University of Valencia, Valencia. Cell cultures were maintained in an incubator (IGO 150, Jouan, Saint-Herblain Cedes, France) at 37 °C in a humidified atmosphere of 5% CO2/95% air (AirLiquide Medicinal, Valencia, Spain). Treatments of Hep3B cells were performed in subconfluent *t*-25 flask cell cultures (1.5×10^6 cells/flask seeded one day before the experiment), whereas primary hepatocytes were cultured and treated in collagen- precoated multi-well plates, "BioCoat" (BD, Madrid, Spain). In vivo, EFV has been shown to have high plasma protein binding. This fact was taken into account and thus 10% serum was present during the entire period of exposure to the drug.

2.3. RT^{2}Profiler™ PCR array

Expression of 84 genes was studied by real time PCR-Array (RT^{2}Profiler™ PCR Array: Human Stress and Toxicity Finder™, SABiosciences, Frederick, MD, Ref. PAHS-003C) using 5 housekeeping genes. For these experiments, Hep3B cells were treated with EFV for 8 h (25 μM, $n = 3$) or 24 h (10 and 25 μM, $n = 2$).

2.3.1. RNA isolation

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and eluted in 30 μl of water. It was then quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.3.2. First-strand cDNA synthesis

First strand cDNA synthesis was achieved using the RT^2 First Strand Kit (SABiosciences, Frederick, MD). One microgram of total RNA was reverse transcribed in a final volume of 20 μl following the manufacturer's instructions and including a genomic DNA elimination step. Reverse transcriptase was inactivated by heating at 95 °C for 5 min. cDNA was diluted to 111 μl by adding RNase-free water and was stored at −20 °C until use.

2.3.3. PCR array

cDNA was mixed with RT^2 SYBR green/ROX qPCR master mix (SABiosciences, Frederick, MD) following the manufacturer's instructions. Thereafter, 25 μl/well were loaded in 96 well plates with pre-dispensed specific primer sets of the RT2 Profiler PCR Array (SABiosciences, catalog number PAHS-003C). PCR array experiments (10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C) were performed using an ABI 7900HT FAST instrument (Applied Biosystems, Foster City, CA).

2.3.4. Data analysis

We employed the ΔΔCt method using the online analysis tool provided by the supplier of the PCR arrays (http://www.sabiosciences.com/pcrarraydataanalysis.php). Genes with Ct values greater than 35 cycles were considered non-detectable. An average of five house-keeping genes [beta-2-microglobulin (*B2M*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), ribosomal protein L12a (*RPL13A*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and beta-actin (*ACTB*)] was used to obtain the ΔCt value for each gene of interest. The ΔΔCt value was calculated according to the difference between the ΔCt of the treatment group and that of the control group. The fold-change was calculated by 2^(−ΔΔCt), which represents the level of the expression of each gene in the EFV-treated sample vs that in the vehicle control sample.

2.4. RT-PCR validation

The array was validated by performing real time RT-PCR (at least 3 times in duplicate) using mRNA of Hep3B cells treated with the vehicle or EFV (10 and 25 μM at 8 and 24 h) and primary human hepatocytes treated with vehicle or EFV (10 and 25 μM at 24 h). Six representative genes of the array were selected according to their level of expression, and RT-PCRs were performed with the same sets of primers used in the gene array (SABiosciences, Frederick, MD).