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RESTRICTING OBESITY: ANTI-S6K1 THERAPY

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1. Summary of the project

The epidemic in obesity is a major worldwide health problem in adults and now children. Importantly, inhibiting the recruitment of adipocyte derived mesenchymal stem cells (ASCs) to fat depots has been identified as an opportunity to treat obesity. In parallel, it is known that mice lacking the S6K1 gene have a lesion in adipogenesis due to a defect in the ability to induce the commitment of ASCs to the adipogenic lineage. Recent studies have now shown that mediators of adipogenesis normally induce S6K1 to translocate into the nucleus of ASCs and trigger an adipogenic transcriptional program mediated by a phosphorylation/methylation cascade of specific histones. This cascade blocks Wnt gene expression and favors the adipogenic lineage. Preliminary analyses of liposuction samples from patients show direct correlation between the extent of obesity and the activation of the phosphorylation/methylation cascade, such that therapeutic intervention with S6K1 inhibitors offers a potential avenue for the treatment of obesity. To perform these studies, we have used a specific inhibitor of S6K1, LY2584702 tosylate (LY). For in vitro cell culture studies in human cells we employed SPF2 and SPF3 pre-adipocytes or bone marrow stem cells (hBMSC). For the in vivo studies we used C57BL6 mice. Due to space limitations only selected experiments are depicted, however, all results have been submitted for publication and those not depicted here are indicated as submitted.

2. Results obtained

We evaluated in C57BL6 mice the impact of LY, an oral inhibitor of S6K1, initially developed for the treatment of solid tumors. This drug increases phosphorylation of S6K1 at T389, due to the competitive inhibition of ATP in molecular docking with the active site of S6K1, thus blocking its kinase activity. In the first experiment, mice were subjected to 25 and 50 mg/kg dosages of LY. Analysis of the adipose tissue on western blots showed that mice reached the same degree of inhibition of S6 phosphorylation with both LY concentrations of 25 and 50mg/kg for 2hs, thus the lower dosage was sufficient (Fig.1, left). Adipose tissue and liver from mice receiving 25mg/kg sacrificed at different time points were extracted and analyzed by western blot, revealed that this response lasted ≤12h (Fig.1, right).

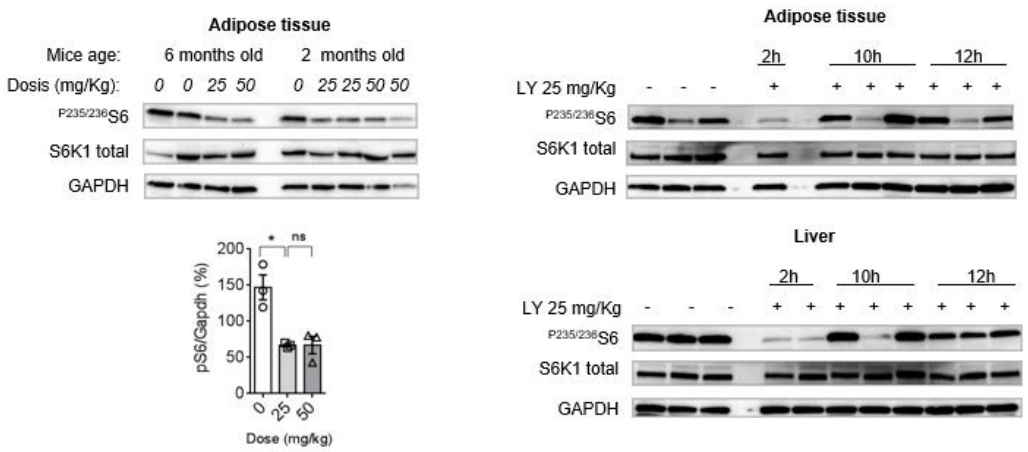


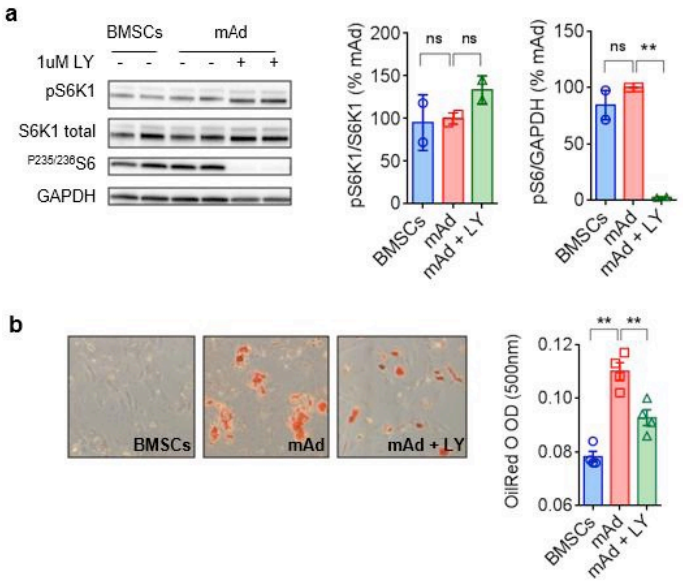
Figure 1. Assessment of the impact two dosages (i.e., 25 and 50 mg/kg) of orally administrated LY on subcutaneous fat samples from 3 and 6 month-old male mice. Phosphorylated (p)S6 and total S6K1, normalized against GAPDH, show no significant differences. Time course assessment of drug-related S6K1 inhibition in adipose and liver indicated ≤12h-lasting effects.

We next tested human cells in culture: The hASCs employed, SPF2 and SPF3 pre-adipocytes were purchased from ZenBio and used to test LY. We found that concentrations of 1μM and 5 μM were sufficient to inhibit S6 phosphorylation. However, despite inhibition of S6K1 these pre-adipocytes differentiated into adipocytes in the presence of 1μM LY as measured by Oil Red O staining. We concluded that these pre-adipocytes were already committed along the adipogenic lineage, so cells at an earlier stage of differentiation were required, i e. mesenchymal stem cells from bone marrow to induce them to commit along the adipogenic lineage.

To analyse the inhibition on fat cell differentiation, human bone marrow stem cells (hBMSC) were induced to differentiate into adipocytes under LY treatment at 1uM to inhibit S6K1. Western blot analysis for the phosphorylation status of S6K1 and S6 confirmed their inhibition (**Fig.2a**). The reduced amount of lipid found under treatment by Oil red O staining showed LY antiadipogenic effect (**Fig.2b**). In parallel, cells from mouse hepatoma cell line Hepa1-6 were induced to accumulate lipid using a combination of palmitate-oleate (PA/OA), mimicking HFD-induced hepatosteatosis, a common side effect of obesity *in vivo*, and were treated with LY (PA/OA+LY) for 24h. Phosphorylation of S6, Akt, the amounts of lipid (O-red Oil staining), intracellular triacylglyceride (TG), and the expression of preselected target genes were quantified. As expected, LY diminished pS6 in these cells, while pS6K1 was not affected as LY is a specific allosteric inhibitor of S6K1, without effects on its ATP binding site (**Fig.2c**),

thus upstream activation as measured by its phosphorylation status is on, but its ability to phosphorylate substrates, including S6, is blocked. In some cases, it appears that S6K1 becomes hyperphosphorylated. Although the macroscopic measurements of lipid staining did not confirm significant variations, there appeared by eye to be a decrease (**Fig.2d**), consistent with the quantification of TG revealing a significant reduction of lipids in treated cells (**Fig.2e**). Of note in agreement with inhibition of S6K1, hepatocytes treated with PA/OA+LY, showed increased amounts of pAkt (**Fig.2c**) and gene expression patterns coincident with results *in vivo*, namely decreased *Cyp4a12a, Ffar4, Lgals1, Mlkl, and Tlr8* (**Fig.2f**).

Next, we assessed the drug in the obese mouse phenotype, comparing control mice fed with normal chow (NC) to mice fed HFD and treated with either vehicle (veh) or LY in a one-week long experiment (NC+veh, NC+LY, HFD+veh, HFD+LY), which confirmed that treatment was well tolerated in all study groups.



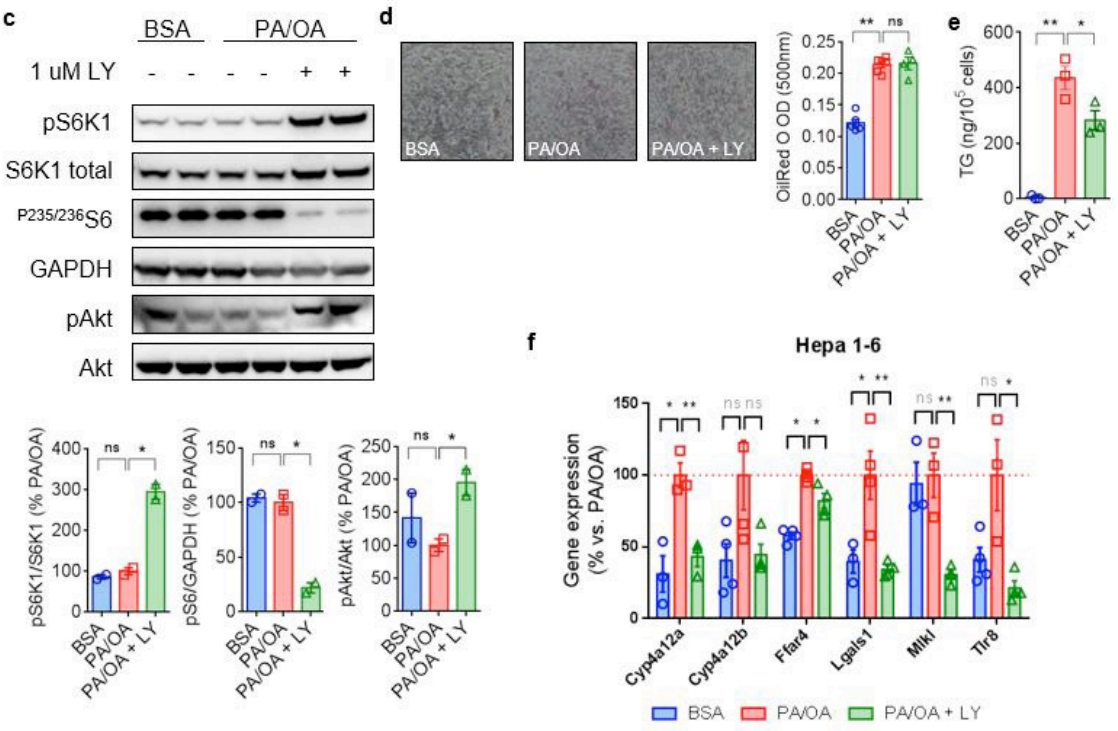


Figure 2. (a) Phosphorylated (p)S6K1, S6K1, pS6, and GAPDH (loading control) levels (WB), and relative quantification, taking "mature" adipocytes (mAd) as control. BMSCs stands for undifferentiated bone marrow stem cells. **(b)** Representative Oil O-Red staining images and quantification of the lipid content of bone marrow stem cells when under non-differentiated conditions (hBMSCs), or differentiated with (mAd+LY) or without (mAd) 1μM LY3584703 added to the differentiation media (n=4). **(c)** In hepatoma Hepa1-6 cells cultured in vitro and challenged with palmitate/oleate (PA/OA), inducing steatosis, 1uM of LY3584703 also compromised p70S6K function, but did not reduced the amounts of lipid, as shown by **(d)** Oil O-Red staining. However, there were significant changes regarding **(e)** the triglyceride content, and **(f)** measures of gene expression for target genes mirroring the impact of the drug in liver of HFD+LY mice. Data is presented as mean ± SEM. Statistical significance was determined by Student t-test. *p-value <0.05, ** p-value <0.01, "ns" as not significant.

We next assessed the pharmacological effect of inhibiting S6K1 in adult mice subjected to diet-induced obesity. In order to characterize the ability of the S6K1 inhibitor LY in counteract high-fat diet (HFD), eight-month-old C57BL6N male mice were challenged with 60% HFD and orally treated with LY (HFD+LY) during 10 weeks. In parallel, mice undergoing either HFD or normal chow (NC) were used as reference groups. Remarkably, HFD-fed mice receiving LY (HFD+LY) gained significantly less body weight and accumulated lower amounts of subcutaneous adipose tissues than their counterparts receiving the HFD **(Fig. 3a)**. To gain insight into the metabolic consequences of this treatment, we measured the concentrations of circulating glucose, triglycerides and cholesterol at fasting. Intraperitoneal glucose tolerance tests (IPGTT) were performed in NC, HFD and HFD+LY mice. Notably, the HFD+LY group

exhibited a significant downregulation of circulating triglycerides (TG) with regard to HFD mice. However, circulating cholesterol, fasting glucose, and the IPGTT revealed no major differences between obese animals under HFD and those treated with the drug (**submitted**). After sacrifice, tissues were extracted and the phosphorylation status of S6, S6K1 and Akt were assessed by western blot, confirming the proper inhibition of S6K1 in all analysed tissues adipose and liver (**submitted**). In parallel, haematoxylin and eosin (H&E) staining was performed in samples of epididymal adipose tissue, and morphometric analysis revealed a rearrangement of adipocyte sizes in HFD-fed mice treated with LY**(Fig.3b)**, as they showed a lower main adipocyte area and less hypertrophy than the HFD group **(Fig.3c)**. Also in liver samples, H&E revealed apparent changes in the lipid content, as further shown by the amounts of triglycerides (TG) and total cholesterol, both being significantly depleted in HFD+LY to the levels found in NC-fed lean mice (**submitted**).

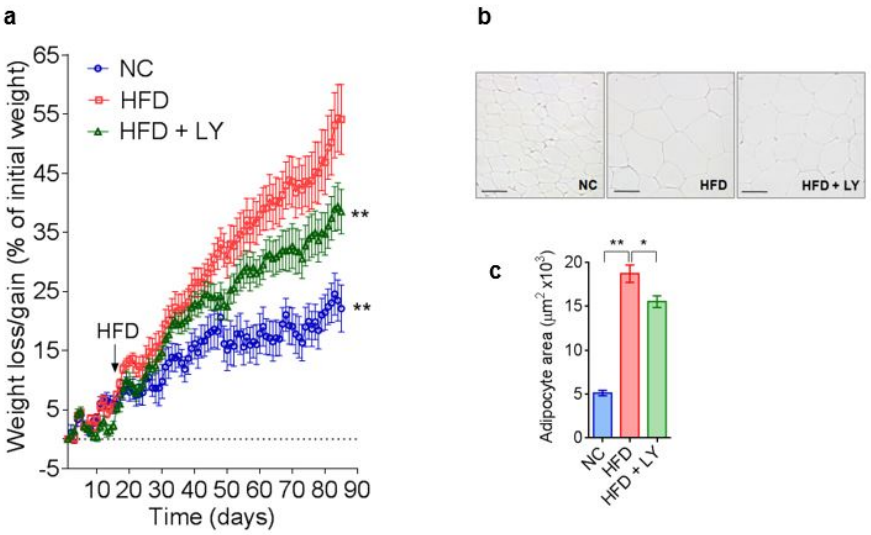


Figure 3: (a) Weight gain curve of mice under normal chow (NC), high-fat diet (HFD), and HFD plus oral gavage (35 mg/kg/12h) of LY (HFD+LY). **(b)** Representative images and **(c)** quantification of subcutaneous adipose tissue (SAT) samples of NC, HFD and HFD+LY mice. Statistical significance was determined by Student t-test. *p-value <0.05, ** p-value <0.01.

A microarray and a gene set enrichment analysis (GSEA) was assessed in adipose tissue (SAT) visceral adipose tissue (VAT) and liver, of mice fed a HFD and treated with the drug (**submitted**). A list of genes differentially expressed was obtained in SAT and liver of obese mice treated with the drug, when compared to obese controls. Using, GSEA and gene ontology (GO) term enrichment, on expression patterns in adipose

tissue, we identified biological pathways that were widely attenuated. Among them, adipogenesis, epithelial-mesenchymal transition, and mTORC1 signalling, were most clearly challenged by the drug. Validation of microarray results by real time-PCR disclosed that genes related to the lipid biosynthesis (e.g., *Cyp2e1*, *Fasn*, *Elovl6*) were inhibited. In addition, genes related to fatty acid uptake (*Cd36*, *Fabp5*), epithelial-mesenchymal transition (*Lgals1*), inflammatory issues (*Gsto1*, *S100a8*, *Tlr8*), and the control of fatty acids biosynthesis (*Srebf1*, *Insig1*), were also less expressed in SAT and VAT of HFD+LY mice, when compared to HFD-fed mice, partially mirroring the expression patterns revealed in NC-fed lean mice (**submitted**). These results confirm the potential of reducing the adipogenic commitment and adipocyte hypertrophy in LY-treated mice, thus inhibiting the onset of obesity by a compound targeting S6K1 in adipose tissue (**submitted**). In contrast, gene expression patterns in liver samples pointed at distinctive variations that included significant effects related to cholesterol homeostasis and mTORC1 signalling (increased), and the downregulation of transcripts associated with epithelial-mesenchymal transition, angiogenesis (**submitted**), and inflammatory response of obese mice under treatment amongst others. Of note, the expression of genes related to fatty acid biosynthesis (e.g., *Cyp4a12a, Cyp4a12b, Cyp2e1*, *Pparg*) and fatty acid uptake (*Ffar4*, *Cd36*) in liver, were compromised by LY. In addition, the synthesis of mRNAs related to the epithelial-mesenchymal transition (*Lgals1*), and those representative of the Inflammatory response (*Mlkl*, *Tlr8*) were also downregulated, mirroring to some extent what we found in fat samples. An apparent overexpression of genes related to mTORC1 signalling in HFD+LY mice was consistent in liver tissue and opposite to the adipose tissue of the same specimens, suggesting the activation of different signalling cascades in fat and liver (**submitted**).

Next a lipidomic analysis was performed on liver samples of these mice by liquid chromatography and mass spectrometry to further evaluate therapeutic changes in the TG content and other lipid species related to fatty liver disease in HFD-fed mice. Strikingly, many TG species that were increased in HFD when compared to NC mice (e.g. TG 49:1-3, 50:1-4, 51:1-2, 53:2, 54:2) were significantly reduced in the HFD+LY group. Indeed, significant reduction of the global amount of TG in HFD+LY mice was consistent with measures taken in serum and liver samples, and also in Hepa1-6 cells (**submitted**). In HFD+LY mice different phospholipids, including phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine-ethers (PC O), showed significant variations that were opposite to those reported in

HFD when compared to NC mice (**submitted**). Specifically, PC O appear to be prevalent in NC-fed mice, as four species were detectable in these liver extracts (PC O-34:1, 36:4, 36:5 and 38:5), and only one species (PC O-38:5) was found in HFD-fed mice. Our results in HFD+LY mice, highlighted the presence of two species of PC O-36:5 and 38:5. Therefore, the ratio PC/PE, which has been related to the progression of steatosis to steatohepatitis, was significantly decreased in HFD-fed mice when compared to NC controls, and partially restored by LY in HFD+LY mice (**submitted**).

These results indicate that LY protects against diet-induced obesity and leads to a decrease in fat mass and deposition of fat in the liver, thus reducing dyslipidaemia in obese mice. Overall, these pioneering findings define the potential of this compound as efficacious therapeutic agent to relieve the burden of dyslipidaemia and fatty liver in obese patients.

3. Relevance and possible future applicability

Obesity is a worldwide epidemic that until recently was largely confined to industrialized countries of the Western world but is now also on the ascent in urban centres of underdeveloped nations. The key factor in promoting the obese state is nutrient overload combined with the innate drive to acquire and store fat in adipose depots. The recent identification of early adipocyte progenitors within the vascular niche of adipose tissue, gave an opportunity to analyse adipocyte progenitors with respect to their molecular makeup and role in adipocyte development. The role of the mTORC1/S6K1 pathway has been extensively studied in the literature, being implicated in the regulation of a multitude of metabolic pathways. Of importance is its role in regulating the processes related to adipogenesis and in the regulation of fat depots. We previously showed that loss of S6K1 led to strong lesion in the ability of stem cells of the adipocytic lineage to be recruited to fat depots, being mice lacking this protein protected against diet-induced obesity.

To date, this is the first study that uses a pharmacological compound (LY3584703 tosylate) directed to S6K1 as a therapeutic target to reverse the onset of obesity, conducting a first study to see its viability in living beings and accompanying these results of studies at the molecular level to assess through which pathways the drug is

having its effect. We can confirm the effectiveness of the compound reducing the formation of fat mass under a 60% high-fat diet and the accumulation of fat in the liver, modifying its lipid species composition, and accompanied with a reduction of the dyslipidaemia that subjects develop under these conditions. The reduction of adipogenesis is consistent with the striking finding that in vitro, human bone marrow derived mesenchymal stem cells differentiating to mature adipocytes under LY treatment, accumulated less amount of lipid compared to control mature adipocytes. These results provide evidence that specific inhibitors of S6K1 could be used for the management and cure of obesity, and its associated pathologies, bringing promising results to the applicability in the clinics. It remains to be seen what future studies should be developed, either with other inhibitory drugs or implementation studies in humans, as possible therapy to help reduce and control the development of this disease and all the complications that derive from it.

4. Generated bibliography

As a result of the realization of this project, which has been possible thanks to the financing of the La Marató de TV3 Foundation, a large number of results and knowledge have been obtained, which have been collected in the manuscript that is currently under review:

"A compound directed against S6K1 hampers fat mass expansion and mitigates diet-induced hepatosteatosis." Aina LLUCH, Sonia R. VEIGA, Jèssica LATORRE, José M. MORENO-NAVARRETE, Núria BONIFACI, Ruth RODRÍGUEZ-BARRUECO, David LLOBET-NAVAS, Gerhard LIEBISCH, You ZHOU, Vesa M. OLKKONEN, George THOMAS, José M. FERNÁNDEZ-REAL*, Sara C. KOZMA*, Francisco J. ORTEGA*.