Lack of mitochondrial toxicity of darunavir, raltegravir and rilpivirine in neurons and hepatocytes: a comparison with efavirenz

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Objectives: Growing evidence associates the non-nucleoside reverse transcriptase inhibitor efavirenz with several adverse events. Newer antiretrovirals, such as the integrase inhibitor raltegravir, the non-nucleoside reverse transcriptase inhibitor rilpivirine and the protease inhibitor darunavir, claim to have a better toxicological profile than efavirenz while producing similar levels of efficacy and virological suppression. The objective of this study was to determine the in vitro toxicological profile of these three new antiretrovirals by evaluating their effects on the mitochondrial and cellular parameters altered by efavirenz in hepatocytes and neurons.

Methods: Hep3B cells and primary rat neurons were treated with clinically relevant concentrations of efavirenz, darunavir, rilpivirine or raltegravir. Parameters of mitochondrial function, cytotoxicity and oxidative and endoplasmic reticulum stress were assessed using standard cell biology techniques.

Results: None of the new compounds altered the mitochondrial function of hepatic cells or neurons, while efavirenz decreased mitochondrial membrane potential and enhanced superoxide production in both cell types, effects that are known to significantly compromise the functioning of mitochondria, cell viability and, ultimately, cell number. Of the four drugs assayed, efavirenz was the only one to alter the protein expression of LC3-II, an indicator of autophagy, and CHOP, a marker of endoplasmic reticulum stress and the unfolded protein response.

Conclusions: Darunavir, rilpivirine and raltegravir do not induce toxic effects on Hep3B cells and primary rat neurons, which suggests a safer hepatic and neurological profile than that of efavirenz.

Keywords: mitochondria, HIV, adverse effects, CNS, hepatotoxicity

Introduction

Efavirenz-containing therapies are frequently prescribed as a first-line treatment for HIV due to their efficacy and availability as co-formulated single tablets administered in a single daily dose.1,2 However, increasing evidence associates this non-nucleoside reverse transcriptase inhibitor (NNRTI) with several adverse events, especially neuropsychiatric incidents, hepatotoxicity, lipid disturbances and rash.1–5 The eminence of efavirenz has been challenged by the arrival of newer antiretroviral agents, such as the integrase inhibitor raltegravir, the NNRTI rilpivirine and the protease inhibitor darunavir. The principal claim of these new drugs is a safer toxicological profile6–8 and similar efficacy and virological suppression with respect to efavirenz.4 Recent in vitro data suggest that efavirenz produces mitochondrial dysfunction in hepatocytes, which culminates in the adaptive response of autophagy and triggers endoplasmic reticulum (ER) stress and the unfolded protein response (UPR).9–12 Efavirenz-induced mitochondrial toxicity has also been described in neurons, where it has been found to specifically affect bioenergetics and viability.13–15 In this study, we have determined the in vitro toxicological profile of the three aforementioned new antiretroviral compounds by evaluating their effects on the mitochondrial and cellular parameters of hepatocytes and neurons altered by efavirenz.

Materials and methods

Reagents and drugs

Chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) and cell culture reagents were supplied by Gibco (Life Technologies, Eugene, OR, USA). Antiretrovirals were purchased from Sequoia Research Products (Pangbourne, UK) and dissolved in DMSO (except efavirenz, which was
Figure 1. Effect of newer antiretroviral drugs on mitochondrial function and cellular physiology in hepatic cells. (a) Quantitative analysis of mitochondrial membrane potential (TMRM fluorescence) and mitochondrial superoxide production (MitoSOX fluorescence) by fluorescence microscopy after 1 h (white
dissolved in methanol). The concentration of methanol employed did not have a significant impact on any of the parameters studied. Clinical concentrations of darunavir (5–25 μM), rilpivirine (0.25–1 μM) and raltegravir (0.5–2 μM) were compared with efavirenz at 25 μM, a clinically relevant concentration that has consistently been shown to modify mitochondrial function. Fluorescent probes were supplied by Molecular Probes (Life Technologies), except Hoechst 33342, which was purchased from Sigma-Aldrich.

Cell culture
Human hepatoblastoma Hep3B cells (ATCC HB-8064) were cultured as described elsewhere. Primary cultures were prepared with cerebral cortex neurons from rat fetuses, as previously reported. Protocols regarding isolation and primary cell culture complied with European Union guidelines and were approved by the Ethics Committee of the University of Valencia. On the basis of previous studies, these cell types were selected as reliable in vitro models to analyse drug-induced hepatotoxic and neurotoxic effects.

Cellular viability
The colorimetric MTT assay (Roche Diagnostics, Mannheim, Germany) was used to study cell viability related to mitochondrial function, as described elsewhere, in cells treated for 24 or 48 h.

Fluorescence microscopy and static cytometry
Fluorescence was detected with an IX81 Olympus microscope (Hamburg, Germany) and quantified by static cytometry software ScanR version 2.0.3.2 (Olympus). Following 1 or 24 h of treatment, the fluorochromes 2.5 μM Hoechst 33342 (to stain the nuclei) and 5 μM MitoSOX® were used to detect mitochondrial superoxide, respectively.

Western blotting
Total cell protein extracts were obtained, quantified and immunoblotted as previously described. The primary antibodies used were anti-GADD153/CHOP mouse monoclonal (Abcam, Cambridge, UK), anti-microtubule-associated protein 1A/1B light chain 3 (LC3) rabbit polyclonal and anti-actin rabbit polyclonal (both from Sigma-Aldrich). The secondary antibodies were peroxidase-labelled anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) and anti-mouse (Thermo Scientific, Rockford, IL, USA).

Presentation of data and statistical analysis
Data (percentages of the control; mean ± SEM) were analysed by one-way ANOVA, followed by a Newman–Keuls multiple comparison test or a Student’s t-test (GraphPad Prism v.3.02 software, La Jolla, CA, USA).

Results
Mitochondrial function of Hep3B cells
None of the new compounds induced significant changes in ΔΨm at 1 or 24 h, although a slight reduction was produced by raltegravir after 24 h. Conversely, cells exposed to 25 μM efavirenz exhibited a significant drop in ΔΨm that was evident at 1 h of treatment and maintained after 24 h (Figure 1a). Fluorescence images (Figure 1b) did not reveal increases in mitochondrial superoxide production in Hep3B cells incubated with any of the new antiretroviral drugs within either period, a result confirmed by cytometric analysis (Figure 1a). In line with previous reports, efavirenz induced a significant increase in superoxide production in a time-dependent fashion, thus indicating hepatic oxidative stress.

Effects on hepatic cell proliferation/survival and viability
Fluorescence microscopy experiments revealed similar cell counts following 24 h of treatment with the vehicle or the various concentrations of the newer compounds, and no alterations in the cell cycle pattern or proliferative capacity of Hep3B cells (data not shown). The MTT assay revealed a slight but non-significant reduction in cellular viability after 24 h (data not shown) or 48 h of treatment with darunavir, rilpivirine or raltegravir (Figure 1c). In sharp contrast, efavirenz induced a cytotoxic effect in hepatic cells, undermining their proliferation and viability.

Analysis of cellular pathophysiological processes linked to mitochondrial function in efavirenz-induced toxicity
Expression of LC3-II, a cleaved form of LC3 indicative of autophagy activation, was significantly augmented at 24 h by efavirenz but not by any of the newer antiretrovirals. Likewise, the expression of CHOP, an indicator of the induction of ER stress and UPR, was significantly enhanced by efavirenz but not by darunavir, rilpivirine or raltegravir (Figure 1d).

Effects on mitochondrial function and cellular viability in neurons
Fluorescence microscopy revealed a significant and concentration-dependent decrease in ΔΨm in primary neurons following incubation with efavirenz, while TMRM levels were similar in neurons exposed to darunavir, rilpivirine, raltegravir or the vehicle. The production of mitochondrial superoxide was not enhanced in any case, suggesting that redox status was unaffected. A completely different response was observed in efavirenz-treated neurons, in which superoxide generation was clearly increased. The differential

bars) or 24 h (grey bars) of treatment (n=4–6). (b) Representative fluorescence microscopy images (×10) of Hep3B cells treated with the highest concentrations of each drug for 24 h, stained with TMRM (top row) or MitoSOX (bottom row). (c) MTT assay of exponentially growing cells after 48 h of culture in the presence of the different compounds (n=5–6). (d) Western blot analysis of the autophagic marker LC3 and the ER stress marker CHOP in Hep3B cells. Representative western blot images showing LC3-II and CHOP protein expression in cells treated for 24 h (n=4). Data (mean ± SEM) were calculated as percentages of the control value (untreated cells) and analysed by one-way ANOVA multiple comparison test followed by Newman–Keuls test. Data for efavirenz were independently analysed with a Student’s t-test versus its own vehicle (methanol, data not shown). Veh, vehicle; DRV, darunavir; RPV, rilpivirine; RAL, raltegravir; EFV, efavirenz. *P<0.05, **P<0.01, ***P<0.001. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
effects of the antiretroviral drugs correlated with cellular viability. None of the new compounds produced changes in MTT absorbance, while efavirenz once again induced a significant reduction in this parameter, similar to that recorded in hepatic cells (Figure 2).

Discussion

The reduction of drug-induced side effects has become a priority in the treatment of HIV, with susceptibility to hepatotoxicity or neurological side effects being a determining factor in the selection of antiretroviral drugs prescribed to patients. Previous studies in our laboratory and by other groups have demonstrated several toxic effects of efavirenz on hepatic cells, involving mitochondrial interference produced through a mechanism that is independent of polymerase γ inhibition and dependent on the inhibition of complex I of the mitochondrial electron transport chain.16,17 As well as compromising cellular viability and survival, efavirenz-induced mitochondrial effects trigger pathophysiological processes such as autophagy, apoptosis and ER stress.9–12 Rilpivirine, another NNRTI, is reported to affect liver enzyme levels to a lesser extent than efavirenz.6,18 In line with this evidence, none of the concentrations of rilpivirine employed in our study altered mitochondrial function in hepatocytes; consequently, no decrease in cell number

Figure 2. Effect of newer antiretroviral drugs on mitochondrial function and cellular viability in primary rat neurons. (a) Quantitative analysis of mitochondrial membrane potential (TMRM fluorescence) by fluorescence microscopy after 24 h of treatment. (b) Quantitative analysis of mitochondrial superoxide production (MitoSOX fluorescence) by fluorescence microscopy after 24 h of treatment. (c) MTT assay of primary neurons after 24 h of culture in the presence of the different compounds. Data (mean ± SEM, n = 3–5) were calculated as percentages of the control value (untreated cells) and analysed by a one-way ANOVA multiple comparison test followed by a Newman–Keuls test. Efavirenz was independently analysed with a Student’s t-test versus its own vehicle (methanol; data not shown). Veh, vehicle; DRV, darunavir; RPV, rilpivirine; RAL, raltegravir; EFV, efavirenz. *P < 0.05, **P < 0.01.
was detected following 24 h of incubation, while the opposite effects were produced by efavirenz. In this context, it is not surprising that autophagy and ER stress induction were similar in vehicle- and rilpivirine-treated cells, as these responses were evidently induced by efavirenz via a mitochondria-dependent mechanism. Together with existing data obtained with a third NNRTI, nevirapine, our results point to a specific mechanism underlying efavirenz-induced liver toxicity.

Interestingly, although warnings have been issued concerning the risk of hepatotoxicity with darunavir, we did not observe any detrimental effects with this protease inhibitor. A possible explanation for this apparent discrepancy is that the aforementioned alert was based on very few clinical cases in which other risk factors were present (viral hepatitis co-infection, administration of other hepatotoxic drugs, etc.). However, it is also possible that the cellular mechanisms involved in darunavir-induced liver toxicity do not involve an acute inhibition of mitochondria.

No alterations were observed after 1 or 24 h of incubation with raltegravir, which is clearly in keeping with existing clinical evidence as hepatic adverse events have rarely been associated with raltegravir in clinical trials. Neuropsychiatric and neurocognitive adverse events have widely been described in efavirenz-treated patients, although the molecular mechanisms involved remain elusive. Recently, we have demonstrated that this compound alters mitochondrial function in primary cultures of neurons and astrocytes, leading to a differential response in the two populations (astrocytes activated glycolysis and restored cell bioenergetics, while neurons did not). These cell-specific mechanisms provide a possible explanation for some efavirenz-related neurotoxic effects. Several clinical trials have been conducted in order to compare the safety of efavirenz in the CNS with that of certain new anti-HIV drugs. The ECHO and THRIVE studies identified fewer neurological and psychiatric adverse events with rilpivirine than with efavirenz in previously untreated patients. Similarly, in a randomized study, patients receiving raltegravir exhibited fewer neuropsychiatric effects than those administered efavirenz. In line with previous research, the in vitro data obtained in the present study support the neurotoxicity-free profile of the more recent antiretroviral drugs assayed.

In summary, our results demonstrate that clinically relevant concentrations of rilpivirine, raltegravir and darunavir do not affect mitochondrial function or compromise cell viability and survival in hepatic cells and neurons in vitro, unlike what is observed with efavirenz. Considering that this basic evidence is in accordance with the results of clinical trials, all three drugs would seem to be safer alternatives to efavirenz in terms of hepatotoxicity and neurological side effects.

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