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Pharmacological modulation of ROR\(\alpha\) controls the beiging of murine and human white adipose tissue

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Running title: ROR\(\alpha\) antagonism promotes fat loss by increased thermogenesis

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Authors contribution: M.A., N.R., E.C and B.A. performed the experiments: M.A. (Western blots, immunohistology and cell culture), N.R. and B.A. (mice experiments), E.C. (Seahorse), B.A. (cell culture). B.F. participated to the fundings of the work and contributed to the manuscript. B.A. designed the study, developed protocols, analyzed the data and wrote the manuscript
Abstract

Beiging is an attractive therapeutic strategy to fight against obesity and its side metabolic complications. The loss of function of the nuclear transcription factor RORα has been related to a lean phenotype with higher thermogenesis in sg/sg mice lacking this protein. Here we show that pharmacological modulation of RORα activity exerts reciprocal and cell-autonomous effect on UCP1 expression ex vivo, in cellulo and in vivo. The RORα inverse-agonist SR3335 up-regulated UCP1 expression in brown and subcutaneous white adipose tissue (scWAT) explants of WT mice, while the RORα agonist SR1078 had the opposite effect. We confirmed the reciprocal action of these synthetic RORα ligands on gene expression, mitochondrial mass and uncoupled oxygen consumption rate in cultured murine and human adipocytes. Time-course analysis revealed stepwise variation in gene expression, first of TLE3, an inhibitor of the thermogenic program, followed by a reciprocal effect on PRDM16 and UCP1. Finally, RORα ligands were shown to be useful tools to modulate in vivo UCP1 expression in scWAT with associated changes in this fat depot mass. SR3335 and SR1078 provoked opposite effects on the WT mice body weight, but without any effect on sg/sg mice. This slimming effect of SR3335 was related to an increased adaptive thermogenesis of the mice, as assessed by the rectal temperature of cold-stressed mice and induction of UCP1 in scWAT, as well as by indirect calorimetry in presence or not of a β3 adrenoreceptor agonist. These data confirmed that RORα ligands could be useful tools to modulate thermogenesis and energy homeostasis.
Introduction

Activating non-shivering thermogenesis in brown adipose tissue (BAT) and white adipose tissue (WAT) to enhance the rate of energy expenditure is a new pharmacological target to protect against obesity. BAT burns glucose and fatty acids, thanks to UCP1 which dissociates substrate oxidation from ATP production (28), while WAT is specialized in energy storage and mobilization. Brown and white adipocytes share several transcription factors critical for the development and maintenance of mature fat cells (16,32). However, the ways used to generate the different adipocyte subtypes are still not fully understood. In brown adipocytes, PRDM16 and PGC1α interact with PPARγ, the master regulator of adipocyte differentiation, to activate the thermogenic program (31). Nevertheless, in white adipocytes, specific transcriptional effectors of white adipocyte gene expression are scarce (20).

Moreover, the ability of some white mature adipocytes to adopt a thermogenic phenotype in response to cold has led to the discovery of “beige adipocytes”, molecularly distinct from the brown ones. These beige adipocytes can emerge either from the differentiation of few “beige” progenitors hidden amongst “white” precursors present within the WAT or, from the direct conversion of existing white adipocytes into beige fat cells (transdifferentiation) (2,19). This latter hypothesis suggests the presence of transcriptional suppressors of the thermogenic program in white adipocytes and such molecular brakes have been described, including Rb, RIP 140, TLE3, or ZFP423 (reviewed in 33).

Elucidating the molecular mechanisms that switch on the beige phenotype could identify new molecular targets to be addressed in the fight against obesity and its metabolic complications. Whereas many extracellular effectors able to modulate WAT browning have been already reported (8), new gene candidates can still emerge from the phenotype of mutated mice. Given that the RORα-deficient (sg/sg) mice exhibited higher rate of energy expenditure both in chow diet (4) and high fat diet (13), a relationship between RORα and thermogenensis has been suspected. Muscat ‘s group was the first to establish an inverse correlation between RORα and UCP1 expression and to suggest that enhanced UCP1 and thermogenic activity may be an important contributor to the lean, obesity-resistant phenotype
of sg/sg mice (18,35). We further confirmed an increased “browning” of all the fat depots in these mice, thus conferring to RORα an inhibitory role on the thermogenic program that could be counteracted ex vivo by the use of a synthetic RORα inverse-agonist (25). Indeed, we showed that a short treatment with this specific RORα ligand, in the absence of other external stimulation, was sufficient to induce UCP1 protein appearance in WAT explants, within a timing compatible with a potential browning process by transdifferentiation or revealing the presence of pre-existing beige adipocytes.

RORα, β and γ constitute a subfamily of nuclear receptors involved in many physiological processes. RORα is the major form expressed in WAT and is increased during adipogenesis (27). The recent characterization of endogenous ligands for these former orphan receptors has stimulated the development of synthetic ligands by pharmaceutical companies since targeting these receptors could represent new opportunities to treat several diseases (14). Several oxysterols have been found to function as RORα inverse-agonists, inhibiting G6PC (Glucose-6-Phosphatase) and Bmal expression (39). The Scripps Research Institute designed several RORα ligands with, for some of them, an in vivo efficiency: SR3335 is a selective RORα inverse-agonist and SR1078, a RORα agonist able to suppress or increase some hepatic target genes in vivo, respectively (15,38).

The aim of this work was 1) to investigate the possibility of a pharmacologically-induced inverse relationship between RORα activity and UCP1 expression in adipose tissue explants by using either a RORα inverse-agonist or a RORα agonist; 2) to more precisely delineate the time-course of the molecular events produced by these RORα ligands in murine adipocyte cell lines and in human adipocytes; 3) to test the ability of in vivo administration of these RORα ligands on non-shivering thermogenesis and body weight in mice.
Material and Methods

**Animals and treatment.** sg/sg mice (a spontaneous mutation in C57BL6/ strain) were obtained by crossing heterozygous sg/+ mice, given by Pr. J. Mariani’s laboratory (IBPS, Paris, France), and compared with +/- littermates. All animals care and use procedures were in accordance with the guidelines of the Charles Darwin Ethics Committee (Ce5/26092 and 26094). Mice were housed in a specific pathogen-free environment in a temperature-controlled room maintained at 24°C, with a 12h/12h light-dark cycle (lights on from 8h to 20h). Water and food (A03, UAR, Epinay-sur-Orge, France) were provided *ad libitum.*

Because sg/sg mice are leaner than WT, the adult mice used in this study were chosen to be weight-matched rather than age-matched because body weight influence thermogenensis (18), average age difference being about 6 months. For *in vivo* studies, mice were i.p. injected with SR3335 or SR1078 (15 mg/kg) or with the excipient (10% dimethylsulfoxide (Dmso), and 10% Tween 80 solution), single-caged, then shifted to 6°C for 3h for cold experiment. Body temperature was measured with a rectal probe (Physitemp, RET3, Clifton, New-jersey, USA) and a reader (Physitemp, BAT-12, Clifton, New-Jersey, USA) every hour during the test. Mice were sacrificed by cervical dislocation. Perigonadal and inguinal (white), and inter-scapular (brown) adipose tissues were cautiously dissected, weighted, snap-frozen in liquid nitrogen and stored at -80°C before RNA extraction or protein homogenate preparation. For *ex vivo* studies, adipose tissues were minced into small pieces (5-10 mg) and incubated in DMEM containing 10 mM glucose, 0.5% bovine serum albumin (BSA) and 10 µM RORα ligands (or the excipient Dmso) for the times indicated in the figure legends.

Metabolic rate was measured by indirect calorimetry in TSE Phenomaster cages (Systems, Chesterfield, Missouri).19 wk-old C57BL6/j mice were housed individually one week before the experiment under a 12h light/12h dark cycle at 22°C. Food (chow diet) and water were provided *ad libitum.* 6 mice were injected daily with SR3335 (15mg/kg) or the excipient at the end of the light period (ZT11) for four days. On the fourth day morning (ZT5), each mouse was challenged with the β3 adrenoreceptor agonist CL316,243 (1 mg/kg) to explore adaptive thermogenesis. Body composition was
measured by nuclear magnetic resonance (NMR) at the beginning and the end of the experiment. Measurements in the metabolic chambers were made from D1 to D5.

**Cell culture.** Murine 3T3-F442A adipocytes were differentiated as previously described (34). Human adipocytes have been obtained from adipose-derived stem cell (hASC) isolated from the abdominal subcutaneous adipose tissue of 3 healthy and pre-menopausal women (BMI< 25 kg/m²) in a context of plastic surgery and with the free and informed consent of the donors, in accordance with the ethical standards of the local ethical committee and the Declaration of Helsinki (1964). The study was approved by the French regulatory authorities (CPP Ile de France V, n° 14964). hASC were isolated according to Zhu et al (43). Briefly, tissue was digested with 0.2% collagenase (Sigma, Darmstadt, Germany). After centrifugation, the stroma vascular fraction was filtered on 100 µM pores, rinsed and seeded. Cells were cultured in αMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1% penicillin-streptomycin and 145 nM basic fibroblast growth factor (Pepro Tech, Neuilly sur Seine, France). Sub-confluent hASC cells were trypsinized and seeded at 10,000 cells/cm². The white differentiation process used was: two days post-confluence, adipogenesis was initiated by culturing hASC in DMEM with 10% FCS, 1 µM rosiglitazone, 1 µ M dexamethasone, 500 µM IBMX (3-isobutyl-1methylxanthine), 1 µM insulin during 5 days, then with 1 µM insulin and 1 µM rosiglitazone for the following 9 days. The impact of repeated doses of RORα ligands during adipogenesis was tested twice with four points in each kind of adipocytes. Treatment with 10 µM RORα ligands in mature adipocytes was performed in DMEM containing 10 mM glucose, 0.5% BSA and for the indicated times in the figure legends; three different experiments including 6 points have been performed for each RORα ligand.

**Confocal immunofluorescence microscopy.** Adipocytes were grown and differentiated on 13-mm glass coverslips. They were fixed and permeabilized in cold methanol for 10 min at −20°C. Primary and secondary antibodies were applied to coverslips in PBS with 1% BSA for 15-30 min at room temperature. Confocal microscopy was performed using a Leica TCS-SP microscope (Lasertechnik, Berlin, Germany) equipped with a 63× objective. Double fluorescence images were acquired in a
sequential mode. Serial optical sections of 1 µm were taken. Selected paired sections were then processed to produce single composite color-merged overlay images using Adobe Photoshop software (version 5.5). The antibody against PLIN1 (ab3526) was from Abcam, Paris, France.

**RNA extraction, cDNA synthesis and quantitative PCR.** Adipocyte and adipose tissue mRNA were extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, Courtaboeuf, France), then reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Carlsbad, CA, USA). Quantitative PCR of the genes of interest was performed using SYBR Green and a Light Cycler 480 Real-Time PCR System (Roche Diagnostics, Meylan, France) and the specific primers listed as followed: for m*Ucp1* ACAGAAGGATTGCCGAAAC (fwd) and AGCTGATTTCCTCTGAATG (rev); for m*PRDM16* ATGTGCTTAAATCCACCTTA (fwd) and GGAGAGGAGTCTTCCAGAG (rev); for m*TLE3* TGGTGAGCTTTGGAGCTGT (fwd) and CGGTTTCCCTCCAGGAAT (rev) and for m*ADRB3* ATCGTGTCGCTGTCGCGT (fwd) and ATCTGGCCCCTACACGCCAC (rev); for m*36B4* GCTGATGGGCAAGAACACCA (fwd) and CCCAAAAGCCTGGAAGAAGGA (rev); for m*HPRT* AGGACCTTCGGAAGTGT (fwd) and TCAAATCCCTGAAGTACTCAT (rev); for PGC1α CAACCGCAGTCGCAACAT (fwd) and TGGGAACCCTTGGGGTCA (rev); for m*cox8b*, GAACCATGAAGCCAACGACT (fwd) and GCGAAGTTCACAGTGGTTCC (rev); for m*cox7a* CAGCGTCATGGTCAGTCTGT (fwd) and AGAAAACCGTGTCGAGAGA (rev); for m*CPTIb* ATCATGTATCGGCCGAAACT (fwd) and CCATCTGGTAGGACACATGG (rev); for m*CS*: GGACAATTTCGCACTCTGC (fwd) and TCGGTTCATTCCTCTCTGCATA (rev); for m*ERRα* GCAGGGCAGTGGGAAGCTA (fwd) and CCTCTTGAGAAGCTTTT (rev); for RIP140 TCAAAAGCCACACCATACC (fwd) and TCGTCTCCACTGTGTCATC (rev); for h*CS* TAGTGCTTCCTCCAGAATTTG (fwd) and CCACCATACTCATGTCACAG (rev); for h*COX2* TACGGCGACTAATCTTCAA (fwd) and CCGGAATTGCATCTTTT (rev).

Gene expression was normalized to 36B4 and HPRT and data analysis was based on the Δ ΔCCT method.
**Protein extraction and western blot analysis.** Frozen adipose tissue was homogenized on ice in Tissue Protein Lysis Buffer (Euromedex, Souffelweyersheim, France) then centrifuged firstly at 10,000 g, 4°C for 8 min to remove lipids, and then at 20,000 g, 4°C for 10 min to remove insoluble material. Supernatants were subjected to SDS–PAGE and western blotted with antibodies against RORα (sc-28612), PPARγ (sc-7273), hUCP1 (sc-293418) (Santa-Cruz Biotechnology, Heidelberg, Germany) and against PRDM16 (ab 106410), mUCP1 (ab 10938) (Abcam, Paris, France), against TLE3 (Proteintech 11372-1-AP, Manchester, UK) and tubulin (T5168, Sigma, Darmstadt, Germany). Bands were measured on unsaturated exposition by densitometry using the software “Image J” (NIH, USA) and expressed vs tubulin used as loading control.

**Mitochondrial bioenergetics analysis.** Mitochondrial mass of the differentiated adipocytes were appreciated by the fluorescent labeling of the Mitotracker Red probe (MTR, M-7512, Molecular Probes, Eugene, OR). Measurement of oxygen consumption was performed using a Seahorse Bioscience XF96 Analyzer (Agilent, Santa Clara, CA, USA), according to the manufacturer’s protocol. OCR measurement were obtained following sequential additions of oligomycin (10 µM), FCCP (6.3µM) and antimycin A (AA)/rotenone (10 µM).

**ROR synthetic ligand.** SR3335 and SR1001 were purchased from Cayman (Interchim, Montigny le Bretonneux, France) and SR1078 from Calbiochem (Merck Chemicals, Nottingham, UK).

**Statistical analysis.** Values are presented as means +/- SEM. Statistical analysis was performed by one-way or two-way ANOVA or by unpaired Student ‘s t test as mentioned in the figures caption (Graph Pad Software, San Diego, CA, USA). P<0.05 was considered the limit for statistical significance.
Results

*UCP1 expression is modulated by RORα inverse-agonist or agonist in scWAT and BAT explants*

A 16h exposure of scWAT explants from WT mice to the RORα inverse-agonist SR3335 enabled a transient appearance of UCP1 protein (Fig 1A); this effect was found to be specific of RORα because explants from RORα-/- (sg/sg) mice, physiologically expressing UCP1, were unsensitive to SR3335 addition (histogram Fig 1A). SR3335 was also able to increase the UCP1 amount in the BAT of WT mice, but with a lower magnitude than in scWAT (x1.8 and x3 in BAT and scWAT, respectively) (Fig 1B). In order to test if the reciprocity was true, BAT explants from both genotypes were treated with a RORα agonist, SR1078. Fig 1C confirms that this ligand-dependent increase of RORα activity led to a half-decrease of UCP1 amount in BAT. Expectedly, SR1078 had no effect on UCP1 expression in explants from sg/sg mice. Our data thus show that the amount of UCP1 in adipose tissue is *ex vivo* modulated by ligands of the nuclear receptor RORα, in the absence of other culture manipulations.

*Beige cells can arise from 3T3-F442A adipocytes treated with RORα inverse-agonists*

Loss of RORα in mice promotes adipose tissue browning and its decreased activity in adipose explants leads to an increase of UCP1 expression (18,25). This shows that RORα exerts a negative effect on UCP1 expression and suggests an indirect mechanism of action. Indeed, experiments of global run analysis have shown that, if the mechanism of gene induction by RORα was direct and transcriptional, the way used by RORα to repress brown fat genes expression was indirect (41). We previously observed that in WAT of sg/sg mice, the higher expression level of brown genes was concomitant with a decreased level of two transcriptional suppressors of the thermogenic program, TLE3 and RIP 140 (25), making them putative direct target genes of RORα. We thus investigated whether and how RORα ligands were able to modulate the thermogenic pathway in a murine white adipocyte cell line, either in differentiating adipocytes or in mature adipocytes (Fig 2).
We first compared the effect of both kinds of RORα ligands (two inverse-agonists and one agonist) on the “white” or “brown” phenotype of 3T3-F442A cells treated during the course of adipose conversion from D4 to D14, and with isoproterenol for the last 6h. Figure 2A shows representative Western blots and histograms averaging the respective protein amount accumulated into the adipocytes from D7 to D14. We first verified if these ligands were able to exert an effect on the RORα protein amount; indeed, ROR gene promoters contained several ROREs suggesting that RORα could regulate its own transcription (9). Consistently, the content of RORα that accumulated during adipogenesis was found to be increased by the treatment with the RORα agonist SR1078 and decreased with the RORα inverse-agonist SR3335, while the other inverse-agonist SR1001 tested had no significant effect (Fig 2A). The RORα inverse-agonist SR3335 was able to increase the UCP1 and PRDM16 protein amount and the mitochondrial mass of the adipocytes (Fig 2D), whereas the number of multilocular lipid droplets into the adipocytes seemed also to be enhanced (sup Fig 1A, red arrows). At the opposite, the RORα agonist SR1078 decreased the UCP1 and PRDM16 protein amount, while increasing that of TLE3, a suppressor of the thermogenic program. Finally, the RORα inverse-agonist SR1001 was not found to exert any significant variation on these proteins. Thus, our data show that the browning of murine adipocytes is modulated by the ligands of RORα, suggesting a putative role for this nuclear receptor in the maintenance of a “white” phenotype.

We next tested whether, in fully mature 3T3-F442A adipocytes, a single exposure to the RORα inverse-agonist SR3335 was also able to induce the onset of the thermogenic program. Fig 2B shows that SR3335 increased PRDM16 and UCP1 protein as soon as 7h after its addition in the medium, with a maximal effect at 16h and a return to almost initial levels after 24h, in agreement with previous results in WAT explants (Fig 1A). Concomitantly, we observed an increase of their mitochondrial mass (Fig 2D); some of these adipocytes already exhibiting a higher number of lipid droplets than in control (sup Fig 1A, red arrows). Time-course analysis of the effect of SR 3335 on Ucp1 and Prdm16 mRNA levels indicated that the increased gene expression preceded that of the related proteins (Ucp1 and Prdm16 mRNA peaking at 9h vs proteins at around 16h) (Fig 2C). Whereas no significant variation of the TLE3
repressor was detectable by Western blot (Fig 2B), a transient decrease of TLE3 mRNA was observed 6h after SR3335 treatment (Fig 2C); reciprocally, the RORα agonist SR1078 was found to induce a transient peak in TLE3 mRNA levels after a 3h exposure. The gene expression of the other suppressor of the thermogenic program, RIP 140, was not changed by RORα ligands (Fig 2C).

Finally, we observed an increased gene expression of PGC1α, Cox8b, Cox7a and CPT1b in F442A cells, suggesting an enhanced β-oxidation capacity in response to SR3335 (Fig 2E). At the opposite, we noted a decrease of Cox7a gene expression by SR1078 in F442A cells (Fig 2E).

These data show that a single dose of SR3335 was able to promote the browning of mature adipocytes. The time-course of the events allows the hypothesis of an initial action of RORα on the level of TLE3 mRNA but does not seem to implicate RIP140 gene expression.

**RORα inhibition induces the emergence of beige characteristics in human adipocytes whereas RORα activation counteracts this effect**

In order to determine whether the regulation of the thermogenic program by RORα was also effective in human adipocytes, we analyzed the effect of RORα ligands on differentiating and mature adipocytes derived from human adipose stem cells (hASC).

hASC were differentiated in white adipogenic medium supplemented with 10 µM RORα ligands from D5 to D13 before being harvested at D14. Protein expression was monitored along the differentiation process and illustrated by a representative Western blot (Fig 3A) and protein amount accumulated from D7 to D14 compared with control cells in side-histograms. At D14, their mitochondrial mass was measured with the MTK dye by fluorimetry and by immunocytochemistry (Fig 3B). The perilipin antibody was used to visualize the size of the lipid droplets (sup Fig 1B). Their oxygen consumption rate (OCR) was analyzed by the Seahorse technology. Data confirmed the results obtained in the murine 3T3-F442A cell line. When added during adipocyte differentiation, SR3335 enabled the emergence of a beige phenotype of human adipocytes with an increase of PRDM16 and UCP1 protein levels (Fig 3A) and with the appearance of smaller multilocular lipid droplets as delineated by the
perilipin immunostaining (sup Fig 1B). The mitochondrial mass was enhanced in parallel, as visualized by the Mitotrack staining (Fig 3B), together with an increase of the uncoupled OCR (proton leak) and no variation of coupled respiration (ATP production) (Fig 3C). In addition, SR3335 decreased the amount of the TLE3 protein. Noteworthy, the RORα agonist SR1078 had the opposite effect on the TLE3 protein (Fig 3A) and on the mitochondrial mass (Fig 3B). Finally, the amount of PPARγ was not affected by the RORα ligands (Fig 3A).

Thereafter, we performed the time-course analysis of a single dose of each RORα ligand on protein expression in differentiated human adipocytes (Fig 4). Western blots from Figure 4A illustrate representative data obtained with SR3335 or with SR1078, whereas kinetics shown in Figure 4A averaged the data obtained by protein density analysis. RORα inverse-agonist SR3335 increased PRDM16 (from 3h to 9h) and UCP1 (peaking at 16h), while decreasing TLE3 (lowest level after a 9h exposure) (Fig 4A). On the opposite, the TLE3 protein amount increased as soon as 3h after the RORα agonist SR1078 addition, while those of PRDM16 and UCP1 progressively decreased (Fig 4A). Some other candidates were analyzed by gene expression (Fig 4B). Concerning the other repressor of thermogenic genes we tested, RIP140 mRNA was decreased by the RORα agonist SR1078, whereas an increase would be expected for a repressor. Finally, we observed an increase of PGC1α, COX2 and citrate synthase mRNAs amount by SR3335 and not by SR1078, suggesting an enhanced β-oxidation capacity by SR3335.

As observed in the murine preadipocyte cell line, these data confirmed that the ligand-dependent RORα inhibition enables the emergence of beige characteristics in human adipocytes as well, whereas RORα activation counteracts it.

*In vivo pharmacological modulation of UCP1 in scWAT by RORα ligands affects the mass of the fat depot, body weight and energy expenditure of the mice.*
Because the two RORα ligands that we *ex vivo* used have been demonstrated to be active *in vivo* (15,38), we then tested their ability to control UCP1 amount in mice adipose tissues after a single I.P. injection of SR3335 or SR1078 (15 mg/kg). Given the average 16h period of time necessary for UCP1 to rise in WAT explants and the short half-life of the compounds, we chose to test the effect of the ligands 18-20h after injection.

The representative Western blot and the side-histograms (Fig 5A) show that SR3335 induced a 4-fold increase in UCP1 amount in scWAT of WT mice, rising an average level similar to that detected in sg/sg mice. Under the same conditions, no significant effect of SR3335 on UCP1 protein amount was observed in the BAT (Fig 5B). By contrast, *in vivo* exposure to the RORα agonist exerted the opposite effect: SR1078 injection reduced by 3-fold the UCP1 amount in the scWAT of WT mice, and by 1.3-fold in the BAT, but without any effect on sg/sg mice (Fig 6 A,B). These data confirmed that RORα ligands could be useful tools to modulate UCP1 protein amount in adipose tissues *in vivo*.

We thus investigated whether these effects of RORα ligands on UCP1 expression have been associated with variations of whole body weight and/or adipose tissue depot mass of the mice. We found that SR3335 and SR1078 exerted opposite effect on the mice body weight. A single injection of SR3335 was able to decrease their body weight (Fig 5C), while that of SR1078 to increase it (Fig 6C), by about 2.8% of their initial weight compared to control animals. Likewise, SR3335 decreased the scWAT relative mass (Fig 5D) of the mice, while SR1078 induced a non-significant increase in the mass of this depot (Fig 6D). The relative BAT and liver mass were not affected by the RORα ligands (Fig 5D and 6D). Body weight and tissues depot mass being unsensitive to both treatments in sg/sg mice, this confirms a RORα-dependent effect of the synthetic ligands (Fig 5 and 6).

To further functionally investigate the origin of the weight loss exerted by SR3335 on WT mice, we tested their energy expenditure by indirect calorimetry in response to the RORα agonist (Fig7). C57BL6/j mice were daily injected by SR3335 or the excipient for 3 days. Fig 7A illustrated the pattern of data acquisition on day 3 and night 3 while Fig 7B showed the averaged data upon 48h (days and nights 2 and 3). Data clearly showed that SR3335 was able to increase energy expenditure, O2 consumption and CO2 production, especially during the night period without affecting neither food and
drink intake nor the locomotor activity of the mice. Finally, given that the respiratory exchange rate (RER) was nightly decreased by SR3335, data suggests an increased lipid oxidation in this period of time at the expense of the other nutrients present in the chow diet.

**The decreased body weight and fat mass induced by the RORα inverse-agonist SR3335 is associated with increased body temperature and browning of scWAT in cold-stressed mice**

To investigate if the SR3335-dependent increase of UCP1 in scWAT was functionally associated with a gain in adaptive thermogenesis, mice from both genotypes were treated or not for 24h with a single dose of SR3335, then challenged by a 6°C cold exposure for 3h. Rectal temperature was measured every hour. Figure 8A confirmed that sg/sg mice maintain higher rectal temperature than WT mice during cold exposure, as previously shown by Lau et al (18). Indeed, while WT mice lost 3.5°C after 2h in the cold, sg/sg mice only lost 2°C. Strikingly, SR3335 injection partially prevented the decrease in the rectal temperature of WT mice that exhibited a rectal temperature pattern similar than sg/sg mice. The measurement of body weight before and after the cold challenge showed that the higher thermogenesis of sg/sg mice was associated with a higher weight loss (-7.5%) than in WT mice (-5%). Remarkably, SR3335 injection allowed the WT mice to lose similar body weight than sg/sg mice (-8%) (Fig 8B). As expected, SR3335 did not induce an additional weight loss in sg/sg mice (Fig 8B).

Mice were sacrificed immediately after cold exposure and their fat depots collected. SR3335 injection in WT mice decreased scWAT relative mass by 30%, at a level comparable to the subcutaneous fat depot of the untreated sg/sg mice (Fig 8C). The scWAT mass of sg/sg mice was unsensitive to SR3335 injection. Of note, the BAT and liver masses of the cold-stressed mice were not influenced by the genotype, or by the SR3335 treatment. The level of UCP1 amount in the scWAT depots of these mice fitted with their respective mass losses; it was increased by 1.7 +/- 0.3 fold in the scWAT of all SR3335-treated vs vehicle-treated cold-stressed WT mice (Fig 8D). Finally, the BAT of these mice were compared at the level of gene expression (Fig 8E): UCP1 and ADBR3 mRNA levels were not different according to the genotype, and only enhanced in WT mice in response to SR3335 and acute cold challenge.
Because the weight-matched sg/sg mice used in our experiments were necessarily six-months older than WT mice, we investigated if the failure of sg/sg response to RORα ligands was not due to the fact that they are much older than WT mice. By examining the absolute weight loss in response to SR3335 injection in old age-matched (65-wk-old) mice from both genotypes after acute cold exposure (4h at 4°C), we could observe that only WT mice lost more weight in response to SR 3335+cold vs cold alone (WT: - 0.77g vs sg/sg -0.06g). Cold-stressed sg/sg mice lost 1.22 +/- 0.27 and 1.16 +/- 0.24 gram with and without SR 3335 injection, respectively, while WT mice lost 2.18 +/- 0.34 and 1.41 +/- 0.14 g with and without SR3335 pre-treatment, respectively (p<0.05), n=4 for sg/sg and 5 for WT mice). These data thus confirm that aged WT mice still respond to SR3335 whereas aged sg/sg mice do not.

Taken together, these data show that during cold exposure, a single SR3335 injection enables WT mice to acquire the same performance than untreated sg/sg mice, and suggests a more efficient adaptive thermogenesis associated with an increased UCP1 expression and a specific loss of scWAT.

The browning effect of RORα inverse-agonist SR3335 affects the non-shivering thermogenesis in WT mice

To unequivocally demonstrate that browning induced by pharmacological modulation of RORα affect non-shivering thermogenesis, we performed indirect calorimetry analysis in C57BL6/j mice in response to a β3-adrenoreceptor agonist. 6 mice having been pre-treated in vivo by SR3335 or not for 3 days were challenged by a CL316,243 injection on D4. Fig 9A and B compared the data 6h after the injection vs the same period of time on D3 and clearly showed that CL316,243 increased the energy expenditure and decreased the RER more importantly in mice pre-treated with SR3335 than in untreated mice. In parallel, their body temperature was increased by 0.3°C (Fig 9C) and, at the end of the experiment, these mice have lost the double of the fat mass loss due to the CL316,243 injection alone (Fig 9D).
Taken together, these data confirm that, as the loss of RORα function in sg/sg mice, pharmacological antagonism of RORα by an inverse-agonist allows the browning of the scWAT depot of WT mice and potentiates the decrease in the mass of this fat pad subsequently to the activation of non-shivering thermogenesis that improves lipid oxidation.

**Discussion**

The regulation of adipose tissue browning was not fully deciphered and required further studies explaining how the regulation of this process may be of interest for tackling obesity and related metabolic disorders. Our data confirmed the involvement of the transcription factor RORα in the regulation of non-shivering thermogenesis, and importantly, revealed the possibility to *in vivo* modulate its activity by synthetic ligands with beneficial consequences on fat mass and body weight of the mice.

The first evidence for the association between RORα signaling and UCP1 mRNA expression was provided by the Muscat ‘s team. They observed a decrease in the expression of *UCP1* mRNA in the BAT of an adipocyte-specific RORα gain of function mouse model (35) whereas, on the opposite, an increased UCP1 expression was found in the BAT and WAT of the RORα-deficient sg/sg model (18) that we further confirmed (25). These findings pointed to RORα as a new transcription factor negatively involved in *UCP1* expression. We previously showed that a synthetic RORα inverse-agonist was a useful tool for increasing UCP1 protein amount and oxygen consumption in scWAT explants of WT mice, thus pharmacologically *ex vivo* mimicking the effect of the *in vivo* RORα deficiency in WAT (25). We confirmed here a similar regulation by the RORα inverse-agonist into BAT explants and also demonstrated that reciprocally, a RORα agonist was able to down-regulate their UCP1 protein amount. Moreover, we could observe an increase of the COX4 protein in BAT explants treated by SR3335 (by 2.4fold, not shown). From these experiments, emerged the possibility to *ex vivo* modulate UCP1 expression (and perhaps mitochondrial function) into adipose tissues by the use of synthetic ligands of
the nuclear factor RORα. Moreover, the action of these ligands appeared to be specific of RORα, since no effect was detectable on adipose explants isolated from sg/sg mice.

We then investigated if this “up and down” browning effect of RORα ligands was reproducible in cultured adipocytes. We observed for the first time that the ligand-dependent RORα modulation can control the emergence of beige characteristics, not only in murine but also in human adipocytes; the RORα inverse agonist increasing thermogenic genes and mitochondrial biogenesis and function, and the RORα agonist exerting the opposite effect.

The first protocol we used was several repeated doses added every 2-3 days along the differentiation process in a way to “optimize” the expected answer. It took into account the kinetics of expression of RORα that appears only significant from D2 or D4 of differentiation (1,7 respectively). Thus, this approach was performed in a way to prevent an interference with the initial differentiation cocktail used for hASC, for example. Our goal was not to decipher the origin of the beige cells, i.e. whether the RORα ligands interfered with the differentiation of specific precursors, but rather to determine whether modulating RORα activity allowed their switch in an active or inactive thermogenic state (12). Our demonstration of a browning effect obtained only few hours after the addition of a RORα inverse-agonist on mature adipocytes would fit with a transdifferentiating effect (2,19). At the opposite, the fact that RORα activation by agonists was still able to rapidly decrease some residual brown gene expression in the human adipocytes was compatible with such hypothesis. However, one cannot exclude the possibility that RORα ligands acted on pre-existing beige adipocytes into the F442A cell line or hASC. In that field, molecules reported as affecting beige activation and differentiation have been most often tested ex vivo on cell systems of differentiation (and by addition as soon as the beginning of the differentiation process) (8). Few studies have been performed on mature adipocytes, (21,29,42), or on explants (10).

Our data revealed that, in both murine and human mature adipocytes, treatment with the RORα inverse-agonist SR3335 resulted in a rapid increase in PRDM16 and UCP1 protein amount reaching a
peak after about 16h before decreasing at 24h. The appearance of the UCP1 protein as soon as 6-7h after the treatment corroborated the time course observed in primary mouse adipocytes exposed to 31°C (40). The maximal increase in their respective mRNA, peaking at 9h before decreasing at 16h, fits with the pattern of UCP1 transcript induction by trans-retinoids (24). The transient effect of SR3335 on UCP1 expression is coherent with the reported half-lives of UCP1 transcript and protein (5h and 10h, respectively), in brown adipocytes treated with the β3-adrenergceptor agonist CL316,243 (22). Our results suggest transcriptional events activated by SR3335 or repressed by SR1078. Given that RORα activation exerts a negative effect on UCP1, and since it has been reported that negative gene regulation by RORα tends to be indirect (41), we focused on the pattern of expression of two different suppressors of the thermogenic program (6,37). We previously found these suppressors to be decreased in sg/sg mice (25), suggesting they could be putative targets for RORα. RIP140 mRNA levels were not changed by the RORα ligands in murine F442A adipocytes and decreased by the RORα agonist SR1078 in hASC, whereas an increase would have been expected for a repressor. At the opposite, TLE3 mRNA levels were reciprocally regulated by the RORα agonist or inverse-agonist. TLE3 mRNA level peaked as soon as 3h after the addition of the RORα agonist SR1078 in the medium, but decreased maximally 6h after the addition of the RORα inverse-agonist SR3335. These data suggest that the suppressor of the thermogenic program TLE3 could better fit as a candidate target of RORα transcriptional activity than RIP140.

A key mechanism of SR3335 action could be to release the brake of RORα on the thermogenic pathway. TLE3 has been described as a white-selective cofactor that acts reciprocally with the brown-selective co-factor PRDM16 to specify thermogenic gene program. Indeed, TLE3 is able to disrupt the physical interaction between PRDM16 and PPARγ (37). Consistently, TLE3 is less abundant in BAT than in WAT (Fig 2A and 37). The same distribution between fat depots was observed for RORα expression (Fig 2A), allowing to hypothesize that these two proteins, whose expression rises during white adipogenesis (1,7,36), could act in concert to maintain a “white” adipocyte phenotype. Finally, the RORα ligands did not affect the expression of the master adipogenic gene PPARγ (Fig 3A), and thus
did not interfere with the global adipogenic program, but rather act downstream to switch the fat cell towards a white or beige phenotype.

There are limitations in the interpretation of our data:

i) we have not tested the expression of other thermogenic brakes than TLE3 and RIP140,

ii) TLE3 transcript could not be strictly RORα-dependent, because it is not entirely absent from scWAT of sg/sg mice (25); however, comparing the circadian rhythms of the thermogenic program in the scWAT of sg/sg compared to WT mice (25), we observed that the highest reciprocal variation of Prdm16 and TLE3 mRNA levels took place at the same period of the day (at the beginning of the dark period), suggesting a higher disproportion in the abundance of these reciprocal actors of thermogenesis in the absence of RORα than in its presence. This suggested that the stoichiometric equilibrium between these two factors was modified in sg/sg mice during the circadian cycle of gene expression. However, only experiments of TLE3 gain and function in the presence of RORα inverse agonist would demonstrate the involvement of TLE3 in the regulation of UCP1 by RORα.

iii) the specificity of synthetic ligands is always questionable. While these RORα ligands were shown to have no activity at other nuclear receptors as defined in a Gal4-chimeric receptor assay (14), some potential interaction within the ROR subfamily existed for some of them. Indeed, while SR3335 is a RORα–specific inverse agonist (15), SR1001 and SR1078 are able to bind both RORα and RORγ (14,38). This is the reason why we previously verified the reciprocal effects of the inverse agonist SR3335 and of the agonist SR1078 on the RORα protein amount in 3T3-F442A adipocytes and then excluded SR1001 because it was not found to decrease the amount of RORα (Fig 2A). In the case of SR1078, its inhibitory effect on UCP1 expression in scWAT and BAT (Fig6) could be due to the inhibition of both RORα and RORγ; this is an option because a specific RORγ inverse agonist (SR1555) has been reported to increase UCP1 expression into the BAT (5).

Another important point to discuss was whether the link between thermogenesis and RORα activity involved β3-adrenoceptor signaling. Cold being the natural stimulus for the activation of
thermogenesis, the prevailing model was that it exerts its effect on adipocytes indirectly \textit{i.e.} via the central nervous system, relayed by the $\beta_3$-adrenoceptor signaling. However, it was reported that the induction of thermogenic genes in the scWAT from $\beta$-adrenoceptor-deficient mice was largely preserved on cold exposure, suggesting that there is a $\beta$-adrenoceptor-independent pathway that controls thermogenic gene expression in scWAT (40). In sg/sg mice, there was no increase of the $\beta_3$-adrenoceptor expression (ADBR3) in BAT and WAT vs WT mice (Fig 8 and 17). Whereas increased levels of norepinephrine have been reported in the BAT of sg/sg mice (4), these mice displayed similar increase of oxygen consumption after i.p. injection of the $\beta_3$-adrenoceptor agonist CL316,243 than WT mice (18). The group of Lau et al (18) concluded that the increased thermogenic activity of sg/sg mice suggested that ROR$\alpha$ deficiency results in a cell-autonomous effect that is not driven by the central nervous system. The ligand-dependent ROR$\alpha$ modulation of thermogenesis we observed here confirmed a cell-autonomous process. Indeed, it was obtained \textit{ex vivo} in the absence of other stimuli. However, during the treatment of differentiating 3T3-F442A adipocytes with the ROR$\alpha$ agonist SR1078, we observed a higher inhibition of UCP1 in presence of the $\beta$-adrenoceptor agonist isoproterenol (Fig 2A) than in its absence (not shown). This suggests a potential interference between ROR$\alpha$ activation and $\beta_3$-adrenoceptor signaling. Nevertheless, our investigations on the possibility for the ROR$\alpha$ ligands to directly impact the cAMP/protein Kinase A signaling, thus downstream the activation of $\beta$-adrenoceptors, have not been conclusive (not shown). Interestingly, it was observed that TLE3 expression inhibited the ability of isoproterenol to induce \textit{UCP1} expression in PRDM16-expressing cells (37). Given that SR1078 was able to increase TLE3 protein amount in 3T3-F442A adipocytes that also expressed PRDM16 (Fig 2A), our data confirmed a similar scenario, but the underlying molecular mechanisms remain to be explored. Finally, we observed that SR3335 was able to increase ADBR3 gene expression into the BAT of WT mice after acute cold challenge (Fig 8); thus perhaps improving the local adrenergic tone that was reduced, but still upgradable by a $\beta_3$-specific agonist in 13-months aged mice (30).

On a physiological point of view, we show that ROR$\alpha$ ligands could be also useful tools to
modulate *in vivo* UCP1 expression in scWAT of WT mice with associated consequences on the mass of this fat depot. We found that SR3335 and SR1078 exert opposite effect on the WT mice body weight, and without any effect on sg/sg mice. Remarkably, a single injection of SR3335 was able to rapidly decrease their body weight (Fig 5C), while that of SR1078 to increase it (Fig 6C), by about 3% of their initial weight at the same period of the day. Indirect calorimetry allowed to demonstrate that SR3335 was *in vivo* able to promote an increase of energy expenditure associated with a decrease of RER, confirming an enhanced lipid oxidation in C57BL6/j mice. We then show that, during cold exposure, a single SR3335 injection enabled WT mice to acquire the same performance than untreated sg/sg mice, suggesting a more efficient adaptive thermogenesis associated with an increased UCP1 expression and a specific loss of scWAT. However, acute cold exposure was not sufficient to assess UCP1-dependent non-shivering thermogenesis since other thermogenic processes, including shivering, participate in the maintenance of body temperature during the first hours following exposure to low temperatures. Thus, to unequivocally demonstrate that browning induced by pharmacological modulation of RORα affects non-shivering thermogenesis, we performed indirect calorimetry in response to β3-specific agonist. Data confirmed that SR3335 increased CL316,243-induced energy expenditure, lipid oxidation and fat mass loss in C57BL6/j mice.

We thus demonstrate that, similarly to the loss of RORα function in sg/sg mice, pharmacological antagonism of RORα by an inverse-agonist improves the browning of the scWAT depot and potentiates the decrease in the mass of this fat pad in cold-stressed WT mice and in global fat mass in response to a β3-specific agonist. It was previously reported that sg/sg mice did not lose more body weight than WT mice after cold exposure (10°C), whereas exhibiting a higher body temperature (18). We thought that this discrepancy with our own data in sg/sg mice could be explained by the fact that the authors expressed their results in gram of weight lost (absolute value), but not as the percentage of variation of the initial body weight. Indeed, Lau *et al* (18) mentioned that the differences in weight between sg/sg and WT mice were <15%. In our case, the significant difference was 14%, but we rather chose weight-related mice than age-related ones (about 10 and 18 months-old for WT and sg/sg mice, respectively). Indeed, sg/sg mice are leaner than their WT counterparts, and we performed our experiments on aged
mice in order to minimize their body weight differences, and also to obtain enough adipose tissue explants to work with. Nevertheless, because age is known to have a deep impact on UCP1 expression that declines precipitously between 3 and 12 months of age in C57BL6/j mice (30), we previously checked the potential effect of age in the differential response to ROR\(\alpha\) ligands between the two genotypes. We actually noticed a declined response towards \textit{in vivo} SR3335 treatment in aged WT mice but, that was nevertheless conserved at the age of 52-65 wk; in 26-wk-old WT mice, the UCP1 protein amount in scWAT being increased by about 4-fold (fig 5), then by 1.82 +/- 0.52 (n=5) in 52-65 wk-old mice. In parallel, in 52-65 wk-old-matched sg/sg mice, UCP1 failed to response to SR3335 (x 0.91 +/- 0.3) (n=5) and thus the difference between the two genotypes remained significant.

Given that aged mice have been shown to be defective in cold-induced beige adipocyte formation due to a cellular aging senescence-like phenotype of beige progenitors (3), this notion strengthens the therapeutic interest of our findings, with the new proposal to activate \textit{in vivo} the white to beige switch in aged individuals, with beneficial effect on body weight. This effect mainly concerned the scWAT in mice and was only tested in adipocytes differentiated from precursors isolated from human abdominal scWAT. However, it was reported that in Human, visceral adipocytes were more prone to beiging (44), thus it would be interesting to test the effect of ROR\(\alpha\) ligands on human deep fat depots. Finally, our \textit{in vivo} study did not allow to distinguish in which proportion the energy balance of the mice was affected by the SR3335-induced beiging of scWAT and by the SR3335-activation of BAT itself. Nevertheless, we only observed a decrease of WAT fat pads and, not of interscapular BAT, by the ROR\(\alpha\) agonist, perhaps in line with the activation of FFA oxidation in these fat depots. In humans, it is difficult to delineate if BAT activity decline with age or obesity. Anyway, it was believed there would be good reason to attempt (pharmacologically) to oppose this development and thus decrease the risk for middle-age obesity (26).

In conclusion, our data highlight insight into i) a new potential mechanism of WAT beiging that will require more investigations for a better knowledge in the specific biology of white and brown adipose tissues and ii) a potential therapeutic target to stimulate adipose tissue beiging in the absence of
cold exposure. The fact that RORα inverse-agonist was also able to decrease neoglucogenesis in the liver \textit{ex vivo} (11) and blood glucose in diet-induced rodent models of obesity (15) confirmed the therapeutic potential of antagonizing RORα to lower body fat percentage and improve glucose homeostasis. However, it should be kept in mind that RORα is an ubiquitous nuclear factor, the inhibition of which probably leading to adverse effects in other tissues. Further long-term investigations are mandatory to assess their efficiency and safety in animal models of metabolic diseases.
Bibliography


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Disclosure Statement

The authors have no conflict of interest to declare.
Figure Legends

**Fig 1: RORα inverse-agonist SR3335 and agonist SR1078 reciprocally modulated the amount of UCP1 protein in adipose explants**

A: Representative Western blot of UCP1 protein appearance in response to *ex vivo* treatment by SR3335 (10 µM) in scWAT explants from WT mice and averaged data obtained by 20h SR3335 treatment in WT and sg/sg mice (side-histogram; n= 5). UCP1 expression follows circadian variation (25) that persists for a few days in explants. ZT= Zeitgeber Time, ZT0 is the beginning of the light phase and ZT12, the beginning of the dark phase. The points 4h and 8h have been frozen at the same period than controls (T0) at ZT10, and the points 16h and 24h at the same period than T0 at ZT2.

B and C: Effect of SR3335 (B) or SR1078 (C) *ex vivo* treatment on BAT explants from WT and sg/sg mice and averaged data after 20h (side-histogram; n= 5). Data are presented as means +/- SEM. *p <0.05 vs excipient-treated WT explants as determined by unpaired two-tailed Student’s test.

**Fig 2: Beige cells can arise from 3T3-F442A adipocytes treated with RORα inverse-agonists: inverse effect by RORα agonist**

A: Representative Western blot of murine differentiating adipocytes treated by two different RORα inverse-agonists (SR3335 or SR1001) and by a RORα agonist (SR1078), from D4 to D14 post-confluence, isoproterenol being added 6h before cells lysis; the sum of respective protein that accumulated along the adipogenic differentiation process was quantified vs tubulin in the side-histograms (n=3 to 5).

B: Representative Western blot of the time-course analysis of a single dose of SR3335 (10 µM) on protein amount in mature 3T3-F442A adipocytes.

C: Time-course analysis of one single dose of SR3335 (red) or SR1078 (green) (10 µM) on gene expression (n=4-5 by point).
D: Estimation of the mitochondrial mass into the adipocytes (as measured by the Mitotracker dye expressed per ng DNA), either 17h after a single dose of SR3335 or after several doses all along adipogenesis (D4-D14) (n=3).

E: Relative gene expression 16 or 24h after the RORα inverse-agonist (SR3335) or agonist (SR1078) addition on differentiated adipocytes. Data are presented as means +/- SEM. *p <0.05 **p <0.01 vs excipient-treated adipocytes as determined by unpaired two-tailed Student’s test.

Fig 3: Effect of RORα inverse-agonist and agonist along the adipogenic process of human adipocyte stem cells (hASC)

A: On the left, representative Western blot of human differentiating adipocytes treated by the RORα inverse-agonist (SR3335) or agonist (SR1078), from D5 to D14 post-confluence; on the right, the sum of respective proteins that accumulated along the adipogenic differentiation process was quantified vs tubulin in hASC from two different donors (n=6).

B: Estimation of the mitochondrial mass into the adipocytes (as measured by the Mitotracker dye expressed per ng DNA) and immunodetection of mitochondria by the mitotracker dye into the human mature adipocytes, pre-treated or not with SR3335, and in presence or not of isoproterenol.

C: The oxygen consumption rate (OCR) was measured by Seahorse analysis at D14 on mature adipocytes, pre-treated or not with SR3335 or SR1078 (n=6). On the left, representative Seahorse run with and without isoproterenol 10 µM. On the right, basal and isoproterenol-stimulated uncoupled (proton leak) and coupled (ATP production) mitochondrial respiration under various RORα ligands treatment. Proton leak and ATP production were defined by the equations: OCR_{oligomycin} – OCR_{AA/rotenone} and OCR_{basal} – OCR_{oligomycin}, respectively . Data are presented as means +/- SEM. *p <0.05 and **p <0.01 vs excipient-treated adipocytes as determined by unpaired two-tailed Student’s test.

Fig 4: RORα inhibition enables the emergence of beige characteristics in human differentiated adipocytes whereas RORα activation counteracted it
A: on the left, representative Western blot of the time-course effect of SR3335 or SR1078 on protein amount in differentiated human adipocytes. On the right, corresponding averaged protein amount (n=3-5); two or three different hASC donors having been used.

B: Time-course analysis of one single dose of SR3335 (red) or SR1078 (green) (10 µM) on gene expression (n=4-5 by point). Data are presented as means +/- SEM *p <0.05, **p <0.01 and ***p <0.005 vs excipient-treated adipocytes as determined by unpaired two-tailed Student’s test.

**Fig 5: Injection of the RORγ inverse-agonist (SR3335) enables the appearance of UCP1 in scWAT and decreases body weight and subcutaneous fat mass of mice**

WT and sg/sg male mice, aged 6 and 12 months respectively, have been IP-injected with SR3335 (or Dmso) (15 mg/kg) 23h before analysis. Data are presented as means +/- SEM. *p <0.05 vs excipient-treated WT mice as determined by unpaired two-tailed Student’s test.

A, B: on the left, representative Western blot showing UCP1 protein amount into the scWAT (A) and BAT (B) of the mice injected with SR3335 or Dmso; on the right, averaged data obtained with 6 mice of each genotype.

C: Analysis of body weight variation of the mice expressed in % of initial weight (n=5-6).

D: Analysis of adipose tissue and liver masses expressed in % of body weight at the same period of the day (n=5).

**Fig 6: Injection of the RORα agonist (SR1078) decreases UCP1 in the BAT and scWAT and increases body weight of mice**

WT and sg/sg female mice, aged 12 and 18 months respectively, have been IP-injected with SR1078 (or Dmso) (15 mg/kg) 18h before analysis. Data are presented as means +/- SEM. *p <0.05, **p <0.01 and ***p <0.005 vs excipient-treated WT mice as determined by unpaired two-tailed Student’s test.

A, B: on the left, representative Western blot showing UCP1 protein amount into the scWAT (A) and BAT (B) of the mice injected with SR1078 or Dmso; on the right, averaged data obtained with 6 mice of each genotype.
C: Analysis of body weight variation of the mice expressed in % of initial weight (n=6).
D: Analysis of adipose tissue and liver masses expressed in % of body weight at the same period of the day (n=6).

**Fig 7: RORα inverse-agonist (SR3335) increases energy expenditure in C57BL6/j mice.**

Six 19 wk-old mice were daily IP injected with SR3335 (15mg/kg) or the excipient at the end of the light period (ZT11) for 3 days. Energy expenditure, O₂ consumption and CO₂ production were measured by indirect calorimetry and related to the lean mass of the mice housed at 22°C.

A: Representative profile of data acquisition on day 3 and night 3. In red and black, mice were treated with SR3335 and the excipient, respectively. Black arrows indicate the time of SR3335 injection.
B: Average data obtained upon 48h (days and nights 2 and 3) and presented per 12h-period of daylight or night. Respiratory exchange ratio (RER) was calculated by the ratio VCO₂/VO₂. Average food and water consumption were measured by differential weight-based sensors. Spontaneous locomotor activity was measured by infrared beam breaks. Data are presented as means +/- SEM. *p <0.05, **p <0.01 vs excipient-treated WT mice as determined by unpaired two-tailed Student’s test.

**Fig 8: RORα inverse-agonist (SR3335) decreases body weight and subcutaneous fat by increasing body temperature and browning of scWAT in cold-stressed mice**

WT and sg/sg male mice aged 10-18 months have been IP-injected with SR3335 (or Dmso) (15 mg/kg) 23h before being cold-stressed for 3h at 4°C in individual cages (n=6 of each genotype and condition). Data are presented as means +/- SEM. *p <0.05, **p <0.01 vs excipient-treated WT mice as determined by unpaired two-tailed Student’s test.

A: Core body temperature appreciated by rectal measure every hour.
B: Body weight lost at the end of the cold exposure and expressed in % of initial weight.
C: Relative fat and liver masses of the mice at the end of the experiment.
D: Western blot illustrating the UCP1 protein amount in the scWAT of some of the mice at the end of the cold exposure.
E: Analysis of some gene expression in the BAT of the mice at the end of the experiment.

Fig 9: RORα inverse-agonist (SR3335) increases non-shivering thermogenesis in C57BL6/j mice in response to a β3 adrenoreceptor agonist.

Six 19-wk-old mice were daily IP injected with SR3335 (15 mg/kg) or the excipient for 4 days (at ZT11), and challenged with a dose of a β3-adrenoreceptor agonist CL316,243 (1 mg/kg) on the day 4 at ZT5. Energy expenditure, O₂ consumption and CO₂ production were measured by indirect calorimetry and related to the lean mass of the mice housed at 22°C.

Energy expenditure (A) and RER (B) were measured upon the 6h-period of time following CL316,243 injection; +CL referred to data measured on day 4 (between ZT5 and ZT11) and were compared with the data obtained on the corresponding period of time on day 3 (called –CL). In red and white, mice were treated with SR3335 or the excipient, respectively.

C: The change in mice body temperature exerted by CL316,243 administration was the difference between rectal temperature measured on D4 and D3 evenings, thus after and before CL316,243 injection.

D: referred to fat mass loss in SR3335 and Dmso-treated mice at the end of the experiment, including the 24h-period of time after the CL316,243 injection. We compared the fat mass (measured by NMR) of each mouse between the end (D5) and the beginning (D1) of the indirect calorimetry experiment.

Data are presented as means +/- SEM. *p <0.05 referred to SR3335 effect (SR3335-treated vs Dmso-treated mice, # p <0.05 ## p <0.01 referred to CL effect vs corresponding pre-treated mice as determined by unpaired two-tailed Student’s test.