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ORIGINAL RESEARCH

# FK506 affects mitochondrial protein synthesis and oxygen consumption in human cells

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**Abstract** FK506 is an important immunosuppressive medication. However, it can provoke neurotoxicity, nephrotoxicity, and diabetes as adverse side effects. The decrease in oxygen consumption of rat cells treated with pharmacologically relevant concentrations of FK506, along with other evidences, has insinuated that some of the toxic effects are probably caused by drug-induced mitochondrial dysfunction at the level of gene expression. To confirm this suggestion, we have analyzed cell respiration and mitochondrial protein synthesis in human cell lines treated with FK506. This drug provokes an important decrease in oxygen consumption, accompanied by a slight reduction in the synthesis of mitochondria DNA-encoded proteins. These results are similar to those triggered by rapamycin, another macrolide with immunosuppressive properties, therefore insinuating a common toxic pathway.

**Keywords** FK506 · Mitochondria · Oxidative phosphorylation · Toxicity

## Introduction

*Streptomyces tsukubaensis* produces FK506. This compound showed antifungal activity against *Aspergillus fumigatus* and *Fusarium oxysporum* and, in mice, had important immunosuppressive properties (Kino et al. 1987). This drug was first introduced as a human immunosuppressive medication in 1989 (Starzl et al. 1989). FK506 binds to FK-binding protein (FKBP) 12. This complex inhibits the phosphatase activity of calcineurin, preventing the dephosphorylation of nuclear factor of activated T cell (NFATc), its nuclear translocation, and its interaction with different genes,

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such as the proinflammatory interleukin-2 gene. In this way, FK506 blocks T cell-mediated immune response because, in these cells, the activation of membrane receptors by antigens induces a calcium signal that activates the calcineurin. The chemical structure of FK506 is entirely different from that of the frequently used immunosuppressive cyclosporine A. Cyclosporine A binds cyclophilin, and this complex also acts as a calcineurin inhibitor (Prokai et al. 2012). Due to their impressive ability to improve short-term outcomes, these drugs are used in most of the immunosuppressive protocols to prevent graft rejection following organ transplantation. However, the toxicity related to calcineurin inhibitors is one of the main reasons for long-term failures. The most limiting adverse side effects of calcineurin inhibitors are neurotoxicity, nephrotoxicity, and diabetes (Serkova and Christians 2003). However, the nephrotoxic and diabetogenic effects of FK506 are five times higher than those of cyclosporine A (Prokai et al. 2012).

Studies using a magnetic resonance spectroscopy-based metabonomic approach indicate that toxicity due to calcineurin inhibitors is caused by drug-induced mitochondrial dysfunction. Indeed, comparison of the typical clinical symptoms of cyclosporine A neurotoxicity and mitochondriopathies, such as mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) and myoclonic epilepsy with ragged red fibers (MERRF), indicates an almost complete overlap. These mitochondrial diseases are usually caused by pathologic mutations in the mitochondrial DNA (mtDNA)-encoded tRNAs that are involved in the mitochondrial synthesis of proteins. Interestingly, mitochondriopathies can also cause kidney dysfunction and diabetes (Serkova and Christians 2003). It has been recently reported that insulin secretion and oxygen consumption were significantly decreased in rat INS-1 cells treated with pharmacologically relevant concentrations of FK506 (Rostambeigi et al. 2011).

Because the electron transport chain (ETC) of the oxidative phosphorylation (OXPHOS) system is the major oxygen consumer in the cell, since this system is the main ATP producer in most of the cells, and mitochondrial ATP is important for insulin secretion, it was insinuated that maybe some of the FK506 toxic effects were mediated by a mitochondrial dysfunction at the level of gene expression (Rostambeigi et al. 2011). To confirm this suggestion in humans, we have analyzed oxygen consumption and mitochondrial protein synthesis in human cell lines treated or not with FK506.

## Material and methods

### Cells and growth conditions

As it has been suggested that FK506 toxicity was mediated by mitochondrial dysfunction, to avoid specific effects of the mtDNA genetic background, we used six human osteosarcoma 143B cell lines with the same nuclear genetic background but different mtDNA genotype (AcH, 612H, 613H, 3K, 8K, and 48K from Gomez-Duran et al. 2010). Cells were seeded in Dulbecco's modified Eagle's medium (DMEM) with glucose (25 mM) and fetal bovine serum (FBS) (5 %). After 8 h, the medium was replaced by DMEM without glucose but with galactose (5 mM), pyruvate (1 mM), and FBS (5 %), and the cells were grown for 72 h. It has been shown that this growth medium stimulates mitochondrial biogenesis and oxidative metabolism and, therefore, would increase the OXPHOS effects of FK506 (Rossignol et al. 2004). We did not use penicillin–streptomycin in the growing media to avoid undesired phenotypic effects. The medium was renewed every 48 h. To evaluate the effects of different calcineurin inhibitors, cyclosporine A 800 ng/ml (665 nM) or FK506 50 ng/ml (62 nM) was incorporated in the culture medium. FK506, PROGRAF© 5 mg/ml Astellas Pharma S.A., was obtained from the Pharmacology Department at HUCA, National code 680678 (http://www.aemps.gob.es/).

### Oxygen consumption

In order to analyze oxygen consumption, we used the high-resolution oxygraph OROBOROS®. Exponentially growing cells were collected by trypsinization, washed, counted, and resuspended at $1.5\times10^6$ cells/ml. Endogenous, leaking (with oligomycin added at 49 nM), and uncoupled (with FCCP added at 1.2 μM) respiration analyses were performed according to previously published protocols (Gomez-Duran et al. 2010). The analysis of oxygen consumption was carried out in parallel for both conditions, non-treated and treated cell lines. Each cell line and condition was measured three times. Results were expressed as picomoles of $O_2$ per minute per million cells.

All non-treated cell lines had shown normal respiration rates (Gomez-Duran et al. 2010).

Analysis of respiratory complexes and citrate synthase

The respiratory complex IV (CIV) specific activity and quantity were measured by using Mitoprofile® Human Complex IV Activity and Quantity kit from Mitosciences according to the manufacturer's instructions. This kit immunocaptures CIV, and activity is determined colorimetrically by following the oxidation of reduced cytochrome c as an absorbance decrease at 550 nm. Subsequently, in the same well, the quantity of enzyme is measured by adding a CIV-specific antibody conjugated with alkaline phosphatase. CIV activities and quantities were normalized per milligram of protein, a surrogate of cell number or volume. Therefore, CIV activities and quantities were expressed as millioptical density per minute per milligram of protein. The p.MT-CO1/SDHA and SDHA/Janus Green ratios were measured using the MitoBiogenesis® In-Cell ELISA kit from Mitosciences according to the manufacturer's instructions. Human cells were seeded in 48-well microplates, and the levels of CIV p.MT-CO1 and CII SDHA subunits were measured simultaneously in each well. All these variables, along with the protein levels, were assayed by triplicate for each cell line and condition in at least three independent experiments following previously described protocols in a NovoStar MBG Labtech microplate reader (Gomez-Duran et al. 2010). The enzymatic activities of complexes I + III (CI + III) and citrate synthase (CS) were assayed, by triplicate for each cell line and condition, following previously described protocols with some modifications and normalized per milligram of protein (Ruiz-Pesini et al. 1998).

Mitochondrial protein synthesis

The mitochondrial protein synthesis was analyzed as described previously (Chomyn 1996; Gomez-Duran et al. 2010). Electrophoresis was performed with a PROTEAN II xi system. As a load control, we dyed the gel for 15 min with fixing solution (30 % methanol, 10 % acetic acid) plus 0.025 % of Brilliant Blue R (Coomassie blue). Then, the gel was washed several times with a 50 % methanol, 10 % acetic acid solution and left overnight in fixing solution. Finally, it was treated for 20 min with Amplify solution, dried, and used for autoradiography. The band intensities from appropriate exposures of the fluorograms were quantified by densitometric analysis with the Quantity One 1-D analysis software (Bio-Rad). Three bands, corresponding to p.MT-ND4, p.MT-CO3, and p.MT-ND3 polypeptides (upper, middle, and lower part of the gel, respectively), were selected for quantification.

Statistical analysis

The statistical package StatView 6.0 was used to perform all statistical analyses. Mean and standard deviation are represented. The normal distribution was checked by the Kolmogorov–Smirnov test. As all variables were normally distributed, the unpaired two-tailed *t* test was used to compare parameters. *P* values of $<0.05$ were considered statistically significant.

## Results

To confirm that FK506 toxic effects could be mediated by a dysfunction of the OXPHOS system, we analyzed the oxygen consumption in six human cell lines treated or not with FK506 (50 ng/ml) during 3 days. Endogenous and uncoupled, but not the leaking, respirations were significantly lower in human cells treated with FK506 than in non-treated cells (Fig. 1). The oxygen consumption was 35.1 (endogenous) and 32.2 % (uncoupled) inferior in treated than non-treated cells.

Oxygen is consumed in mitochondria. Therefore, a lesser number of mitochondria could be responsible for lower oxygen consumption. To get an idea of mitochondrial number or volume, we determined the specific activity of citrate synthase, a nucleus-encoded mitochondrial enzyme, commonly used for this purpose. There were no differences between treated and non-treated cells (Fig. 2).

Oxygen is reduced to water in CIV by electrons flowing through the ETC. ETC includes respiratory complexes I (CI), II (CII), III (CIII), IV (CIV, cytochrome oxidase or COX), and the protein cytochrome c. CI, CIII, and CIV, but not CII or cytochrome c, contain mtDNA-encoded polypeptides (7 out of 44, 1 out of 11, and 3 out of 14, respectively) (Balsa et al. 2012). FK506 is a macrolide. Some reports suggest that macrolides, such as erythromycin, can provoke inhibition of the mitochondrial protein synthesis when interacting with mitochondrial rRNAs (Doersen and

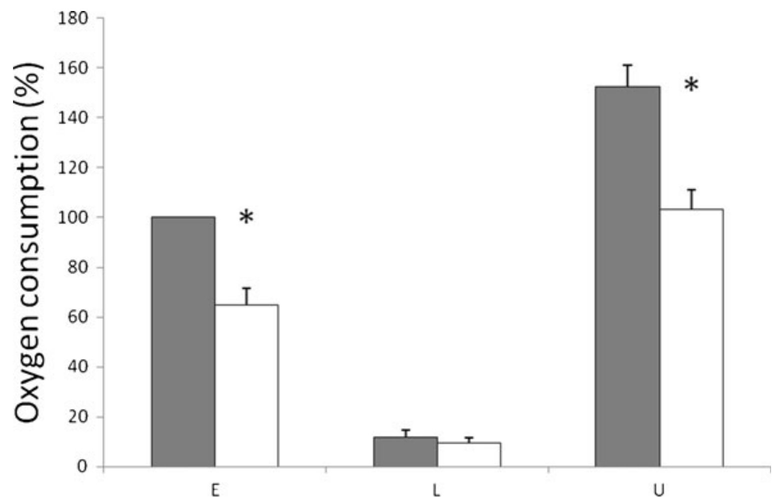


**Fig. 1** Effects of FK506 on oxygen consumption. *Gray* and *white bars* represent mean values for six non-treated and six FK506-treated cell lines, respectively. *E*, *L*, and *U* stand for endogenous, leaking, and uncoupled respiration, respectively. The mean value for endogenous respiration in non-treated cell lines was set to 100 % in order to assess respiration rate derived from the treatment. *$P<0.0001$ (unpaired two-tailed *t* test)

Stanbridge 1979, 1982; Luca et al. 2004). Therefore, it would be possible that FK506 would affect oxygen consumption by decreasing the translation of mtDNA-encoded ETC subunits. Because important subunits of CI, CIII, and CIV are coded in the mtDNA, we determined CI + III and CIV activities in cells treated with FK506. However, we did not find differences in the specific activities of these ETC complexes when compared to non-treated cells (Fig. 2); neither did we found them in CIV quantity or SDHA levels, a CII polypeptide (Fig. 2). This result suggested that FK506 had no effect on mitochondrial translation of proteins.

To confirm that the synthesis of mtDNA-encoded polypeptides was not altered, we performed mitochondrial protein synthesis assays before and after treatment with FK506. Surprisingly, we observed a small but significant decrease in the amount of mitochondrial translation products (Fig. 3). Metabolic labeling to estimate mitochondrial protein synthesis in cultured

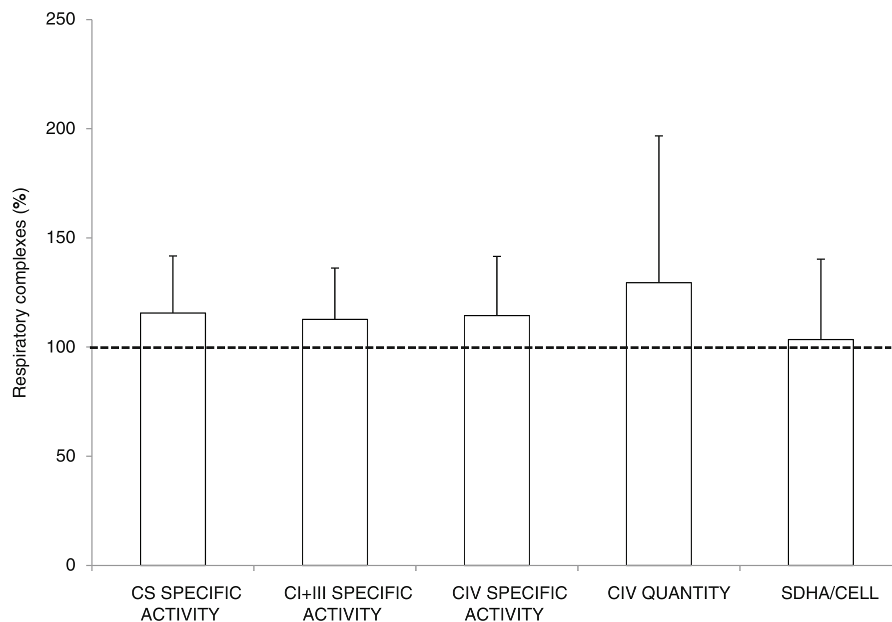


**Fig. 2** Effects of FK506 on citrate synthase and respiratory complexes. *White bars* represent the percentage of change in the respiratory complexes and citrate synthase in six FK506-treated cell lines. The mean value for six non-treated cell lines was set to 100 % and is showed as a *dashed horizontal line*

cells is a semiquantitative technique (Moreno-Loshuertos et al. 2011). Therefore, with the aim of ratifying this result, we quantitatively determined the levels of p.MT-CO1, the CIV subunit that directly reduces the oxygen to water, and normalized them to those of SDHA. This p.MT-CO1/SDHA ratio showed, again, a small but significant decrease, thus confirming a slight effect of FK506 on mitochondrial protein synthesis (Fig. 3).

To check whether this action of FK506 on OXPHOS function was, in part, responsible of its more toxic effect than cyclosporine A, we determine the p.MT-CO1/SDHA ratio of cells treated with cyclosporine A (800 ng/ml). This cyclosporine A concentration had a comparable impact, to that of FK506, on cell proliferation and viability and insulin secretion and cell content of INS-1 cells (Rostambeigi et al. 2011); however, it did not appear to affect oxygen consumption in HepG2 cells (Luo et al. 2012). This p.MT-CO1/SDHA ratio in cells treated with cyclosporine A was not different from that of control cells (Fig. 3).

The effect of FK506 on ETC has been studied in six cybrid cell lines from two different mtDNA genetic backgrounds, haplogroups H (AcH, 612H, and 613H) and Uk (3K, 8K, and 48K). To check if these mtDNA genotypes influenced the ETC effects of FK506, we compared all previously analyzed parameters in both haplogroups, but we did not find significant differences between them (data not shown).

## Discussion

An analysis of $CO_2$ production from D-[$^{14}$C]glucose or [2-$^{14}$C]pyruvate in FK506-treated versus control rat enterocytes showed significant reductions in $CO_2$ released from treated cells, thus indicating a mitochondrial dysfunction (Madsen et al. 1995). Moreover, the oxygen consumption in rat INS-1 cells after 72 h of treatment with pharmacologically relevant concentrations (50 ng/ml) of FK506 was approximately a third lower than that from the non-treated cell line (Rostambeigi et al. 2011). We have found a similar decrease of oxygen consumption in human cell lines treated during 72 h with FK506 50 ng/ml. The lack of differences in leaking respiration indicates that this drug does not affect electron slipping or proton leaking processes. On the other hand, the significant differences in endogenous and uncoupled respiration suggest that treated cells contain lesser ETC capacity because of a lower ETC quantity or a direct inhibition of the electron transport.

The toxic effects of the macrolide FK506 could be due to its interaction with the mitochondrial ribosome and the inhibition of organelle protein synthesis, decreasing the ETC quantity and then impairing oxygen consumption and many other cell functions dependent on the OXPHOS system (Martínez-Romero et al. 2011). In fact, the amount of mtDNA-encoded subunits and the p.MT-CO1/SDHA ratio of human cell lines treated with FK506 is 5 % lower than that of non-treated cell lines. A small decrease in mitochondrial protein synthesis accompanied by a big decrease in the oxygen consumption has been previously reported for other drugs, such as calmodulin antagonists (Vijayasarathy et al. 1993). A decrease in the level of expression of a given subunit did not necessarily lead to a proportionate (if any) decrease in the activity of the corresponding respiratory chain complex (Rossignol et al. 2003). The expression and steady-state amounts of a particular respiratory complex subunit are, maybe in some conditions, higher than those required to assemble a normal amount of that complex. This fact would explain why a decrease in p.MT-CO1 expression and steady-state amounts in FK506-treated cells are not mirrored in a difference in CIV activity or quantity. However, the absence of differences in CI + III and CIV specific activities or CIV levels between treated and untreated cells, similar to previous experiments in human umbilical endothelial cells (Illsinger et al. 2011), appears to rule out that a lower ETC quantity was responsible for the decrease in oxygen consumption. As CII has no mtDNA-encoded polypeptides, then SDHA levels can be used as a marker of mitochondrial number or volume. Thus, our results suggest that the mitochondrial density was not affected by the FK506 treatment, contrary to that observed in rat INS-1 cells (Rostambeigi et al. 2011) but similar to that observed in human umbilical endothelial cells (Illsinger et al. 2011). This fact was also confirmed by the absence of differences in CS specific activity. On the other hand, the significant differences in respiration could be due to a direct inhibition of the ETC by FK506. In fact, it had been previously shown that FK506 partly blocked CIII activity (Simon et al. 2003; Zini et al. 1998). However, our results on CI + III specific activities rule out this possibility.

Despite an important decrease in oxygen consumption, our results appear to preclude an important implication

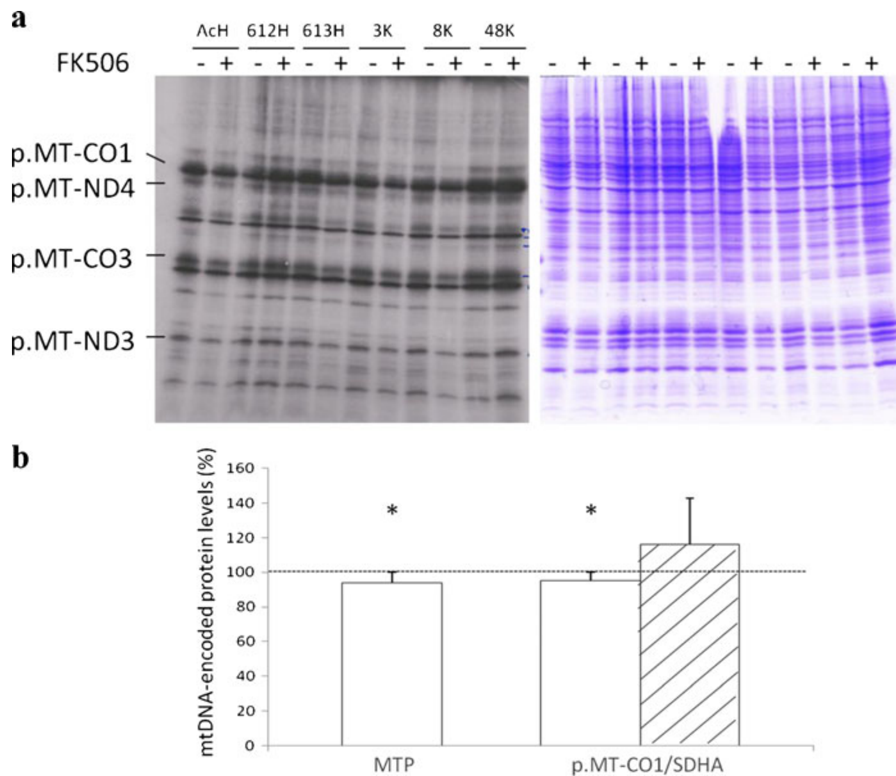


**Fig. 3** Effects of FK506 on mitochondrial protein synthesis. **a** *Left panel*. Gel showing the electrophoretic patterns of mitochondrial translation products (*MTP*), in which the bands of three mtDNA-encoded polypeptides used for quantification (*p.MT-ND4*, *p.MT-CO3*, and *p.MT-ND3*) are indicated. We also show the p.MT-CO1 band to compare it with the quantitative assay results (see Fig. 3b). The name of each cell line used is provided. *Right panel*. Loading control from the six non-treated (−) and FK506-treated (+) cell lines. **b** Quantification of mitochondrial protein synthesis. The MTP (Fig. 3a, *left panel*) were normalized using a densitometry of the Coomassie staining pattern (Fig. 3a, *right panel*). *White* and *stripped bars* represent mean values for FK506- and cyclosporine A-treated cell lines, respectively. The mean value for non-treated cell lines was set to 100 % and was shown as a *dashed horizontal line*. *$P \leq 0.0433$ (unpaired two-tailed *t* test)

of ETC in the side effects of FK506. However, in addition of ETC, the OXPHOS system also includes the ATP synthase or complex V (CV). Individuals with mtDNA mutations affecting CV subunits often show normal specific activities of the ETC complexes (Thorburn et al. 1993). However, cells with these mutations have lower oxygen consumption (Trounce et al. 1994). Very interestingly, it has been shown that FK506 inhibits oxygen consumption and CV activity of mitochondria from the rat brain (Zini et al. 1998). FK506 also decreased the expression of rat aortic ATP synthase gamma chain (Zamorano-Leon et al. 2012). Therefore, it would be possible that some FK506 side effects were mediated through its interaction with CV.

Rapamycin is another macrolide with immunosuppressive activity. FK506 and rapamycin share a chemical domain that allows them to bind FKBP12. Other parts of these molecules are different and enable FK506 or rapamycin, in conjunction with surface residues from FKBP12, to interact with calcineurin or the mammalian target of rapamycin (mTOR), respectively (Dumont and Su 1996). Rapamycin, an mTOR inhibitor, decreases endogenous and uncoupled respiration in Jurkat T cell leukemia clone E6-1 cells (Ramanathan and Schreiber 2009; Schieke et al. 2006). Moreover, in the C2C12 mouse myoblast cell line, rapamycin decreased the expression of several transcriptional regulators important for mitochondrial biogenesis, such as PGC-1$\alpha$, resulting in a decrease in the expression of nucleus-encoded mitochondrial proteins, such as cytochrome c, COX5a, or ATP5g1, and oxygen consumption (Cunningham et al. 2007). In human prostate cancer cell lines, rapamycin also decreases the

expression of ATP5g1 gene that codes for a subunit of CV (van der Poel et al. 2003). Interestingly, PGC-1$\alpha$ was also downregulated in FK506-treated rat INS-1 cells (Rostambeigi et al. 2011). Thus, perhaps the FK506 toxic effects may be due to a mitochondrial dysfunction mediated by the mTOR pathway. In fact, it has been already shown that chronic administration of FK506 decreased mTOR activity (Yu et al. 2013).

It has been previously shown that side effects were noted in connection with 76 % of FK506 concentrations above 30 ng/ml. Thus, FK506 concentrations should preferably be kept below 20 ng/ml (Bottiger et al. 1999). In humans, an increased ratio of blood 3-hydroxybutyrate to acetoacetate observed in liver transplant recipients treated with FK506 relative to the healthy control subjects implied a more reduced hepatic intramitochondrial redox state, i.e., a lower capacity of NADH oxidation by the ETC. The mean plasma FK506 level at the time of these metabolic studies was 0.5 nM (Krentz et al. 1993). These results, along with the lower oxygen consumption and mitochondrial translation of human cells treated with FK506, make it reasonable to suggest that OXPHOS defects contribute to FK506 toxicity.

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