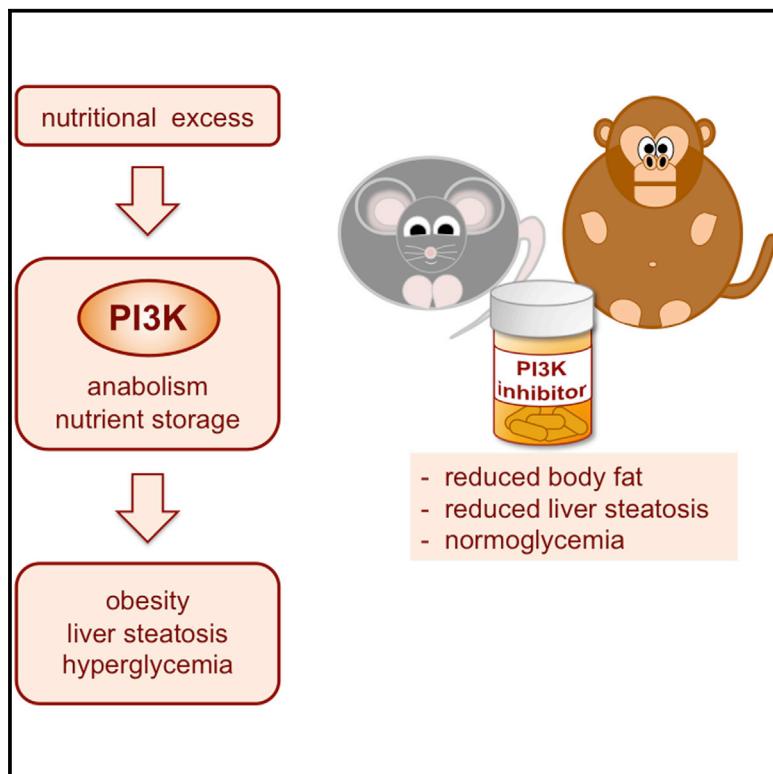


Cell Metabolism

Pharmacological Inhibition of PI3K Reduces Adiposity and Metabolic Syndrome in Obese Mice and Rhesus Monkeys

Graphical Abstract



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In Brief

Ortega-Molina et al. investigate pharmacological PI3K inhibition in obese mice and rhesus monkeys, and conclude that pharmacological inhibition of PI3K is an effective and safe anti-obesity intervention that reverses metabolic syndrome.

Highlights

- Treatment of obese mice with PI3K inhibitors reduces obesity and metabolic syndrome
- Weight loss induced by PI3K inhibitors is due to a decrease in adiposity
- Chronic PI3K inhibition did not result in drug resistance or toxic effects
- Treatment of obese monkeys with PI3K inhibitors is safe and reduces adiposity

Pharmacological Inhibition of PI3K Reduces Adiposity and Metabolic Syndrome in Obese Mice and Rhesus Monkeys

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SUMMARY

Genetic inhibition of PI3K signaling increases energy expenditure, protects from obesity and metabolic syndrome, and extends longevity. Here, we show that two pharmacological inhibitors of PI3K, CNIO-PI3Ki and GDC-0941, decrease the adiposity of obese mice without affecting their lean mass. Long-term treatment of obese mice with low doses of CNIO-PI3Ki reduces body weight until reaching a balance that is stable for months as long as the treatment continues. CNIO-PI3Ki treatment also ameliorates liver steatosis and decreases glucose serum levels. The above observations have been recapitulated in independent laboratories and using different oral formulations of CNIO-PI3Ki. Finally, daily oral treatment of obese rhesus monkeys for 3 months with low doses of CNIO-PI3Ki decreased their adiposity and lowered their serum glucose levels, in the absence of detectable toxicities. Therefore, pharmacological inhibition of PI3K is an effective and safe anti-obesity intervention that could reverse the negative effects of metabolic syndrome in humans.

INTRODUCTION

The phosphatidylinositol 3-kinase type I (PI3K) family is at the center of the most conserved aging-inducing pathway in evolution. Indeed, partial genetic attenuation of the PI3K signaling cascade at multiple levels results in lifespan extension in worms, flies, and mice (Barzilai et al., 2012; Fontana et al., 2010; Kenyon, 2010). In mammals, the PI3K family comprises four related lipid kinases (α , β , γ , δ) that respond to multiple receptors by generating the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which in turn has a wide range of cellular

effects (Vanhaesebroeck et al., 2010). In support of the role of PI3K in promoting aging, genetically engineered mice with decreased $\text{PI3K}\alpha$ activity or with decreased overall PI3K signaling (due to overexpression of the PIP_3 phosphatase PTEN) are long lived (Foukas et al., 2013; Ortega-Molina et al., 2012). Furthermore, dietary restriction (DR) extends lifespan in most tested animal models, and this is partly mediated in worms and flies by reduced PI3K pathway activity (Fontana et al., 2010). In rhesus monkeys, DR extends healthspan (Mattison et al., 2012), and it may also extend longevity (Colman et al., 2009). Finally, in humans, there is evidence that DR improves health (Cava and Fontana, 2013), and it also downregulates the PI3K pathway (Mercken et al., 2013).

At the opposite end of the beneficial effects of reduced PI3K and DR are the detrimental effects of hypercaloric and fat-rich diets. Hypercaloric diets initially result in obesity due to the storage of the extra energy in the adipose tissue. However, the continuous caloric overload eventually results in the aberrant accumulation of lipids in non-adipose tissues (Virtue and Vidal-Puig, 2010). The direct pathological consequence of chronic hypercaloric diets is a multi-systemic deterioration known as metabolic syndrome, which is characterized by insulin resistance, liver steatosis, atherogenic cardiovascular disease, dyslipidemia, and systemic inflammation (Kaur, 2014). Of note, the comorbidities associated with metabolic syndrome overlap with some of the most important aging-associated diseases, namely diabetes, cardiovascular and cerebrovascular diseases, and cancer (Gurevich-Panigrahi et al., 2009; Pi-Sunyer, 2009).

A substantial amount of evidence indicates that PI3K plays an important role in setting the balance between nutrient storage and nutrient consumption. In particular, mice with systemic overall reduction of PI3K signaling (due to *PTEN* overexpression) have increased energy expenditure and are protected from obesity and from metabolic syndrome (Garcia-Cao et al., 2012; Ortega-Molina et al., 2012). The inhibition of single PI3K isoforms may also achieve similar metabolic effects, as is the case of mice with partial decrease of $\text{PI3K}\alpha$ activity (Foukas et al., 2013), complete loss of $\text{PI3K}\gamma$ activity (Becattini et al., 2011; Kobayashi et al., 2011), combined complete loss of

PI3K γ and PI3K β activities (Perino et al., 2014), and liver-specific complete loss of PI3K α (Chattopadhyay et al., 2011). Finally, the role of PI3K in human obesity has received direct experimental support from the observation of a strong association between hyperactive PI3K signaling due to germline *PTEN* haploinsufficiency and obesity (Pal et al., 2012).

In summary, separate lines of research on longevity, dietary restriction, obesity, and metabolic syndrome have converged on the concept that moderate downregulation of PI3K signaling activity has the potential to improve health and provide protection from obesity and from its associated diseases. Following on this, it is of great importance to determine the potential benefits of pharmacological treatments that reduce PI3K activity. Here, we show that PI3K inhibitors, at low doses, can safely reduce obesity and ameliorate metabolic syndrome in obese mice and monkeys.

RESULTS

Effects of CNIO-PI3Ki on Glucose Homeostasis

In this work, we use two small compounds with high inhibitory potency and selectivity against PI3K. GDC-0491 (also known as pictilisib) is a well-characterized inhibitor of PI3K α (IC_{50} 3 nM), PI3K δ (IC_{50} 3 nM), PI3K β (IC_{50} 33 nM), and PI3K γ (IC_{50} 75 nM), with no relevant inhibitory activity against other kinases, minimal access to the brain, and a good pharmacokinetic profile (Salphati et al., 2012; Workman et al., 2010). We also use CNIO-PI3Ki, an inhibitor developed at the Spanish National Cancer Research Center (CNIO) and already used in previous reports (Muñoz-Espín et al., 2013; Ortega-Molina et al., 2012). CNIO-PI3Ki inhibits PI3K α (IC_{50} 2.4 nM), PI3K δ (IC_{50} 2.5 nM), and PI3K γ (IC_{50} 44 nM) (Figure S1A), it shows no relevant inhibitory activity against 284 tested kinases including mTOR (Figure S1A; Table S1), has minimal access to the brain (Figure S1B), and has a good pharmacokinetic profile in mice (Figure S1C). Of note, the half-life of the elimination phase of CNIO-PI3Ki by oral administration is 4 hr in mice, thus allowing for a substantial period of activity upon administration (Figure S1C).

The beneficial metabolic effects of overall PI3K signaling reduction, or isoform-specific genetic inhibition of PI3K γ and PI3K β , have been attributed to an increase in energy expenditure, and thereby to an attenuation of nutrient storage in favor of nutrient consumption (Becattini et al., 2011; Garcia-Cao et al., 2012; Ortega-Molina et al., 2012; Perino et al., 2014). Several tissues and mechanisms can conceivably contribute to the increased energy expenditure associated with PI3K inhibition. We have previously shown that GDC-0941 and CNIO-PI3Ki increase thermogenesis (i.e., energy expenditure) in cultured brown adipocytes (Ortega-Molina et al., 2012). Given the fact that GDC-0941 and CNIO-PI3Ki inhibit several PI3K isoforms, we used shRNA inhibition to identify the isoform responsible for the regulation of thermogenic activity in brown adipocytes. Only shRNA inhibition of PI3K α , but not of other PI3Ks, resulted in upregulation of *Ucp1* and *Pgc1 α* mRNAs in forskolin-stimulated brown adipocytes (Figure S1D). This finding reinforces the concept that PI3K α inhibition is a relevant mediator of the link between PI3K and metabolism, which is in agreement with previous observations in mice with partial decrease of

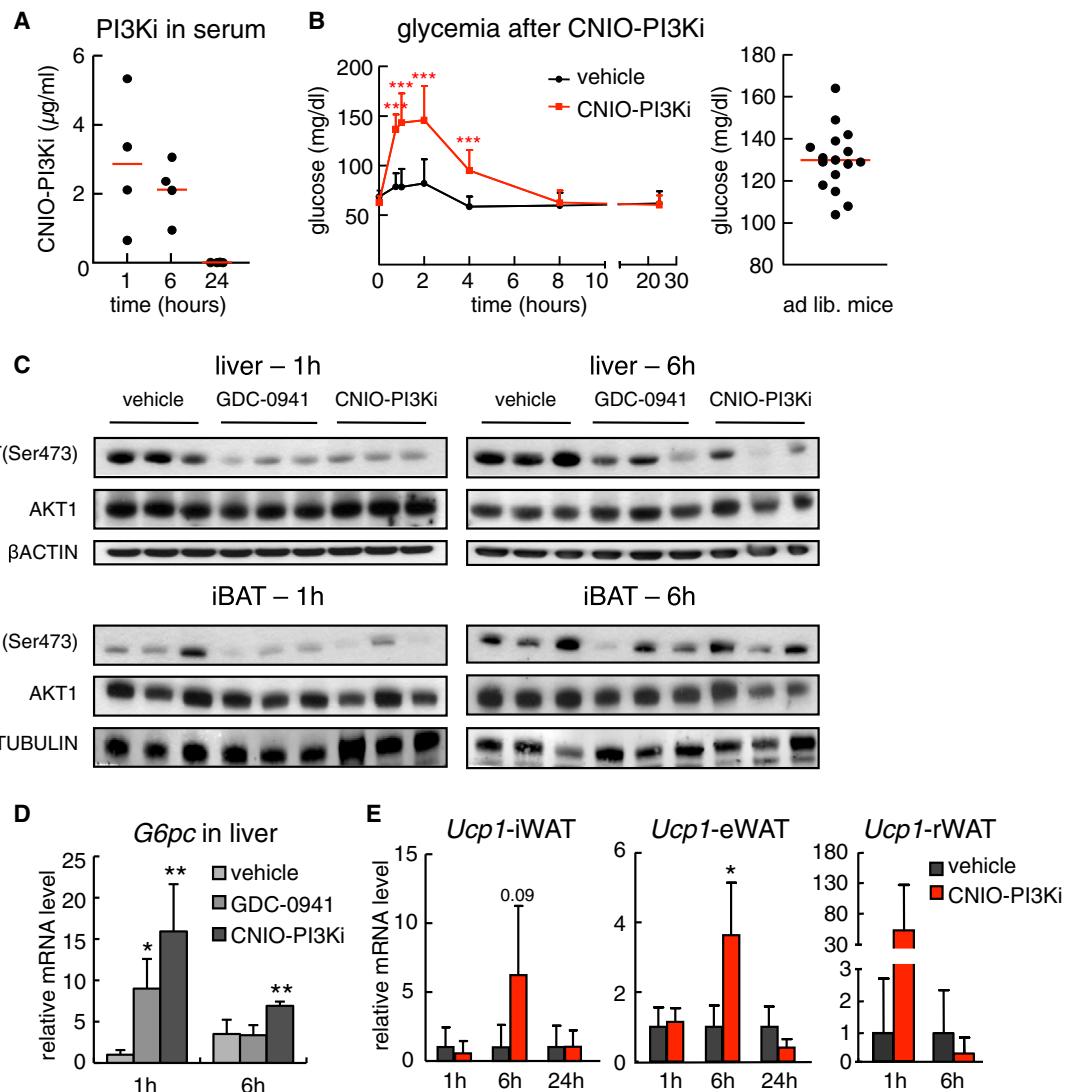
PI3K α activity (Foukas et al., 2013). This does not preclude that inhibition of other PI3K isoforms, particularly PI3K γ and possibly also PI3K β , could contribute to increased energy expenditure through mechanisms other than brown adipocyte thermogenesis (Becattini et al., 2011; Perino et al., 2014).

As a first step to use CNIO-PI3Ki in mice, we began by measuring its serum levels by mass spectrometry. After a single oral dose of 15 mg/kg, the drug reached a serum concentration of 2–3 μ g/ml (4–6 μ M) during 1–6 hr post-administration, and it was completely cleared after 24 hr (Figure 1A). Given the direct role of PI3K in insulin signaling, we examined the effect of CNIO-PI3Ki on glucose levels in fasted C57BL6 mice. A peak of glucose of about 150 mg/dl was observed between 30 min and 2 hr post-administration of the drug (Figure 1B). These levels of glycemia are comparable to the normal levels of glucose in ad libitum-fed mice (Goren et al., 2004) (see also Figure 1B, right panel). As expected, both GDC-0941 (75 mg/kg) and CNIO-PI3Ki (15 mg/kg) produced a notable reduction in the levels of phosphorylated AKT (P-Ser473-AKT) in liver and interscapular brown adipose tissue (iBAT) at 1 hr post-administration (Figure 1C). Inhibition of P-AKT was less prominent, although still detectable, at 6 hr post-administration (Figure 1C). Reductions in phosphorylated FOXO1, FOXO3, and 4EBP1 were also notable at 1 hr post-administration particularly in iBAT (Figure S1E). Similarly, PI3K inhibition resulted in a remarkable upregulation of the hepatic gluconeogenic transcriptional program (measured by *G6pc* mRNA) at 1 hr post-treatment, but *G6pc* levels returned close to basal conditions after 6 hr (Figure 1D). Therefore, at the doses used, PI3K inhibitors produce a transient glycemic response within physiological range.

In agreement with our previous data (Ortega-Molina et al., 2012), the levels of protein UCP1 increased in the BAT at 6 hr post-administration of CNIO-PI3Ki (15 mg/kg) (Figure S1F). As expected from the kinetics of CNIO-PI3Ki clearance, the levels of P-AKT and P-FOXO1 were normalized in the iBAT after 24 hr, but the levels of UCP1 were still elevated (Figure S1F). This is consistent with the long half-life of protein UCP1 (Puigserver et al., 1992). Extending our previous findings on the ability of CNIO-PI3Ki to activate the brown adipocytes interspersed within white adipose depots (Ortega-Molina et al., 2012), we also observed an increase in the expression of *Ucp1* in subcutaneous white adipose tissue (WAT) (inguinal or iWAT) and in visceral WAT (epididymal or eWAT, and perirenal or rWAT) at 6 hr post-administration, but this effect disappeared after 24 hr (Figure 1E).

PI3Ki Reduces Obesity

To test whether pharmacological inhibition of PI3K could revert obesity we began by using a short-term assay with daily administration of a relatively high dose of inhibitors. Male mice of 10 months of age that had been fed with high-fat diet (HFD) since 2 months of age were used for these assays. High-fat-fed mice had a body weight that was 44% above the weight of mice fed with standard diet (SD) (Figure S2A), and this increase in body weight was exclusively due to an increase in adiposity (and not in lean mass) (Figure S2B). As expected after 8 months in HFD, these mice had developed insulin resistance (Figure S2C). Additional parameters, such as serum leptin, free fatty acids, and triglycerides (Figures S2D–S2F) were consistent with the HFD

**Figure 1. Effects of PI3K Inhibition In Vivo**

(A) Detection of CNIO-PI3Ki in serum. CNIO-PI3Ki 15 mg/kg was orally administered by gavage (C57BL6 males n = 4 per group; 3–4 months old). Detection was made by mass spectrometry at the indicated times. Dots correspond to individual values and red lines to the average.

(B) Left panel, glucose serum levels at the indicated times after CNIO-PI3Ki 15 mg/kg orally administered by gavage (C57BL6 males n = 8–9 per group; 4 months old). Mice were fasted overnight, treated by gavage, and maintained under fasting. Values correspond to the average \pm s.d. Right panel, glucose serum levels of ad libitum-fed mice (C57BL6 males n = 16; 3 months old). Dots correspond to individual values and the red line to the average.

(C) Immunoblot analyses of the indicated proteins in liver and iBAT extracts 1 or 6 hr after oral administration by gavage of vehicle, GDC-0941 75 mg/kg, or CNIO-PI3Ki 15 mg/kg.

(D) G6pc mRNA expression relative to β -actin in liver of mice treated by gavage with vehicle or CNIO-PI3Ki 15 mg/kg at the indicated times.

(E) Ucp1 mRNA levels in inguinal (iWAT), epididymal (eWAT), and perirenal (rWAT) white adipose tissue of mice treated by gavage with vehicle or CNIO-PI3Ki 15 mg/kg at the indicated times. Values are relative to the levels in vehicle-treated mice. Bars correspond to the average \pm s.d.

Mice were under ad libitum feeding all the time in the assays shown in (A), (C), (D), and (E). Statistical significance was determined by the two-tailed Student's t test: *p < 0.05, **p < 0.001. See also Figure S1 and Table S1.

regime and the obese status of these mice. Food and water intake were also measured (Figure S2G).

Diet-induced obese mice were dosed daily by gavage with 10 or 15 mg/kg of CNIO-PI3Ki or with 10 or 75 mg/kg of GDC-0941 for a period of 10 days (see Figure 2A). During the assay, mice remained fed ad libitum with HFD, and food intake did not change during the treatment period (Figure 2B).

Interestingly, despite maintaining their normal high-fat food intake, body weight decreased significantly upon treatment with the PI3K inhibitors (Figure 2C). In the most effective case (CNIO-PI3Ki at 15 mg/kg), mice reached the same body weight as non-obese mice fed with SD in a period of 10 days. Treatment with CNIO-PI3Ki at 10 mg/kg or with GDC-0941 at 75 mg/kg decreased body weight less abruptly, but

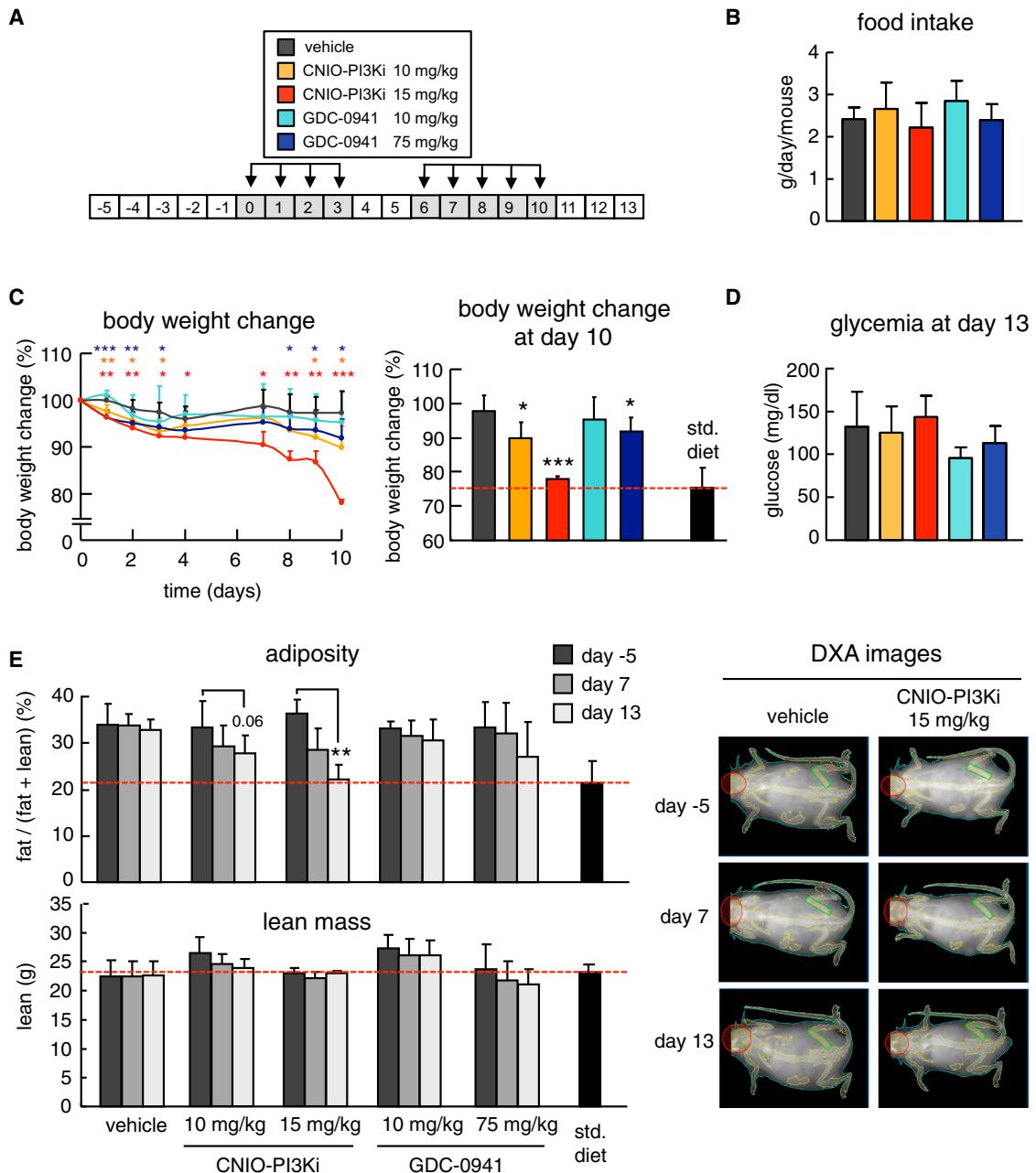


Figure 2. Body Weight Reduction and Decreased Adiposity after CNIO-PI3Ki Treatment

(A) Schematic diagram showing the administration of PI3K inhibitors or vehicle by gavage at the indicated days (gray boxes) to diet-induced obese mice (C57BL6/CBA males n = 4–7 per group; 10 months old).

(B) Average food intake per day during treatment with PI3K inhibitors (days marked in gray in A). Color code as in (A).

(C) Body weight change relative to day 0 during the treatment (left) or at the last day of treatment (right). For reference, the panel to the right includes a dotted red line corresponding to the relative body weight of control male mice fed with SD. Color code as in (A).

(D) Ad libitum glucose serum levels at the end of treatment (day 13). Color code as in (A).

(E) Adiposity (top) and lean mass (bottom) at the indicated times according to the diagram in (A) measured by dual-energy X-ray absorptiometry (DXA). Adiposity values correspond to the percentage of fat relative to the sum of lean and fat masses. For reference, the graphs include dotted red lines corresponding to adiposity and lean mass of control male mice fed with SD. Right panel, representative DXA images of two individual mice treated with vehicle or CNIO-PI3Ki (15 mg/kg), respectively.

All values correspond to average ± s.d., and statistical significance was determined by the two-tailed Student's t test: *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S2.

significantly (Figure 2C). In agreement with the transient effect of CNIO-PI3Ki on glycemia (see above Figure 1B), the ad libitum glucose levels in the treated mice were normal 3 days after the end of the treatment (Figure 2D). To distinguish whether PI3K inhibitors result in loss of fat mass or loss of lean mass, we employed dual-energy X-ray absorptiometry (DXA) before, during, and after treatment (Figure 2E, right panel). Importantly, obese mice treated with CNIO-PI3Ki 15 mg/kg showed a progressive reduction in adiposity that at the end of the treatment was comparable to the adiposity of non-obese mice (Figure 2E, left upper panel). A tendency to decrease adiposity was observed with CNIO-PI3Ki 10 mg/kg and with GDC-0941 75 mg/kg (Figure 2E, left upper panel). In all cases, lean mass remained stable (Figure 2E, left lower panel), thereby indicating that the weight loss produced by PI3K inhibition is selectively due to the loss of fat mass. Collectively, these data indicate that pharmacological inhibition of PI3K induces a significant decrease in adiposity and body weight in diet-induced obese mice.

PI3Ki Decreases Visceral Fat and Hepatic Steatosis

Examination of tissues from the above-described mice (at day 13) revealed that mice treated with CNIO-PI3Ki 15 mg/kg presented a normal coloration of the liver, which was in contrast to the pale color characteristic of obese mice (Figure 3A). More dramatically, vehicle-treated obese mice presented large masses of adipose tissue in the thoracic cavity around the heart (pericardial fat), and this abnormal accumulation of fat was essentially absent in mice treated for 10 days with CNIO-PI3Ki 15 mg/kg (Figure 3A). Mice treated with CNIO-PI3Ki 10 mg/kg and GDC-0941 75 mg/kg presented a partial improvement compared with vehicle-treated mice (Figure 3A). Histological analysis of the liver indicated a clear reduction in lipid accumulation in mice treated with CNIO-PI3Ki, 10 mg/kg or 15 mg/kg, or with 75 mg/kg GDC-0941 (Figure 3B). Examination of eWAT, perirenal, and pericardial adipose tissue revealed focal areas of brown-like adipocytes (Figure 3B), which reflects a process known as browning (Wu et al., 2013). Browning was particularly prominent in mice treated with CNIO-PI3Ki 15 mg/kg, intermediate in the case of CNIO-PI3Ki 10 mg/kg and GDC-0941 75 mg/kg, and absent in the case of GDC-0941 10 mg/kg (Figure 3B). Reduced lipid content was also observed in the iBAT following the same trends among treatments as in the other tissues (Figure 3B).

The arcuate nucleus of the hypothalamus (ARC) is a master regulator of metabolism (Sohn et al., 2013; Yeo and Heisler, 2012) exposed to peripheral circulation through the median eminence, a circumventricular organ that lacks brain-blood barrier (Cone et al., 2001). We wondered whether administration of CNIO-PI3Ki (15 mg/kg) or GDC-0941 (75 mg/kg) could affect the expression of the main orexigenic (NPY and AgRP) and anorexigenic (CART and POMC) neuropeptides in the arcuate nucleus. However, we did not detect any significant alteration at 1 hr or 6 hr post oral administration (Figure S3). These results indicate a lack of a hypothalamic effect, at least at the level of the ARC, which is the hypothalamic nucleus most exposed to circulating factors.

We conclude that short-term treatment with PI3K inhibitors reduces abnormal lipid accumulation, induces browning within

white adipose depots, and activates the iBAT, without affecting the ARC.

Loss of Adiposity by Long-Term Treatment with Low-Dose CNIO-PI3Ki

A desirable feature for an anti-obesity treatment is to retain activity at low doses administered during prolonged periods of time. This not only increases the safety margins, but will presumably produce a gradual loss of body weight more amenable to medical supervision. With this in mind, we formulated CNIO-PI3Ki for administration in the drinking water or mixed with the food (see *Experimental Procedures*). By mass spectrometry, we confirmed stability (> 90%) of the compound when dissolved in water (for at least 1 week at room temperature) and also when mixed with the food (for at least 4 months at 4°C).

Male mice fed with HFD (starting at 2 months of age) or with SD, all of 10 months of age, were used for these assays (see Figure S2). HFD-fed and SD-fed mice were randomly separated into two groups that were treated with CNIO-PI3Ki or its vehicle. For administration through the drinking water we chose a CNIO-PI3Ki concentration of 0.1 mg/ml, which corresponds approximately to an accumulated daily dose of 10 mg/kg. Mice had continuous and ad libitum access to water (with vehicle or drug) and to food (HFD or SD). This administration regime resulted in detectable, albeit highly variable, serum levels of CNIO-PI3Ki (63 ± 54 ng/ml, average \pm standard deviation [s.d.], n = 12 pooled SD and HFD mice after 2.5 months of continuous treatment; Figure S4A). Variability was expected given the fact that drinking was ad libitum and thereby heterogeneous from mouse to mouse. No evidence of drug accumulation was obtained in the inguinal WAT, iBAT, or in the liver after 2.5 months of treatment (Figure S4A). The administration regime used in this long-term assay is notoriously different from the short-term assay shown before; nonetheless, it is worth mentioning that the average serum concentration of drug achieved by ad libitum drinking (CNIO-PI3Ki 0.1 mg/ml) is around 40-fold lower than the peak of drug achieved 1–6 hr post-gavage (CNIO-PI3Ki 15 mg/kg).

A progressive decrease in body weight was noticeable in obese mice soon after exposing them to CNIO-PI3Ki (Figure 4A). Body weight loss lasted for 50 days, and then it stabilized at a level that was 20% lower than the body weight of vehicle-treated mice. Importantly, food intake remained unaltered during the entire observation period (Figure 4B). This behavior is compatible with the idea that CNIO-PI3Ki increases energy expenditure, thus lowering the body weight at which mice are in energetic balance. Of note, mice fed with SD did not show any alteration in their body weight in the presence of CNIO-PI3Ki (Figure 4A). The fact that CNIO-PI3Ki is only active on HFD-fed mice, but not on SD-fed mice, is an attractive safety feature, and it suggests that CNIO-PI3Ki may act selectively on energy expenditure induced by nutritional overload. Longitudinal analyses of body composition by DXA indicated that weight loss of obese mice treated with CNIO-PI3Ki was exclusively due to a loss of adiposity, with no alterations in lean mass (Figure 4C), and this was confirmed by the weight of epididymal and inguinal adipose depots, as well as by the macroscopic observation of pericardial and perirenal fat (Figure S4B). Further supporting the decreased adiposity, leptin serum levels were also significantly reduced in

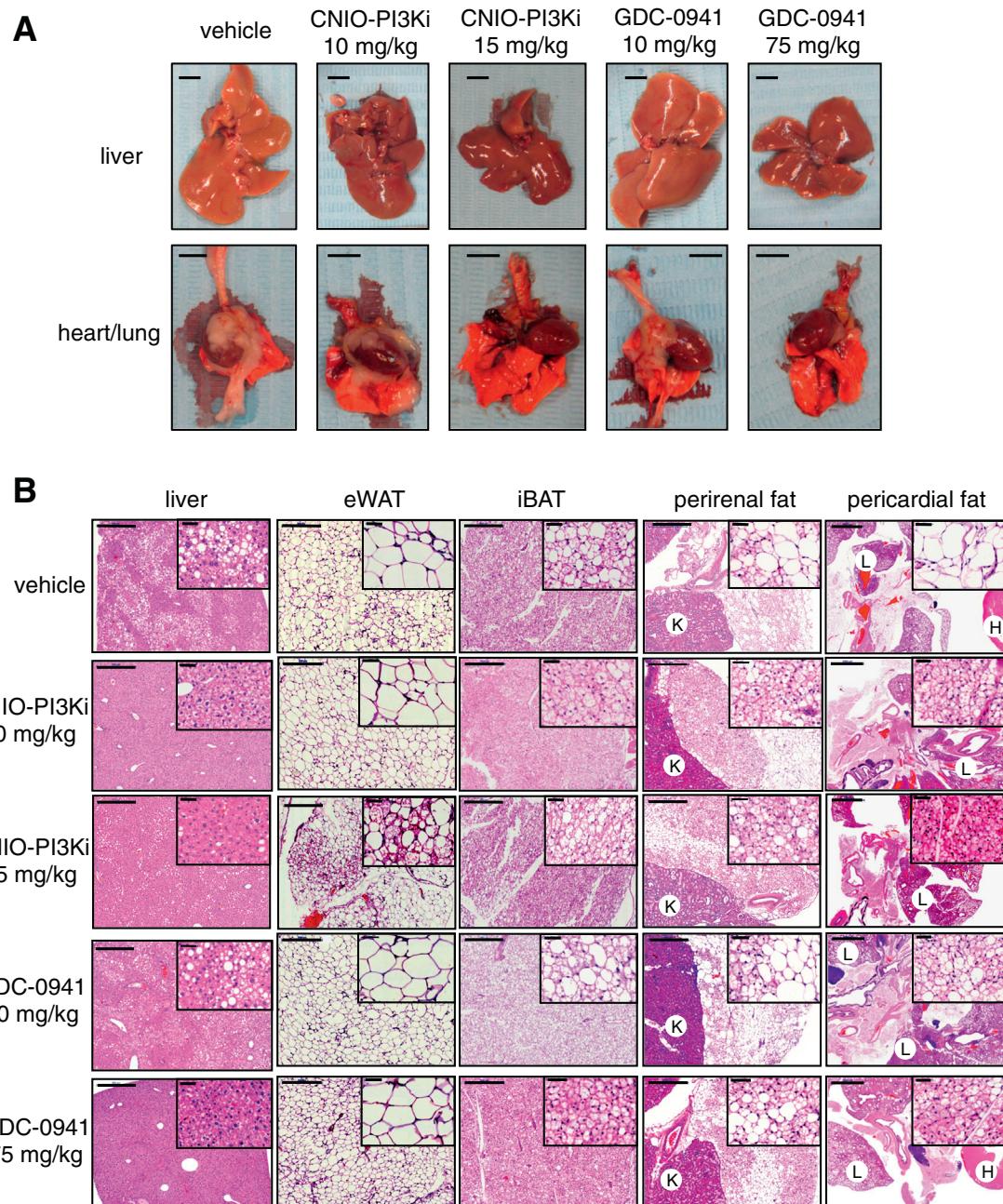


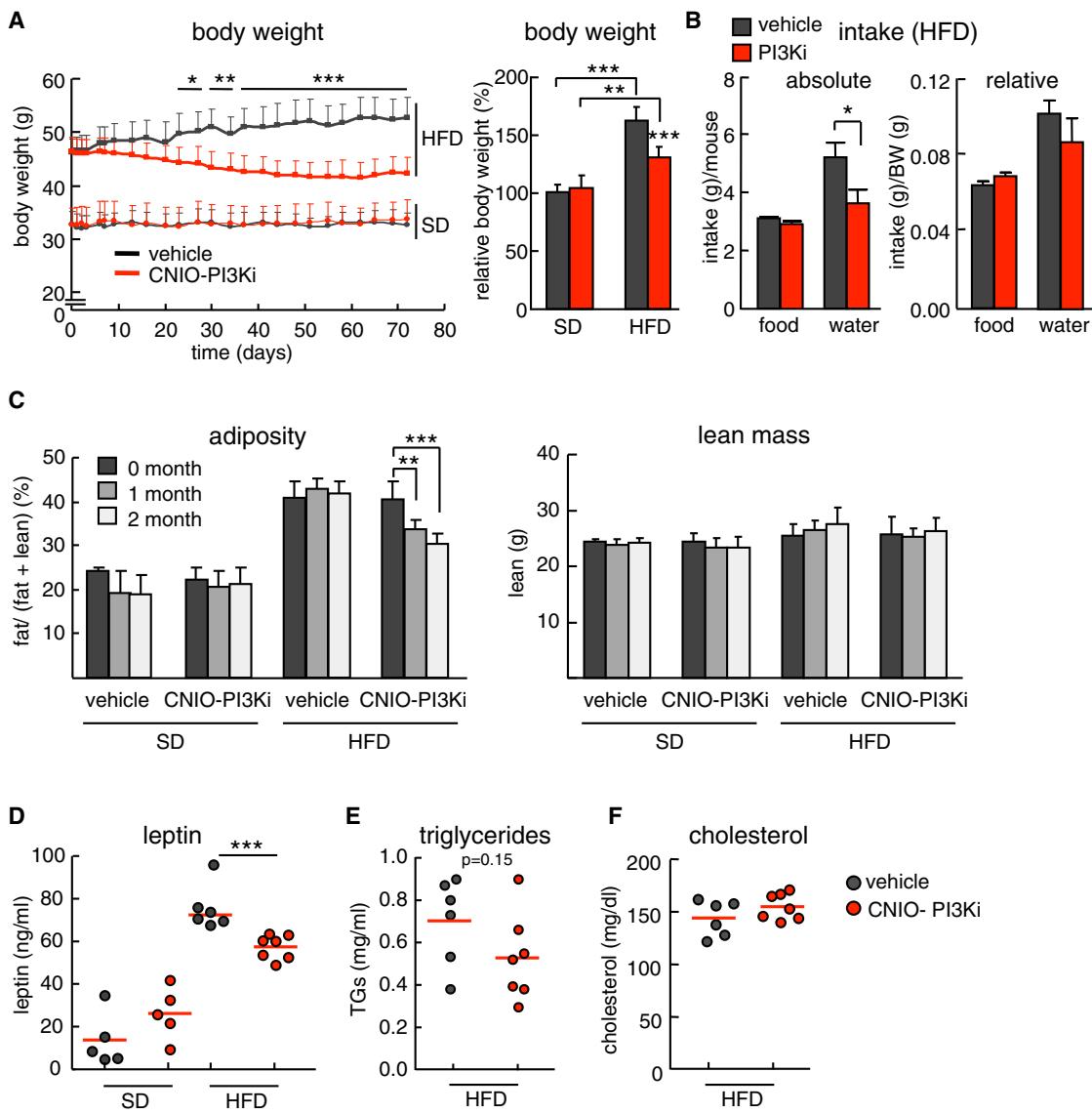
Figure 3. Reduced Liver Steatosis and Increased Browning after CNIO-PI3Ki Treatment

(A) Representative pictures of liver and fat around lung and heart (pericardial fat) in vehicle- and PI3Ki-treated mice at day 13. Bars correspond to 0.5 cm. (B) Representative pictures of H&E-stained sections of the indicated tissues at day 13. eWAT is epididymal WAT, and iBAT is interscapular BAT. In the perirenal fat, K indicates the kidney; in the pericardial fat, L indicates the lungs and H indicates the heart. Bars in the low-magnification pictures correspond to 500 μ m, with the exception of pericardial fat pictures, where bars correspond to 2 mm; all bars in the high-magnification insets correspond to 50 μ m. Mice are the same as in Figure 2. See also Figure S3.

obese mice at the end of the long-term treatment with CNIO-PI3Ki (Figure 4D). Also, we observed a tendency toward decreased levels of serum triglycerides (Figure 4E), and no alteration in cholesterol levels (Figure 4F). In summary, long-term pharmacological treatment of obese mice with a low dose of CNIO-PI3Ki produces a progressive loss of adiposity that stabilizes at a lower body weight.

Correction of Metabolic Syndrome by Long-Term Low-Dose CNIO-PI3Ki

Examination of the liver of the mice from the above assay (see Figure 4A) revealed a complete histological correction of steatosis in long-term CNIO-PI3Ki-treated HFD-fed mice (Figure 5A) and a tendency to have a lower liver weight (Figure S4B). This was accompanied by a significant decrease in serum alanine

**Figure 4. Reduction of Obesity by Long-Term CNIO-PI3Ki Treatment**

(A) Left, body weight curves during 2.5 months of treatment with vehicle or CNIO-PI3Ki (0.1 mg/ml) in the drinking water. All mice were 11 months old at the beginning of the treatments. HFD, mice fed with high-fat diet since 2 months of age; SD, mice fed standard diet (C57BL6 males n = 5–7 per group). Right, body weights at the end of the treatment relative to the weight of SD-fed and vehicle-treated mice.

(B) Absolute (left) and relative (right) food and water intake during treatment of HFD-fed mice. Intake was measured in periods of 3 days, and in a total of four periods distributed during the entire treatment period.

(C) Adiposity (left) and lean mass (right) measured by dual-energy X-ray absorptiometry (DXA) at the indicated times. Adiposity values correspond to the percentage of fat relative to the sum of lean and fat masses.

(D) Ad libitum leptin serum levels at the end of treatment. Dots correspond to individual values and red lines to the average.

(E) Ad libitum triglyceride serum levels at the end of treatment of HFD-fed mice. Dots correspond to individual values and red lines to the average.

(F) Ad libitum cholesterol serum levels at the end of treatment of HFD-fed mice. Dots correspond to individual values and red lines to the average.

Values correspond to the average \pm SEM (B) or \pm s.d. (C), and statistical significance was determined by the two-tailed Student's t test: *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S4.

aminotransferase (indicative of hepatic damage) and a tendency toward decreased interleukin-6 (*IL-6*) expression (indicative of hepatic inflammation) (Figures 5B and 5C). Regarding the adipose tissues of HFD-fed mice, the iBAT and the epididymal WAT (eWAT) of CNIO-PI3Ki-treated mice showed a

clear reduction in lipid droplets compared with vehicle-treated mice (Figure 5A). No differences were discernable in SD-fed mice treated or not with CNIO-PI3Ki. Regarding the WAT, lipid overload due to HFDs also results in an inflammatory reaction that includes macrophage infiltration within the WAT

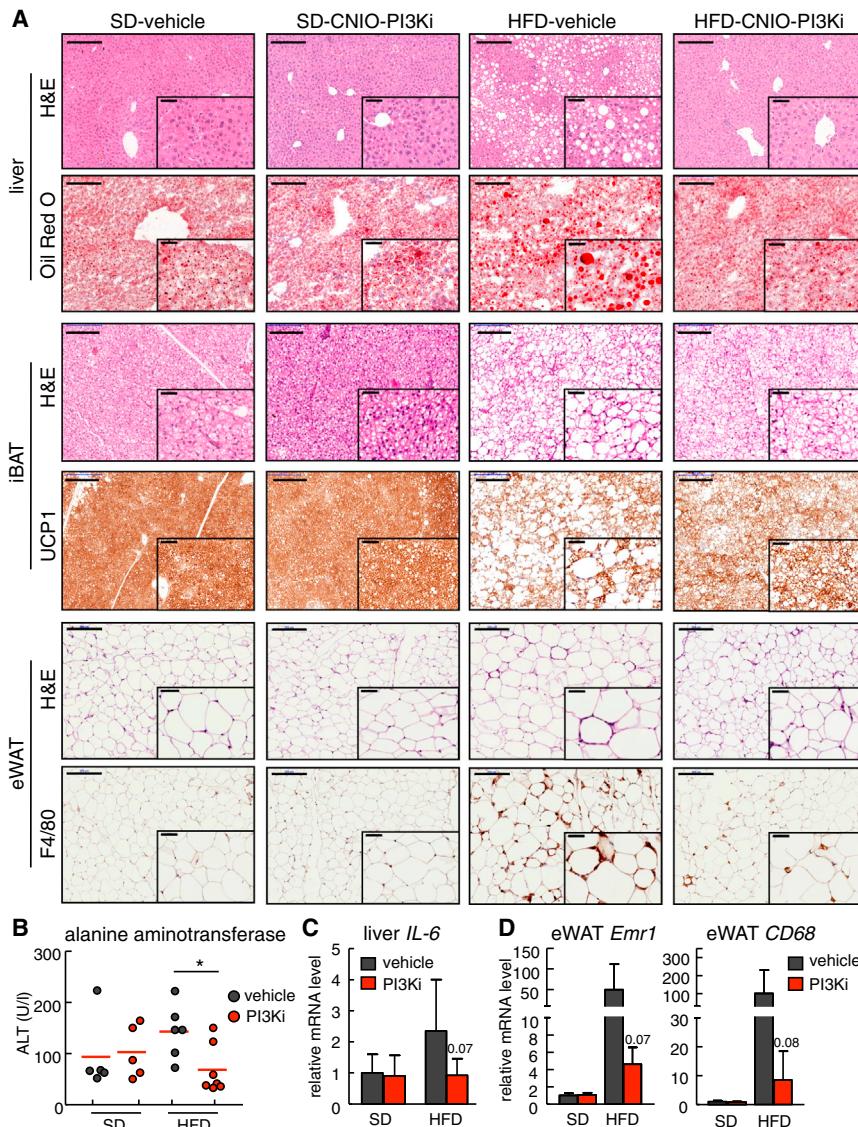


Figure 5. Reduction of Liver Steatosis and Macrophage Infiltration in eWAT by Long-Term CNIO-PI3Ki Treatment

(A) Representative microscopic pictures of liver sections stained with H&E or oil red O (upper panels), interscapular BAT (iBAT) sections stained with H&E or anti-UCP1 (middle panels), and epididymal WAT (eWAT) sections stained with H&E or anti-F4/80 (lower panels). Bars in the low-magnification pictures correspond to 200 µm; bars in the high-magnification insets correspond to 50 µm.

(B) Ad libitum alanine aminotransferase (ALT) serum levels at the end of long-term treatment. Dots correspond to individual values and red lines to the average.

(C) *IL-6* expression in liver. Values are relative to the levels in SD-fed and vehicle-treated mice. Values correspond to the average ± s.d.

(D) *Emr1* and *CD68* mRNA levels in eWAT. Values are relative to the levels in SD-fed and vehicle-treated mice. Bars correspond to the average ± s.d.

Statistical significance was determined by the two-tailed Student's t test: **p* < 0.05. Mice in this figure are the same as in Figure 4, and all the analyses were performed at the end of the long-term treatment.

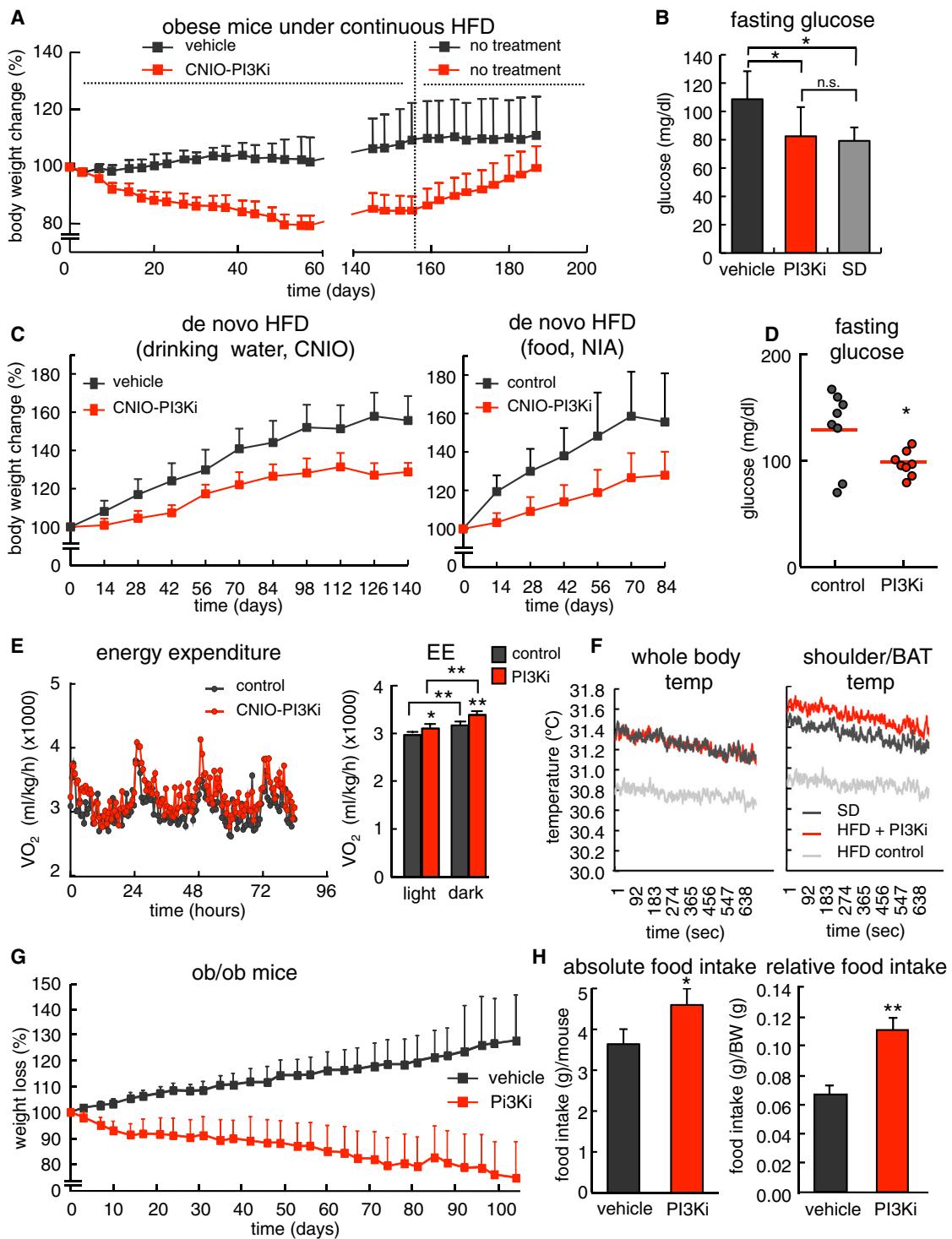
(Strissel et al., 2007). Interestingly, the eWAT of CNIO-PI3Ki-treated HFD-fed mice presented less macrophage infiltration compared to vehicle-treated and HFD-fed control mice, as observed using the macrophage marker F4/80 (Figure 5A). A similar tendency was observed when measuring macrophage mRNA markers in the eWAT (Figure 5D). Of note, we did not observe browning in the WAT (Figure 5A). This is in contrast to the short-term assay where the drug was administered at higher doses, and we surmise that this is due to the low dose of drug used in the long-term assay (40-fold lower serum concentration compared to the peak of drug achieved by gavage). Finally, no changes in markers of slow- and fast-twitch fibers were observed in the gastrocnemius after 2.5 months of treatment with CNIO-PI3Ki (Figure S4C).

We also examined glucose homeostasis in the above groups of mice after 2.5 months of treatment. However, at this time, glycemia, insulinemia, glucose tolerance, and the insulin resistance index HOMA-IR were not affected in CNIO-PI3Ki-treated

HFD-fed mice compared to their controls (Figures S4D and S4E). Given the fact that we are using mice with a profound diabetic profile due to sustained HFD feeding (8 months), we reasoned that 2.5 months of treatment might not suffice to improve glucose homeostasis. To address this, a new assay was performed in which obese mice (that had been on HFD for 8 months) were exposed for 5 months to the same dose of CNIO-PI3Ki (0.1 mg/ml in ad libitum drinking water).

As before, obese mice treated with CNIO-PI3Ki lost weight during 50 days and then body weight stabilized at a set point that was 20% lower than vehicle-treated obese mice (Figure 6A). This reduction in body weight was maintained during the following 100 days, as long as the treatment was maintained, thereby implying that chronic treatment does not result in drug resistance. Of note, withdrawal of treatment resulted in steady body weight gain, thus indicating that long-term treatment with CNIO-PI3Ki does not produce irreversible metabolic alterations. Regarding glucose resistance, HFD-fed mice that had been treated for 5 months with CNIO-PI3Ki (and then released from the drug for 1 month) had completely normalized their fasting glucose levels, being indistinguishable from those of SD-fed mice (Figure 6B).

Together, the above observations demonstrate that long-term treatment of HFD-fed mice with a low dose of CNIO-PI3Ki decreases liver steatosis and adipose tissue inflammation and improves glucose homeostasis.

**Figure 6. Properties of CNIO-PI3Ki on Obesity and Energy Expenditure**

(A) Body weight change relative to day 0. Mice of 2 months of age were fed with HFD during 8 months. At this time (day 0), mice were divided into two groups and treated with vehicle or CNIO-PI3Ki (0.1 mg/ml) in the drinking water during 5 months. At day 155, treatment with vehicle or CNIO-PI3Ki was removed from the water for the rest of the assay. Mice were fed with HFD during the entire assay. Values correspond to the average \pm s.d. C57BL6 males n = 9–10 per group. Significant weight difference between groups lasted from day 7 to day 180.

(B) Fasting glucose level of HFD-fed mice at the end of the procedure (5 months with treatment and 48 days treatment-free) compared to SD-fed control mice.

(C) Left, performed in the CNIO, body weight change relative to day 0. Mice of 2 months of age were put simultaneously on a HFD with CNIO-PI3Ki (0.1 mg/ml) or vehicle in the drinking water for 20 weeks. Values correspond to the average \pm s.d. C57BL6 males n = 8–10 per group. Significant weight difference starts at day 3.

(legend continued on next page)

Increased Energy Expenditure by Long-Term Low-Dose CNIO-PI3Ki

Next, we asked whether the effects of CNIO-PI3Ki on body weight were noticeable upon de novo HFD feeding of lean mice. We took advantage of this new assay to further challenge the robustness of our observations. Specifically, SD-fed lean mice were changed to HFD together with the administration of CNIO-PI3Ki via drinking water (at the CNIO, Madrid) or via food pellets (at the NIA, Baltimore). In the latter case, the presence of the drug did not affect food intake (Figure S5A). In both assays, mice had ad libitum access to water and food, and the total accumulated daily dose of CNIO-PI3Ki was of approximately 10 mg/kg (in the case of water the drug was at a concentration of 0.1 mg/ml, and in the case of food it was 0.17 g of drug per kg of food). Interestingly, the rate of weight gain produced by the HFD regimen was clearly slowed down in CNIO-PI3Ki-treated mice compared to vehicle-treated mice, both via drinking water (CNIO) and via food pellets (NIA) (Figure 6C). Also, CNIO-PI3Ki-treated mice (food, NIA) had lower fasting glucose levels, thereby implying that the drug protects from the development of glucose intolerance (Figure 6D). These observations indicate that the beneficial effects of PI3K inhibition are apparent as soon as high-fat feeding starts in healthy lean mice, and therefore do not require pre-existent metabolic damage by high-fat feeding.

We have previously shown that CNIO-PI3Ki (15 mg/kg via oral gavage) elevates energy expenditure (Ortega-Molina et al., 2012). This increase in energy expenditure can be explained, at least in part, by the activation of thermogenesis in brown adipocytes, both *in vivo* and *in vitro* (Ortega-Molina et al., 2012). We wondered if low-dose CNIO-PI3Ki (food, NIA) had a measurable impact on energy expenditure. Interestingly, HFD-fed mice treated with CNIO-PI3Ki via food pellets showed higher constitutive levels of energy expenditure (Figure 6E), oxygen consumption, and CO₂ production (Figure S5B). To evaluate iBAT thermogenesis, we measured whole-body and shoulder temperature with a thermographic camera. Mice were anesthetized to eliminate physical activity, and readings were taken immediately after anesthesia. As expected, body temperature progressively dropped, but, interestingly, shoulder temperature was consistently higher in CNIO-PI3Ki-treated mice compared to controls (Figure 6F). Of note, we also observed higher locomotor activity in CNIO-PI3Ki-treated mice (Figure S5C), although it remains debatable to what extent an increase in locomotor activity translates into an increase in total energy expenditure (Virtue et al.,

2012). We conclude that low-dose CNIO-PI3Ki augments energy expenditure in mice.

Low-Dose CNIO-PI3Ki Treatment Reduces Obesity in Hyperphagic Mice

Mice deficient in leptin (ob/ob mice) constitute a relevant model of obesity, which in this case is not due to a high intake of fat, but to a high intake of standard food due to hyperphagy. Administration of low dose of CNIO-PI3Ki (via ad libitum drinking water with the drug at 0.1 mg/ml) to ob/ob mice for 3 months had a dramatic impact on body weight (Figure 6G). The magnitude of this effect was even more striking when considering that CNIO-PI3Ki-treated ob/ob mice were even more hyperphagic than vehicle-treated ob/ob mice (Figure 6H). In support of our previous findings with diet-induced obese mice, CNIO-PI3Ki-treated ob/ob mice had lower levels of serum transaminase ALT (Figure S5D), decreased hepatic steatosis, and decreased adipocyte size in perirenal and pericardial fat, as well as in iBAT (Figure S5E). No improvement on glucose homeostasis was observed in the drug-treated ob/ob mice after 3 months of treatment (Figure S5F). The latter is in line with our previous observations in diet-induced obese mice, where CNIO-PI3Ki treatment improved glucose homeostasis after 5 months of treatment, but not after 2.5 months (see above Figures 6B and S4E). These results indicate that CNIO-PI3Ki is effective in obese mice fed with a standard diet.

Low-Dose CNIO-PI3Ki Treatment Reduces Adiposity in Rhesus Monkeys

Based on the above data in mice, we decided to test the effect of CNIO-PI3Ki on rhesus monkeys (*Macaca mulatta*) at the NIH Animal Center in Poolesville, Maryland. During a preliminary dosing study (see *Supplemental Experimental Procedures*), intravenous administration of 2.1 mg/kg of CNIO-PI3Ki resulted in a serum concentration of 400 ± 224 ng/ml at 2 hr post-administration (Figure S6A). This dose was well tolerated based on stable glucose levels (Figure S6B) and heart rate (Figure S6C), and the monkeys did not experience any ill effects following the procedure. Based on this, a study was performed on 19 naturally obese monkeys (see *Supplemental Experimental Procedures*) that were randomly divided into a control group (n = 9) and a CNIO-PI3Ki-treated group (n = 10). Drug treatment consisted of daily oral administration of 2.1 mg/kg of CNIO-PI3Ki for 12 weeks. CNIO-PI3Ki concentration in the serum 2 hr post oral administration was 35 ± 20 ng/ml (Figure S6D), which was ~2-fold lower

Right, performed in the NIA, body weight change relative to day 0. Mice of 28 weeks of age and fed with SD were put on a HFD or HFD supplemented with CNIO-PI3Ki (0.17 g of drug per kg of food) for 12 weeks. Values correspond to the average ± s.d. C57BL6 males (control n = 18; CNIO-PI3Ki n = 24). Differences in body weight were significant after first weight measurement.

(D) Fasting glucose levels of mice after 12 weeks with or without CNIO-PI3Ki treatment in their HFD food. Animals (control n = 8; CNIO-PI3Ki n = 8) are from the assay in (C) (right panel, performed in the NIA). Dots correspond to individual values and red bars to the mean.

(E) Energy expenditure (EE) recorded during 80 hr (left) and mean energy expenditure (right) measured during light and dark period of control and CNIO-PI3Ki-treated mice. Animals (control n = 7; CNIO-PI3Ki n = 8) are from the assay in (C) (right panel, performed in the NIA).

(F) Whole-body and shoulder area temperature of mice on HFD with or without CNIO-PI3Ki treatment (same animals as in C, right panel, performed in the NIA) and of control lean mice on SD (n = 6–9 per group). Temperature was recorded every second during 10 min.

(G) Body weight change relative to day 0. Hyperphagic ob/ob male mice (12 weeks old) fed with SD and treated with vehicle or CNIO-PI3Ki (0.1 mg/ml) in the drinking water during 16 weeks. C57BL6 males ob/ob n = 10 per group. Significant weight difference between groups starts at day 3.

(H) Absolute (left) and relative (right) food intake of ob/ob mice treated with vehicle or CNIO-PI3Ki relative to their body weight. Intake was measured in periods of 4 days, and a total of two periods distributed in the middle of the treatment.

Values correspond to the average ± s.d. Statistical significance was determined by the two-tailed Student's t test: *p < 0.05, **p < 0.01. See also Figure S5.

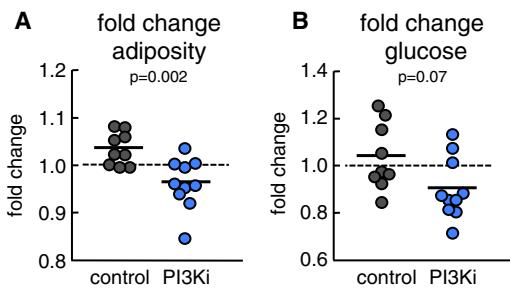


Figure 7. Effects of CNIO-PI3Ki on Obese Rhesus Monkeys

(A) Fold change of adiposity after 12 weeks of treatment with vehicle ($n = 9$) or CNIO-PI3Ki ($n = 10$). Values correspond to the ratio between adiposity at week 12 and adiposity at baseline, for each monkey. Adiposity was measured in the trunk (adiposity = fat/(fat + lean)%).
(B) Fold change of fasting glucose (same representation as in A) for each monkey at week 12, relative to baseline.
Statistical significance was determined by the two-tailed Student's t test. See also Figure S6.

than that observed in mice treated via drinking water with a drug concentration of 0.1 mg/ml. The obese monkeys were weight stable at the onset of the treatment, and intake of standard monkey chow did not change during the trial (Figure S6E). However, during the trial, all monkeys received a daily palatable treat that facilitated the dosing of the drug (control monkeys received the same treat), which represented a 16% increase in the total calorie intake (Figure S6E). These additional calories resulted in a small, but significant, increase in the trunk adiposity of the control group at the end of the 12-week trial (Figure 7A). Interestingly, in contrast to the control monkeys, adiposity decreased in the CNIO-PI3Ki-treated monkeys (Figure 7A). The difference in adiposity between vehicle-treated and CNIO-PI3Ki-treated monkeys was of 7.6%. No changes were detected in total body weight in any of the groups (Figure S6F). Additionally, drug-treated monkeys did not show any sign of discomfort, altered behavior, or toxicity during the 12-week trial.

Metabolic parameters and blood chemistry variables were measured before, during, and after the trial (see *Supplemental Experimental Procedures*). Importantly, a tendency to decrease fasting glucose levels was observed (Figure 7B), although no changes were apparent in the glucose tolerance test (Figure S6G), fasting insulin levels (Figure S6H), or in the insulin resistance index (HOMA-IR) (Figure S6I). Furthermore, CNIO-PI3Ki did not affect any of the other metabolic and blood chemistry parameters tested (see *Supplemental Experimental Procedures*), thereby indicating its safety and lack of toxicity at the specified dose.

We conclude from this trial that CNIO-PI3Ki, at low doses, reduces adiposity and may also decrease serum glucose levels in obese rhesus monkeys, without detectable toxic effects after 12 weeks of daily dosing.

DISCUSSION

Based on previous work by others and us (Becattini et al., 2011; Chattopadhyay et al., 2011; Foukas et al., 2013; Garcia-Cao et al., 2012; Kobayashi et al., 2011; Ortega-Molina et al., 2012), we hypothesized that pharmacological treatment with PI3K

inhibitors could be effective against obesity and metabolic syndrome. Given the important role of PI3K in insulin signal transduction, we first measured the acute effects of CNIO-PI3Ki on fasting glycemia. As expected, oral CNIO-PI3Ki (15 mg/kg) had a clearly detectable glycemic effect on fasting mice, thus providing a bona fide biomarker for the activity of the PI3K inhibitor on insulin signaling. The magnitude of the glycemic peak induced by CNIO-PI3Ki was comparable to a normal post-prandial glycemia, and it was rapidly reversed after 6 hr, which reflects the operation of homeostatic control mechanisms. The liver is responsible for the production of glucose during fasting, and, indeed, we observed reduced AKT and FOXO1 phosphorylation and elevated expression of the gluconeogenic gene *G6pc*. The BAT is another relevant target of PI3K inhibition (Ortega-Molina et al., 2012) and, consistent with this, we also observed reduced AKT and FOXO1 phosphorylation together with an increase in the levels of UCP1 in the iBAT. Increased expression of *Ucp1* was also observed in white adipose depots suggesting an increased thermogenic activity by the brown adipocytes interspersed within WAT, which are known as brite or beige adipocytes (Wu et al., 2013). Therefore, PI3K inhibition produces a transient increase in glycemia within physiological range, together with an increase in thermogenesis.

We have compared in parallel the anti-obesity activity of two PI3K inhibitors, CNIO-PI3Ki and GDC-0941, both being highly specific inhibitors of class IA PI3Ks p110 α and p110 δ , with similar inhibitory efficacies in vitro, and none of them crossing the brain-blood barrier (our current data; Ortega-Molina et al., 2012; Workman et al., 2010). Importantly, both PI3K inhibitors reduced the weight of obese mice in a dose-dependent manner and in a short period of 10 days, without affecting their normal intake of high-fat food. The reduction in body weight was entirely due to a loss of adiposity, which, upon necropsy, was particularly prominent in the pericardial fat. Moreover, in the case of the highest dose of CNIO-PI3Ki, there was evident browning of the epididymal, pericardial, and perirenal fat depots. Liver steatosis also decreased in parallel with the normalization of adiposity.

To simulate a hypothetical clinical application, we tested the effects of long-term treatment with a low dose of CNIO-PI3Ki administered through the drinking water, which resulted in average serum drug levels of 63 ng/ml. This drug concentration is 40-fold lower than the peak of drug achieved 1–6 hr after oral gavage of 15 mg/kg. A number of relevant observations were made. Obese mice treated with CNIO-PI3Ki through the drinking water lost weight progressively during a period of 50 days, while maintaining their normal high-fat food intake. Notably, all the body weight loss was due to a reduction in adiposity, while maintaining a stable lean mass. A slow and progressive weight loss is a desirable feature because, if translated to humans, it would allow better medical supervision. Importantly, after ~50 days, treated mice stabilized their body weight at a set point that was 20% below non-treated obese mice, and this was maintained for as long as the treatment was continued. Mice under chronic treatment with low doses of CNIO-PI3Ki presented increased energy expenditure, and this can explain the resetting of energy balance at a lower body weight. The stability of the new energetic balance also suggests that chronic treatment does not elicit compensatory changes to defend body weight, and it does not result in drug resistance. Importantly, long-term treatment

with CNIO-PI3Ki produced a range of beneficial effects beyond body weight loss. In particular, the liver of treated mice presented decreased steatosis, reduced *IL-6* expression, and lower liver damage (measured by serum transaminases); also, the WAT had lower levels of inflammation, and serum glucose levels were normalized after 5 months of treatment. We could not observe evidence of browning in the WAT when mice were treated with low doses of drug. Interestingly, the effects of CNIO-PI3Ki on body weight were reversible upon withdrawal of the treatment, and mice readjusted their body weight to the same level as control high-fat-fed mice. This reversibility indicates that long-term exposure to CNIO-PI3Ki does not produce irreversible alterations in metabolism. In line with this, we did not observe any evidence of toxic effects in mice even after 5 months of continuous treatment with CNIO-PI3Ki.

In addition to being active in obese high-fat-fed mice, CNIO-PI3Ki was also active in hyperphagic ob/ob mice fed with standard diet. This suggests that the drug is effective against obesity produced by nutritional overload, independently of the fat content of the diet. Importantly, CNIO-PI3Ki did not affect the body weight of healthy lean mice under standard diet, which is an attractive safety feature for a hypothetical human anti-obesity treatment. Also, CNIO-PI3Ki significantly delayed body weight gain and glucose intolerance when healthy lean mice were put de novo on a high-fat diet. This indicates that to be effective the treatment does not require a pre-existent context of chronic high-fat feeding. All together, these three sets of observations (efficacy on ob/ob mice, lack of effect on mice with standard diet, and efficacy upon de novo high-fat feeding) point to nutritional overload as the key requisite for the anti-obesity activity of PI3K inhibition.

Finally, we have extended the efficacy of CNIO-PI3Ki treatment to rhesus monkeys. Obese monkeys were treated for 3 months with a single daily oral dose of CNIO-PI3Ki that resulted in serum levels of 35 ng/ml at 2 hr post-dosing. This concentration of drug can be considered low and comparable to the concentration used in the long-term assays in mice (63 ng/ml). Importantly, treatment with CNIO-PI3Ki produced a modest, but significant, decrease in adiposity (7.6% reduction) and a trend toward lowered serum glucose. No toxic effects were observed in any monkey during the 12 weeks of treatment.

Based on the evidence presented here in mice and monkeys, we propose that moderate pharmacological inhibition of PI3K can be an effective and safe therapeutic strategy against human obesity and metabolic syndrome.

EXPERIMENTAL PROCEDURES

PI3K Inhibitors

The low-molecular-weight compound CNIO-PI3Ki is described in patent WO2010/119264 (files available at the World Intellectual Property Organization, <http://patentscope.wipo.int/search/en/detail.jsf?docId=WO2010119264>). The kinase activity of PI3K isoforms was measured by using the commercial PI3-kinase (h) HTRF assay available from Millipore, following the manufacturer's recommendations. PI3K α (p110 α /p85 α) and PI3K δ (p110 δ /p85 α) were used at 100 pM; and PI3K β (p110 β /p85 α) and PI3K γ isoforms (p110 γ) at 500 pM and 4 nM, respectively. ATP concentration was 50 times K_m^{ATP} : 200 mM for PI3K α and PI3K δ , 250 mM for PI3K β , and 100 mM for PI3K γ . PIP₂ was held at 10 mM. Values were normalized against the control activity included for each enzyme (i.e., 100% PI3K activity, without compound). These values were plotted against the inhibitor concentration and were fitted to a sigmoidal dose-response (variable slope) curve by using GraphPad software. In addition,

a total of 284 kinases (Table S1) were measured by ProQinase. Details of assay conditions can be found at <http://www.proqinase.com>.

Mouse Experimentation

All animal procedures done at the CNIO were performed according to protocols approved by the CNIO-ISCIII Ethics Committee for Research and Animal Welfare (CElyBA). All animal protocols done at the NIA were approved by the Animal Care and Use Committee of the National Institute on Aging (Baltimore). Mice were housed under specific pathogen-free (SPF) conditions, at 22°C, and with 12-hr dark/light cycles (light cycle from 8:00am to 8:00pm). Mice were fed either with a standard chow diet (Harlan Teklad 2018; 18% calories from fat) or, when indicated, with a HFD (Research Diets D12451; 45% of total calories from fat). For gavage administration, CNIO-PI3Ki and GDC-0941 were dissolved in PEG-300 and 10% N-methyl-2-pyrrolidone. For administration in the drinking water, CNIO-PI3Ki was dissolved in 1.8% cyclodextrin (Sigma). For administration in the food, HFD (AIN-93G diet modified to provide 60% of calories from fat) supplemented with CNIO-PI3Ki at a dose of 0.17 g of drug per kg of food was manufactured by Dyets (Bethlehem).

Monkey Experimentation

All procedures were approved by the Animal Care and Use Committee of the NIA Intramural Research Program. Rhesus monkeys (*Macaca mulatta*) were housed at the NIH Animal Center in Poolesville, Maryland. Monkeys were fed commercially prepared monkey chow that was distributed twice daily along with daily food enrichment, and water was available ad libitum. During the study, animals were supplemented with 5 or 6 PRIMA-treat wafers (Bio-Serv; assorted fruit flavors, F0345; each wafer weighs 5 g with 3.28 kcal/g). The PRIMA-treat wafers were used as vehicle for CNIO-PI3Ki. Individually weighed doses of CNIO-PI3Ki were reconstituted in water and then distributed, via syringe, onto the PRIMA-treats. Once absorbed, they were fed to the animals. Drug administration continued daily (weekends included) for 12 weeks.

Immunohistochemistry, Protein, RNA, Cellular, and Metabolic Analyses

Immunohistochemistry, protein, RNA, cellular, and metabolic analyses were performed following standard procedures as detailed in [Supplemental Information](#).

Statistical Analysis

Unless otherwise specified, data are presented as average \pm s.d. Statistical significance was assessed in most cases using the two-tailed unpaired Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.cmet.2015.02.017>.

AUTHOR CONTRIBUTIONS

A.O.-M. initiated the study, performed part of the mouse studies at the CNIO, and contributed to experimental design, data analysis, discussion, and writing of the paper; E.L.-G. completed the mouse studies at the CNIO, participated in the monkey studies at the NIA, and contributed to experimental design, data analysis, discussion, and writing of the paper; M.M.-M. and G.I. helped with mouse experimentation at the CNIO; S.J.M. and V.M.G. performed mouse studies at the NIA; J.A.M., K.L.V., and M.D.S. contributed to the experimental design, experimentation, data analysis, and interpretation of the monkey studies; D.C. quantified the drugs by mass spectrometry and performed the pharmacokinetic assays; I.G.-G. and M.L. performed the analysis of hypothalamic neuropeptides in the arcuate nucleus; S.M. and J.P. designed, synthesized, and characterized the small organic compound CNIO-PI3Ki; R.D.C. designed, supervised, and secured funding for the studies at the NIA and contributed to writing of the paper; M.S. designed and supervised the study, secured funding for the studies at the CNIO, and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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