



# Efavirenz causes accumulation of intracellular lipids in hepatic cells.

Ana Blas-García<sup>1</sup>, Daniel Ballesteros<sup>3</sup>, Daniel Monleón<sup>2</sup>, José M. Morales<sup>2</sup>, Milagros Rocha<sup>3</sup>, Víctor M. Víctor<sup>3</sup>, Nadezda Apostolova<sup>1</sup> and Juan V. Esplugues<sup>1</sup>.  
<sup>1</sup> Departamento de Farmacología, <sup>2</sup> Laboratorio de Imagen Molecular (Facultad de Medicina) y <sup>3</sup> Fundación Hospital Universitario Dr. Peset, Universidad de Valencia y CIBERehd (Spain)

## ABSTRACT

**Background.** Efavirenz (EFV) has recently been associated with changes in lipid and body fat composition characteristic of lipodystrophy. In this study we have analyzed the molecular mechanisms that could be implicated in these alterations of lipid metabolism, and the role of the master switch of the regulation of cellular bioenergetics, AMP-activated protein kinase (AMPK).

**Methods.** Non-infected Hep3B cells were stained with the fluorescent probe Nile Red after 24h incubation with EFV (10, 25 or 50  $\mu$ M) and measured by static cytometry. In order to define the nature of these accumulated lipids, cells were treated for 4h with EFV (10 and 25  $\mu$ M) and intracellular lipid content was determined by Nuclear Magnetic Resonance (NMR). The expression of the fatty acid transporters CD36 and FATP was analyzed by semi-quantitative PCR. Selected experiments were performed in cells pretreated (30 min) with the AMPK inhibitor Compound C (20 $\mu$ M). Data (n $\geq$ 3) were analyzed by ANOVA (one-way or repeated measures).

**Results.** EFV induced neutral lipid accumulation in hepatic cells after 24h incubation (10 $\mu$ M 124.2 $\pm$ 3.2%, 25 $\mu$ M 125.6 $\pm$ 7.6%, 50 $\mu$ M 147.1 $\pm$ 9.8%\*\* Vs Control 106.1 $\pm$ 1.3%). In NMR experiments, the effects of EFV were also significantly evident following 4h incubation (values of relative intensity signal in arbitrary units, a.u.; 10 $\mu$ M 0.1154 $\pm$ 0.007\*\* a.u., 25 $\mu$ M 0.1126 $\pm$ 0.0082\* a.u. Vs Control 0.097 $\pm$ 0.012 a.u.), and the profile of the lipids suggested that they did not originate in the membranes. Alteration of lipids by EFV was not observed when fatty acids were removed from the culture media, thus demonstrating that they were the result of fatty acid uptake from extracellular sources. Likewise, these changes did not occur in the presence of Compound C, pointing to a key role of AMPK in this lipid uptake. This enzyme was also implicated by the fact that EFV increased CD36 and FATP mRNA expression, two of its downstream targets.

**Discussion.** Our results demonstrate that EFV increases fatty acid uptake in hepatic cells, leading to the accumulation of lipids. These lipids have the same chemical composition as those that form droplets. Given the long-term treatment of patients with EFV, such effects on lipid content suggest that this drug exerts a pro-steatotic role in the liver and could alter lipid metabolism in this organ. Due to the importance of the liver in the general regulation of lipids, EFV may also be implicated in some of the alterations that are characteristic of lipodystrophy.

## INTRODUCTION

Recent studies have demonstrated an acute effect of the NNRTI Efavirenz (EFV) on the mitochondria that is independent of any effect on mtDNA and that reduces mitochondrial function and activates AMP-activated protein kinase (AMPK), which is a master switch for regulation of cellular energetics in health and disease.

Clinical evidence suggests that the EFV contributes to changes in lipid composition. Lipodystrophy and associated metabolic alterations are the most prevalent adverse effects in patients taking HAART, but the specific drugs responsible for these effects and the mechanisms involved are still unclear. As liver plays a major role in general metabolism we have evaluated the effect of EFV on several parameters related to lipid uptake and metabolism in Hep3B cells, in order to determine whether EFV-induced reduction in mitochondrial function could be implicated in these changes in lipid metabolism, and the role of AMPK and its downstream targets, fatty acid transporters CD36 and FATP, in these processes.

## MATERIALS AND METHODS

Non-infected Hep3B cells, cultured in Minimum Essential Medium supplemented with BSA fatty acid free 1%, L-carnitine 50  $\mu$ M, and palmitic acid 0.1 mM, were stained with the fluorescent probe Nile Red after 24h incubation with EFV (10, 25 or 50  $\mu$ M) and measured by static cytometry with a fluorescence microscope (Olympus Ix81). This probe stains neutral lipids.

mRNA expression of fatty acid transporters CD36 and FATP was analyzed by semi-quantitative PCR and quantified by densitometry.

Intracellular lipid content following 4h treatment with EFV was determined by Nuclear Magnetic Resonance.

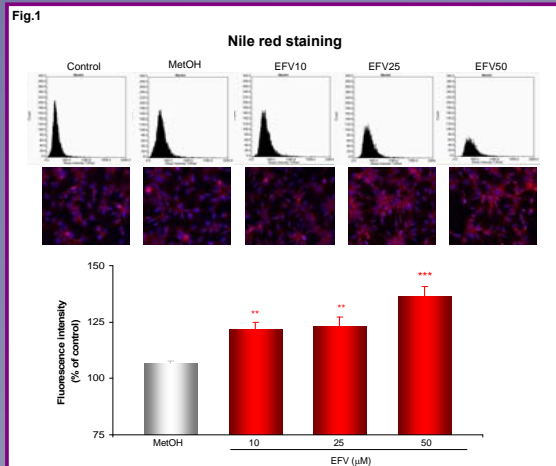
Selected experiments were performed in cells pretreated (30 min) with the AMPK inhibitor Compound C (20 $\mu$ M).

Data (n $\geq$ 3) were analyzed using one-way ANOVA. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001 (vs control)

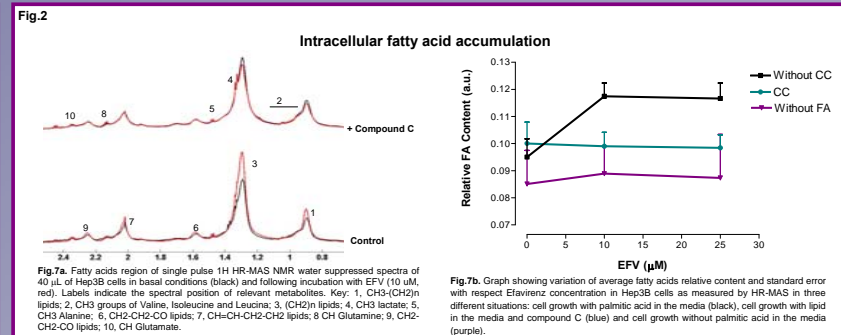
## REFERENCES

Forntz M. et al. *Diabetes*, 54: 1331-1339 (2005); *Hardie D.G.*, *Nat Rev Mol Cell Biol*, 8: 774-785 (2007); *Pettit F. et al.*, *Trends Pharm Sci*, 26: 258-264 (2005); *Villarroya F. et al.*, *Trends Pharm Sci*, 26: 88-93 (2005); *Viollet B. et al.*, *J Physiol*, 574: 41-53 (2006).

## RESULTS



Lipid droplets formation in Hep3B cells after 24h of incubation with EFV (10, 25 and 50  $\mu$ M), detected by static cytometry employing the fluorescent probe Nile red. (A) Representative histograms and images and (B) fluorescence intensity values obtained by static cytometry (n  $\geq$ 9).

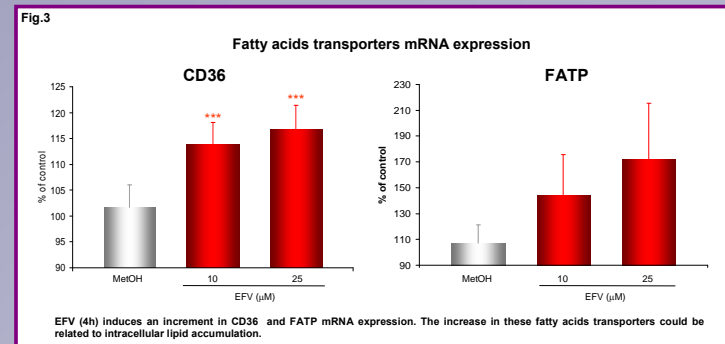


**Fig.7a.** Fatty acids region of single pulse 1H HR-MAS NMR water suppressed spectra of 40  $\mu$ L of Hep3B cells in basal conditions (black) and following incubation with EFV (10  $\mu$ M, red). Labels indicate the spectral position of relevant metabolites. Key: 1, CH<sub>3</sub>-(CH<sub>2</sub>)<sub>n</sub> lipids; 2, CH<sub>3</sub> groups of Valine, Isoleucine and Leucine; 3, (CH<sub>2</sub>)<sub>n</sub> lipids; 4, CH<sub>3</sub> lactate; 5, CH<sub>3</sub> Alanine; 6, CH<sub>2</sub>-CH<sub>2</sub>-CO lipids; 7, CH=CH-CH<sub>2</sub>-CH<sub>2</sub> lipids; 8, CH Glutamine; 9, CH<sub>2</sub>-CH<sub>2</sub>-CO lipids; 10, CH Glutamate.

**Fig.7b.** Graph showing variation of average fatty acids relative content and standard error with respect Efavirenz concentration in Hep3B cells as measured by HR-MAS in three different situations: cell growth with palmitic acid in the media (black), cell growth with lipid in the media and compound C (blue) and cell growth without palmitic acid in the media (purple).

Incubation of Hep3B cells with EFV for 4h induced a significant and moderate increase in total fatty acid. Other signals related to lipids components, like choline-containing compounds or unsaturated fatty acids, were not affected by EFV thus suggesting that the changes observed arise from an increase in saturated fatty acids moieties and do not respond to an alteration in membrane lipids metabolism (Fig.6a).

The spectral changes in fatty acids induced by the two doses of EFV employed (10 and 25  $\mu$ M) were not present if the media contained the inhibitor of AMPK Compound C. In addition, these increases were absent if Hep3B cells had been incubated in a media without palmitic acid (Fig.6b).



EFV (4h) induces an increment in CD36 and FATP mRNA expression. The increase in these fatty acids transporters could be related to intracellular lipid accumulation.

## CONCLUSIONS

Clinically used concentrations of EFV upregulate CD36 and FATP mRNA expression, thus facilitating fatty acids uptake. This drug also increases intracellular lipid content, which could have been a result of the formation of lipid droplets. This intracellular lipid increase was not present in cells treated with Compound C, suggesting a key role of AMPK in this processes.

Given that EFV treatment is usually prolonged, these mechanisms may affect the general regulation of lipid metabolism and could cause the alterations that are characteristic of lipodystrophy.