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AM-879, a PPARγ non-agonist and Ser273 phosphorylation blocker, promotes insulin sensitivity without adverse effects in mice

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ABSTRACT

Obesity is one of the main risk factors for type 2 diabetes, and peroxisome proliferator-activated receptor γ (PPARγ) is considered a promising pathway on insulin sensitivity and adipose tissue metabolism. The search for molecules acting as insulin sensitizers have increased, especially for molecules that block PPARγ-Ser273 phosphorylation, without reaching full agonism. We evaluated the *in vivo* effects of AM-879, a PPARγ non-agonist, and found that AM-879 exerts different effects in mice depending on the dose. At lower doses, this ligand decreased BAT, increased leptin and Crh expression. However, at a higher dose, it promoted improvement on insulin sensitivity, ameliorates expression of metabolism-related genes, decreased the expression of genes related to liver toxicity, maintaining body weight and adipocyte size. These results present a new lead molecule to ameliorates insulin resistance and confirm AM-879 as a PPARγ non-agonist which blocks Ser273 phosphorylation as a good strategy to modulate insulin sensitivity without developing the adverse effects promoted by PPARγ full agonists.

1. Introduction

Obesity and type 2 diabetes (T2D) are common conditions in the Metabolic Syndrome and have achieved pandemic proportions nowadays. They are related to the increased accumulation of adipose tissue, which is considered multifactorial and is a result of a disbalance between energy intake and expenditure, causing several damages, as inadequate response of the organism to insulin, changes in carbohydrate and fat metabolisms, and increased triglycerides accumulation in muscle [1,2]. Previous studies report the importance of peroxisome proliferator-activated receptor γ isotype (PPARγ), a transcription factor belonging to the nuclear receptors (NRs) superfamily, in lipid and carbohydrate metabolism, and in insulin sensitivity regulation, which makes this receptor an important target to fight these comorbidities [3–5].

PPARγ is responsible for the transcriptional regulation of different genes. On glucose metabolism, this receptor acts recruiting it from the blood through insulin action [6]. Concerning lipid metabolism, PPARγ promotes adipocytes differentiation, increasing adiponectin secretion, neuroprotector effects and regulation of inflammatory process by NF-κβ pathway [7,8]. Moreover, this NR upregulates genes as *Cd36, Fabp4* (fatty acid binding protein 4) and *Gk* (glycerol kinase) [9]. On adipocytes, PPARγ activation leads to an increase on adiponectin expression, *IRS2* (insulin substrate 2), *Glut4* (glucose transporter type 4) and *CAP* (Cbl associated protein), which are related to insulin sensitivity and glucose uptake [9].

All these effects are promoted by PPARγ ligands, which may be endogenous, as fatty acids, or exogenous, like Rosiglitazone or other PPARγ agonists, being responsible for activating this NR, and resulting on gene target transcription [10,11]. On this scenario, agonist ligands can fully activate the receptor, recruiting coactivators, as SRC1 and PGC1-α, that increase the expression of genes involved on glucose and lipid metabolism. However, this agonism may consequently cause some undesired effects, as fluid retention, body weight gain, bone loss, and heart failure, among others. Alternatively, partial- or non-agonists, which promotes low or no PPARγ activation, have been described as insulin sensitizers, inducing few adverse effects [12–14], and have been searched in recent studies focusing on weak agonists or non-agonists

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molecules that do not activate this receptor, as an approach to avoid adverse effects of PPARγ full activation.

In 2010, a phosphorylation on Serine 273 of PPARγ was related to insulin resistance, modifying the regulation of the expression of a set of genes involved on lipid and glucose metabolisms [15]. According to reports, the modulation of this receptor activity through some ligands might result in the blockage of the phosphorylation site, promoting insulin sensitivity, and avoiding the transcriptional activation of genes related to lipid metabolism. Based on this, the modulation of PPARγ with specific ligands might prevent the side effects caused by full agonists treatment, as those caused by thiazolidinediones (TZDs) [16–20].

In this context, some PPARγ agonists were described presenting few negative effects after receptor binding, as YR4-42, which shows weaker affinity and activation of PPARγ, similar to pioglitazone, causing improved insulin sensitivity on diet-induced obese (DIO) mice, reduction of serum triglycerides and of body weight [17]. In addition, WSF-7 was also described as PPARγ agonist and Ser273 blocker, promoting less potent adipogenesis than rosiglitazone *in vitro,* increasing glucose uptake and adiponectin expression [18]. Other ligands, as EPA-PC and EPA-PE sea cucumber phospholipids, were reported as PPARγ agonists, promoting increased adipogenesis and lipid accumulation *in vitro,* but *in vivo* these ligands decreased adipocytes size and lipid droplets, improving glucose tolerance and insulin resistance [19].

Besides these weak agonists, other ligands that do not fully activate PPARγ and block Ser273 phosphorylation, as partial or non-agonists, have been reported. The GQ-16 is a partial agonist that decreases visceral adiposity and induces thermogenic genes expression [20,21]. CMHX008 and GQ-11, cause positive effects on reducing the risk of bone loss, and improve lipid profile, respectively [22,23]. MRL-24 is a compound with high affinity for PPARγ, but with poor agonist properties, mainly on adipogenesis context [15]. AM-879 [24], a PPARγ non-agonist, revealed positive effects on not promoting adipocytes differentiation, blocking Ser273 phosphorylation *in vitro,* and inducing changes in gene expression in comparison to Rosiglitazone [25]. Interestingly, this ligand was first described in 2012 as a modulator of the corticotrophin-releasing hormone (CRH) pathway (PubChem AID 651639 e CID 1209375), which interacts negatively with neuropeptide y and positively with leptin, regulating food consumption and satiety [26–29].

As AM-879 was reported as a PPARγ ligand, potentially not inducing the side effects of a full agonist, the aim of this study was to investigate its physiological effects as a new modulator for obesity and diabetes, through a diet-induced obesity (DIO) mouse model, comparing its effects with Rosiglitazone. Our results show that AM-879 is a promising lead molecule, presenting interesting effects in insulin sensitivity and lipid metabolism without promoting weight gain.

2. Methods

2.1. Animals

Male C57BL/6J mice (JAX#664) were housed in the pathogen-free animal facility at the Model Organism Laboratory at Brazilian Biosciences National Laboratory (LNBio), Brazilian Center for Research in Energy and Materials (CNPEM). Animals were maintained on a photoperiod of 12:12 light/dark cycle at 21–24 °C, with free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee (CEUA/CNPEM, protocols 58 and 72).

2.2. Diets and experimental design

Male mice were fed with a control diet (5% kcal as fat; Nuvilab CR1, Quimtia) until six-week-old. After that, they were maintained on a high-fat diet (HFD, 60% Kcal as fat; PragSoluções, Jaú, SP-Brazil) for 18 weeks to promote obesity and hyperglycemia (diets compositions on Suppl. Table 1). In the last two weeks, they were randomly divided into four groups: vehicle, AM-879 (4-({2-[(1,3-dioxo-1,3-dihydro-2H-inden-2-ylidene)methyl]phenoxy} [30]nethyl) benzoic acid, AM-879-40965082, Specs) 40 mg/kg/day (AM-40) and 100 mg/kg/day (AM-100) and Rosiglitazone (Rosi- R2408, Sigma-Aldrich) 4 mg/kg/day [21]. AM-879 doses were chosen based on its affinity for PPARγ, which is 10 times lower than Rosiglitazone [25]. Moreover, after a pilot insulin tolerance test (ITT) assay, we also increased the dose to 25-fold more concentrated in order to enhance its effects.

To avoid the stress caused by gavage, we choose the voluntary oral administration method [31], using a jelly composed by a 20% sucralose solution (Linea®, EIC do Brasil Indústria e Comércio Alimentos S/A-Anápolis, GO), 21% colorless and flavorless jelly (Royal®) and a chocolate essence (Arcolor®, Arco Íris Brasil Indústria e Comércio de Produtos Alimentícios Ltda.- São Lourenço, São Paulo-SP), which was given once a day. The drugs were diluted on the vehicle solution (60 °C), which is sucralose 20% with 0,01% of Tween 80. Body weight and food intake were measured weekly from 6 to 22 weeks of age, and daily during drug treatment (22–24 weeks of age), for weight gain and energy intake calculations.

On the euthanasia day, blood samples were collected in the morning, at the fed state and animals were fasted for 6 h and euthanized by decapitation, with the collection of truck blood, which was centrifuged (1200 g for 20 min at 4 °C) and serum was stored at −80 °C for measurement of insulin, adiponectin, and corticosterone. Epididymal white adipose tissue (eWAT), brown adipose tissue (BAT), heart, kidney, liver and gastrocnemius muscle were dissected and weighted. The length of the right tibia was used as a normalizing parameter. Samples of eWAT and hepatic caudate lobe were processed for histological analysis, and the other organs were stored at −80 °C for further analysis. Liver samples were also used for determination of hepatic triglyceride content. A timeline describing all the experiments is available on Suppl. Figure 1.

2.3. Insulin tolerance test (ITT)

Before starting the treatment and on the thirteenth day of treatment, animals were fasted overnight followed by 1 h of feeding to perform insulin tolerance test (ITT), with insulin dose of 0.75 UI/kg. The collection of blood samples was made from the dorsal tail vein for blood glucose measurements using the Accu-chek Performa® (Roche) blood glucose monitor, at: 0, 4, 8, 12, 16, 20 and 24 min. KITT was measured as described previously [32].

2.4. Plasma hormones quantification

Tail blood samples on fed state were collected with heparin, and plasma was collected by centrifugating it at 2000g for 20 min, 4 °C. Plasma insulin, adiponectin and corticosterone were measured by ELISA kits (Ultrasensitive Mouse Insulin #90080; Adiponectin Mouse #80569 - Crystal Chem®, Elk Groove Village, IL-USA; Corticosterone Competitive ELISA Kit -EIACORT- Thermo Fisher Scientific®), and triglycerides were analyzed using enzymatic assay kits (LaborLab Liquid Stable - # 1770290- LaborLab, SP-Brazil), according to the manufacturer's instructions.

2.5. Liver and white adipose tissue (eWAT) histology

Hepatic caudate lobe and right eWAT were dissected and fixed in 4% paraformaldehyde for 24 h. After that, they were dehydrated, embedded into a paraffin block, cut into 5 μm sections, stained with hematoxylin-eosin by standard procedures for histological analysis. The brightfield images of each tissue slice were captured with a digital camera mounted on upright light microscope (Leica FS DM6), using 20X objectives for liver and 10x, for eWAT. Five distinct regions were captured of each liver slide, with three slices, separated by 50 μm, for each animal, being analyzed by the segmentation method using Weka Segmentation and quantification with MorphoLibJ Plugin from Fiji®.

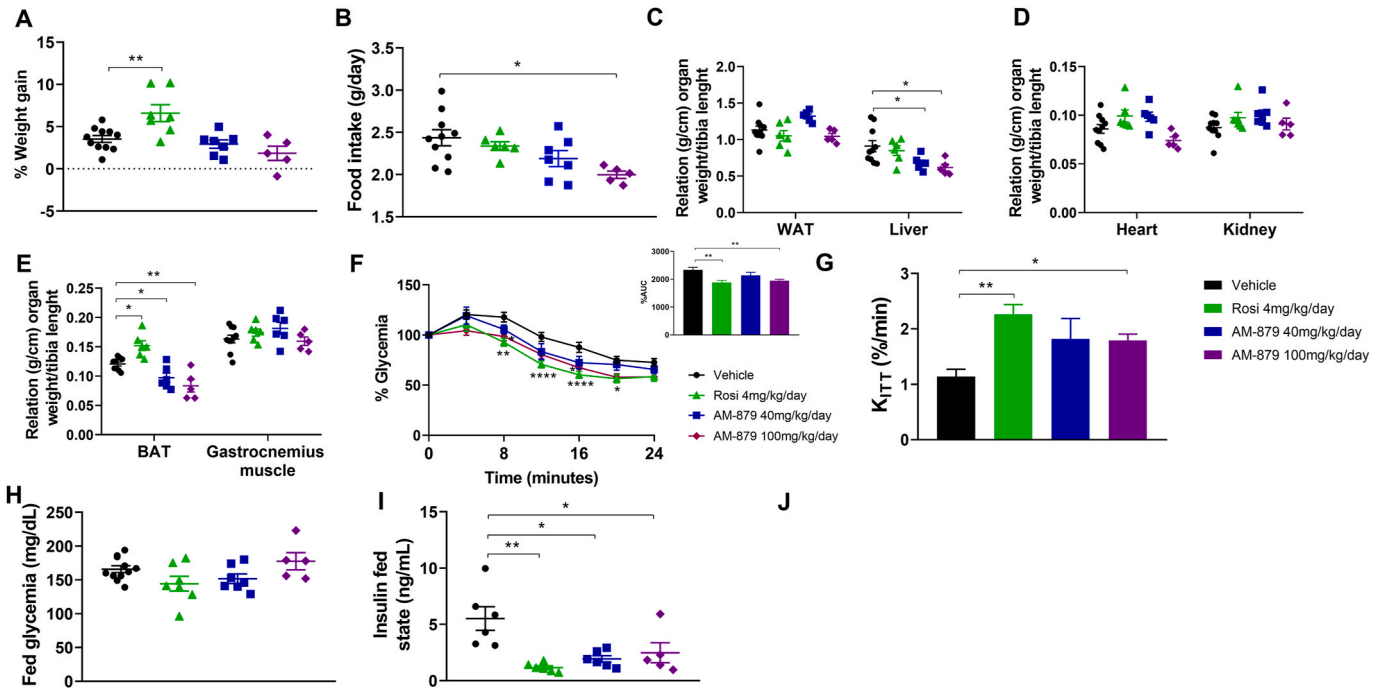

Fig. 1. Physiological effects during two weeks of treatment and phenotypic parameters at the end of two weeks of treatment on high-fat diet (16 weeks) fed animals. (A) Percentage of weight gain considering weight at the first and one day after the last day of treatment. (B) Daily food intake of each animal during two weeks of treatment. (C), (D) and (E) Relation of each organ weight (white adipose tissue- WAT-liver, heart, right kidney, brown adipose tissue- BAT- and gastrocnemius muscle, and tibia length, collected at euthanasia day. (F) Insulin tolerance test (ITT) represented as glycemia percentage considering time zero as 100%, with its respective area under the curve (AUC). (G) Glucose disappearance rate (KITT), represented in percentage per minute. (H) Glycemia on fed state on euthanasia day. (I) Plasma insulin levels on fed state on euthanasia day. (J) Plasma adiponectin levels on fed state on euthanasia day. Data are represented as mean ± SEM. Statistical analysis was done using Dunnett's T3 Multiple Comparison ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$. $n \geq 3$ per group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

For eWAT, the whole tissue was captured in a panoramic picture to analyze all the adipocytes. We developed a Macro workflow on Fiji® for image segmentation and adipocyte identification which were posteriorly used as input for a Python script optimized to perform shape analysis (i.e. area and perimeter) for every single cell on each treatment (n).

2.6. Hepatic triglyceride content quantification

Hepatic triglycerides were quantified using an enzymatic assay kit, as descried above, using the lipid extracted from the liver by the chloroform:methanol:PBS method, as described previously [21]. 100 mg of frozen tissues were homogenized in 4:3:0.8 of the mixture using a Polytron® (PT1200 E) and then centrifuged to extract the organic layer, where the lipid content was measured by an enzymatic method in spectrophotometer using Triglycerides LaborLab GOD-PAP Liquid Stable Kit (LaborLab – SP/Brasil).

2.7. Quantitative real-time PCR analysis

Total ribonucleic acid (RNA) from tissues (eWAT, BAT, liver and hypothalamus) was isolated using TRIzol™ reagent (Ambion- Thermo Fisher Scientific) and the chloroform-isopropanol extraction method, following the manufacturer's protocol and using a Polytron® (PT1200 E) to homogenate the tissue. RNA concentration and quality were checked by Nanodrop 2000 (ThermoScientific, 260/280 ratio higher than 1.8) and cDNA synthesis was carried out using 1 μg of RNA with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™). Quantitative real time PCR (qPCR) reactions were performed with SYBR Real Time PCR master mixes (ThermoFischer Scientific) in a 7500 Real Time PCR system (Applied Biosystems). Relative mRNA expression was determined by the ΔΔ-Ct method [33] normalized to *36b4* and *Rpl27* levels. Primers sequences used in this study are presented in Suppl. Table 3.

2.8. Western blotting

Proteins were extracted from mice white adipose tissue through a solution composed by EDTA 0.02 % (v/v) 0.5 M pH8, 0.1 % (v/v) Tris 1 M pH7.5, 0.0045 % (m/v) sodium fluoride, 0.0019 % (m/v) sodium orthovanadate, Triton X-100 10%, 0.02 % (v/v) PMSF (serine protease inhibitor), 0.04 % (v/v) Cip 25X (protein inhibitor cocktail) and water q. s., incubation during 1 h on ice, after Polytron® (PT1200E) maceration. After that, samples were centrifuged, and supernatant was collected for protein quantification using Pierce™ 660 nm Protein Assay Reagent (Thermo Scientific ™). 15 μg of protein was used for electrophoresis gel and the following primary and secondary antibodies: anti-PPARγ (dilution 1:1000, Anti-PPARγ Antibody (E−8): sc-7273- Santa Cruz Biotechnology, Inc.), anti-Ser273 PPARγ (dilution 1:200, BS-4888R, Bioss), anti-vinculin (dilution 1:1000, ab18058- Abcam), anti-rabbit IgG (dilution 1:5000, A0545, Sigma-Aldrich) and anti-mouse IgG (dilution 1:5000, 401253, Calbiochem®). Membranes were revealed using peroxidase reaction through Amersham ECL Prime Western Blotting Detection Reagent®-GE in ImageQuant™ LAS 500 (GE Health Care Life Sciences).

2.9. Statistical analysis

Data analyses were carried out in the GraphPad Prism 8.0 statistical package. All results are presented as mean ± SEM. Analysis were performed using two-tailed unpaired Student's t-test or Multiple Comparisons one-way ANOVA followed by Dunnett's post hoc test for comparing the means of two or multiple groups, respectively. The ITT test was analyzed by a Two-way ANOVA followed by Dunnett's post hoc test was

also performed to compare the interaction of rows and columns (groups). Statistical significance was noted when $p < 0.05$.

Statistical analysis of WAT was implemented on R-Studio. Outliers were considered as values over the IQR (interquartile range) multiplied by 1.5 and were excluded from analyses. Descriptive statistics and statistical tests 'ggstatsplot' library considering non-parametric distribution (Wilcoxon rank sum test). This test considers H0 (null) and H1 (alternative) hypothesis. Bayesian tests were also implemented due to large sample number, where the Bayes factor (BF_{10}) gives the evidence for H1 over H0. On this case, higher BF_{10} indicates higher evidence in favor of alternative hypothesis, which means sigher and stronger difference between groups (between 3 and 20 is positive, 20 and 100 is strong and over 100 is extremely strong) [34,35].

3. Results

3.1. AM-879 reduced food consumption without affecting body weight and promoted reduction in liver and BAT weights in HFD fed animals

AM-879 is a novel non-agonist of PPARγ that blocks Ser273 phosphorylation and does not induce adipocyte differentiation *in vitro* [25]. These data suggest that AM-879 could potentially be used as a novel insulin sensitizer in T2D associated with obesity, but its *in vivo* effects are unknown. Here, we tested two different doses of AM-879 (40 and 100 mg/kg/day) and compared them to the insulin sensitizer Rosiglitazone (Rosi). It is important to note that the treatment was performed using a voluntary oral administration, in which the ligand was dissolved in jelly, to provide a less stressful drug delivery compared to other methods.

After 18 weeks of HFD feeding and administration of the ligand in the last two weeks, Rosiglitazone group present an increase on body weight gain as expected (Fig. 1A) [36,37]. AM-879 did not lead to weight gain (Fig. 1A), while it led to a decrease in food intake (Fig. 1B). We also observed a reduction in liver weight with both doses of AM-879 (AM-40- 25.3 %, 0.68 ± 0.04 g/cm *vs* 0.91 ± 0.08 g/cm; AM-100- 31.9 %, 0.62 ± 0.05 g/cm *vs* 0.91 ± 0.08 g/cm) (Fig. 1C). Regarding BAT, Rosiglitazone promoted an increase on its weight (25 %, 0.15 ± 0.008 g/cm *vs* 0.12 ± 0.003 g/cm), while both doses of AM-879 reduced the weight of this organ (AM-40- 19.2 %, 0.097 ± 0.008 g/cm *vs* 0.12 ± 0.003 g/cm; AM-100- 30.8 %, 0.083 ± 0.01 g/cm *vs* 0.12 ± 0.003 g/cm). No differences were observed in heart, kidney, and gastrocnemius muscle among all groups (Fig. 1C–E).

3.2. AM-879 improved insulin sensitivity, did not change hepatic steatosis, and promoted the reduction of liver triglyceride content

Insulin tolerance test (ITT) showed that both doses of AM-879 partially improved insulin sensitivity in comparison to the potent insulin sensitizer Rosiglitazone (Fig. 1F). All groups, except the lower dose of AM-879, increased the glucose disappearance rate (KITT) (Fig. 1G). No alteration in glycemia in the fed state was observed (Fig. 1H), although a reduction in fed plasma insulin levels was shown in all treated groups (Fig. 1I), and increased plasma adiponectin levels were observed in the Rosiglitazone and AM-879 100 mg/kg/day groups (Fig. 1J). These results are consistent with improved insulin sensitivity [38–42] due to inhibition of PPARy Ser273 phosphorylation [15,25]. In the liver, both doses of AM-879 maintained the level of hepatic steatosis similar to the vehicle group, while Rosiglitazone promoted an increase in steatosis (Fig. 2A and B). The higher dose of AM-879 also decreased the hepatic triglyceride content in 59.3% (Fig. 2C). These results are further evidence of an improvement in insulin sensitivity, reduction in the side effects of AM-879 and may be related to the decreased liver weight [43], also observed with AM-879 treatment (Fig. 1C).

3.3. AM-879 did not alter adipocyte size but affected the expression of genes regulated by Ser273 phosphorylation and involved in lipid metabolism

Analysis of adipose tissue (Fig. 3) showed that both AM-879 treated-groups maintained adipocyte size (area and perimeter) as observed in the vehicle group, with a slight increase in size. In parallel, Rosiglitazone promoted a decrease in area and perimeter, with an increase in small adipocytes density (area <2500 μm^2 and perimeter <200 μm), suggesting hyperplasia of adipose tissue. The AM-879 100 mg/kg/day-treated animals presented higher number of intermediate adipocytes with perimeter between 250 and 500 μm (Fig. 3A–C and Suppl. Table 2).

To functionally evaluate the S273 phosphorylation blockage, we

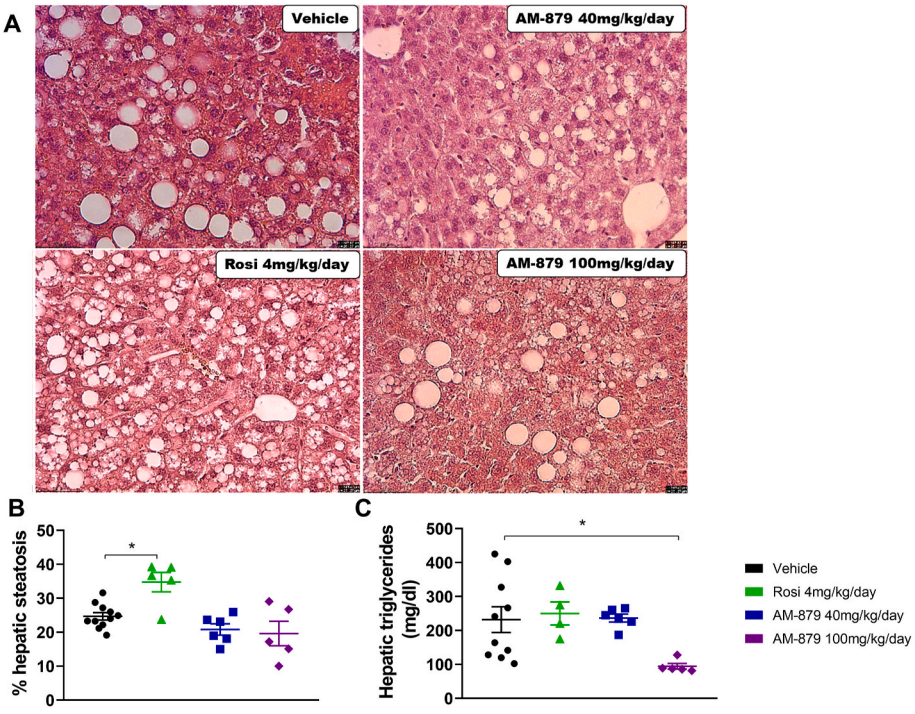

Fig. 2. Liver analysis after 16 weeks of high-fat diet feeding and two weeks of treatment. (A) Liver histology stained with Hematoxylin-Eosin and lipid droplets in white. (B) Quantification on hepatic lipid droplets by Weka Segmentation in Fiji-ImageJ. (C) Liver triglycerides levels quantified by an enzymatic assay and normalized to 100 mg of tissue. Data are represented as mean ± SEM. Statistical analysis was done using Dunnett's T3 Multiple Comparison ANOVA test. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. n ≥ 4 per group.

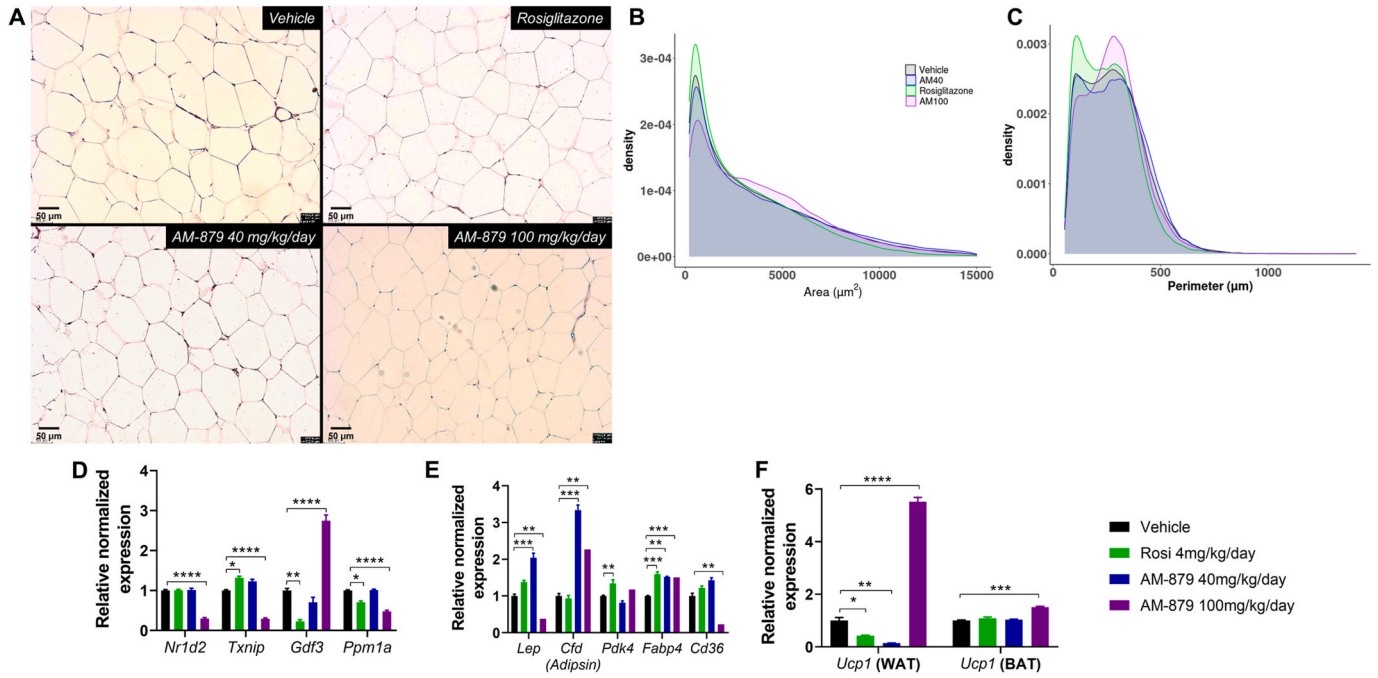

Fig. 3. White adipose tissue (WAT) analysis after 16 weeks of high-fat diet feeding followed by two more weeks of ligand treatment with HFD. (A) WAT histology after embedding it in paraffin and slides were done with 5 µm cuts. Analysis of WAT considering density of adipocytes on each (B) area and (C) perimeter, divided into each group. Relative normalized expression of (D) genes regulated by Ser273 phosphorylation in WAT: *Nr1d2* and *Txnip* described by Choi in 2010, *Gdf3* and *Ppm1a* described in 2020. (E) Genes involved on PPARγ and lipid metabolism pathways in WAT: leptin (*Lep)*, Adipsin (*Cfd), Pdk4* (pyruvate kinase dehydrogenase), *Fabp4* (fatty acid binding protein 4) and *Cd36* (scavenger receptor). (F) *Ucp1* expression. In WAT and BAT. Data were normalized to the expression of reference genes: *36b4* and *Rpl27*. Data are represented as mean ± SEM. Statistical analysis was done using Dunnett's T3 Multiple Comparison ANOVA test. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. n ≥ 5 per group.

measured the expression of genes regulated by this phosphorylation in WAT. The expression of *Nr1d2* and *Txnip* were decreased only by the higher dose of AM-879. In addition, we tested the expression of *Gdf3* and we observed that this gene can also be modulated by PPARy ligands (Fig. 3D). While Rosiglitazone treatment decreased *Gdf3* expression, consistent with reported data [44], surprisingly, AM-879 increased it, suggesting that this modulation acts in an opposite manner for these ligands. Another phosphorylation target, the phosphatase PPM1A (enzyme that promotes Ser273 dephosphorylation), showed reduced expression in the Rosiglitazone and higher dose of AM-879 groups (Fig. 3D).

Regarding expression of genes related to adipogenesis, lipid and glucose metabolism (Fig. 3E), we observed that the different doses of AM-879 played opposite effects on the expression of leptin (*Lep)*, as the lower dose of AM-879 increased it and the higher dose decreased it. Both doses of AM-879 significantly increased the expression of adipsin (*Cfd*). The fact that the expression of *Pdk4* was not changed by AM-879 treatment confirms its non-agonism on PPARγ (Fig. 3E) [25,45], and the increase in *Pdk4* expression by Rosiglitazone is in accordance as previously reported [15,25] (Fig. 3E). Both doses of AM-879 and Rosiglitazone significantly increased the expression of *Fabp4,* while only the AM-879 100 mg/kg/day group decreased the *Cd36* expression (Fig. 3E).

To evaluate a thermogenic marker, we tested *Ucp1* in white and brown adipose tissues. We observed that AM-879 100 mg/kg/day-treated animals had an increase in its expression in both tissues (Fig. 3F), also decreasing BAT weight (Fig. 1E). Interestingly, the lower dose of AM-879 decreased *Ucp1* expression only in eWAT (Fig. 3F).

3.4. AM-879 reduced the expression of genes related to liver toxicity

To investigate possible liver injury due to AM-879 treatment, we analyzed the expression of 6 genes related to liver toxicity: AST (aspartate aminotransferase), ALT (alanine aminotransferase), ALPL (alkaline phosphatase), GGT1 (gamma glutamyl-transpeptidase), ALB (albumin) and LDHA (lactate dehydrogenase). The lower dose of AM-879 reduced the expression of ALPL and GGT1, while the higher dose reduced the expression of all analyzed genes (Fig. 4A and B), indicating no promotion of liver toxicity through AM-879.

3.5. AM-879 altered the expression of genes involved in the CRH pathway without affecting circulating hormone

To investigate the involvement of AM-879 in the CRH pathway, we analyzed the expression of *Crh* and *Npy* in the hypothalamus. Our data showed that AM-879 40 mg/kg/day group had an increase in *Crh* expression, while the higher dose (AM-879 100 mg/kg/day) affected only *Npy* expression, increasing it (Fig. 5A). These results may suggest that the lower dose of AM-879 affected the CRH pathway. Meanwhile, no significant alterations in plasma corticosterone were observed after all treatments (Fig. 5B), suggesting minor physiological effects related to the possible activation of this pathway.

4. Discussion

The search for molecules that promote insulin sensitization is still relevant due to the increased number of type 2 diabetes new cases. Here we evaluated the effects of a promising PPARγ non-agonist in the context of insulin sensitivity as a blocker of PPARγ Ser273 phosphorylation [25], and in the development of adverse effects through modulation of genes regulated by this nuclear receptor.

Our results indicate that AM-879 non-agonist effects are dose-dependent, since the higher dose was more effective in regulating PPARγ pathways, and the lower dose may have some effects on the CRH pathway. These results are consistent with the previously reported Kd for AM-879 - PPARγ binding, which is on the order of 4 µM [25], suggesting that PPARγ modulation *in vivo* requires high concentration of

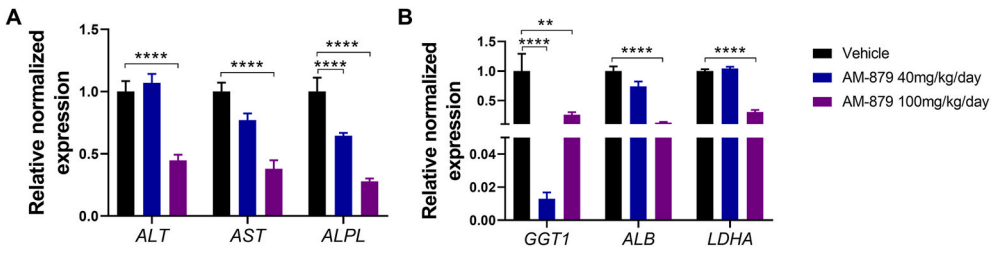

Fig. 4. Analysis of genes related to liver toxicity after 16 weeks of high-fat diet (HFD) and two weeks of each treatment. (A and B) Relative normalized expression of genes related to liver toxicity: AST (aspartate aminotransferase), ALT (alanine aminotransferase), ALPL (alkaline phosphatase), GGT1 (gamma glutamyltransferase), ALB (albumin) and LDHA (lactate dehydrogenase). Data were normalized to the expression of reference genes: 36b4 and Rpl27. Data are represented as mean ± SEM. Statistical analysis was done using Dunnett's T3 Multiple Comparison ANOVA test. $^{**}p < 0.01$ and $^{****}p < 0.0001$. $n \geq 5$ per group.

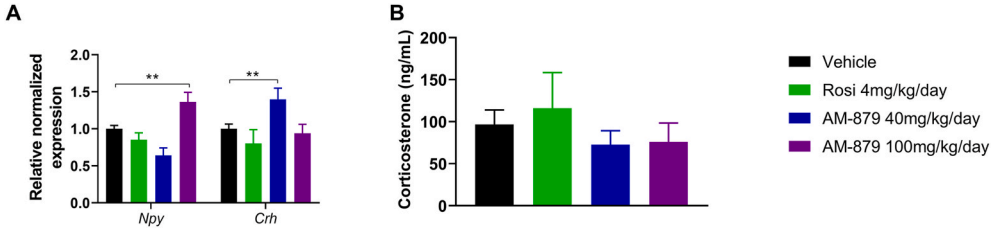

Fig. 5. Analysis of corticotrophin-releasing hormone (CRH) pathway after 16 weeks of high-fat diet (HFD) and two weeks of each treatment. (A) Relative normalized expression of genes involved on CRH pathway in hypothalamus: *Crh* (corticotrophin releasing hormone) and *Npy* (neuropeptide Y). Data were normalized to the expression of reference genes: *36b4* and *Rpl27*. (B) Plasma corticosterone measured using ELISA kit. Data are represented as mean ± SEM. Statistical analysis was done using Dunnett's T3 Multiple Comparison ANOVA test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. $n \geq 5$ per group.

circulating AM-879. Moreover, we show that AM-879 did not promote weight gain and reduced food intake (Fig. 1A and B). The changes in food consumption observed after AM-879 treatment may be related to a possible modulation of AM-879 in the CRH pathway, since it was first reported as a CRH modulator (PubChem AID 651639 e CID 1209375), acting through leptin inhibition, decreasing food intake, and altering energy balance [26,46]. It was previously reported that leptin is the main regulator on this pathway, acting by stimulating CRH and inhibiting neuropeptide Y (NPY), which may control food intake and energy expenditure [47]. Our results show increased *Npy* and decreased *Lep* expression after treatment with AM-879 in higher dose. Because of this we investigate some clues of CRH pathway, and we observed no alterations on *Crh* expression and plasma corticosterone levels (Fig. 4A and B; Fig. 3E). These data indicate no physiological effects related to AM-879 modulation in the CRH pathway [47,48], however, as food intake was kept lower in this condition, our results suggests alternative pathways regulated by PPARγ and/or AM-879 which might regulate food intake.

As expected due to the inhibition of Ser273 phosphorylation [15], insulin sensitivity was improved after treatment with the higher dose of AM-879, with increased glucose disappearance rate (KITT) and decreased area under the curve (AUC) on insulin tolerance test (ITT) (Fig. 1F and G). In accordance, AM-879 also decreased plasma insulin levels and increased plasma adiponectin without changing glycemia (Fig. 1H–J), which are also related to insulin sensitivity [38–42] due to inhibition of PPARγ Ser273 phosphorylation [25].

Concerning liver, AM-879 decreased its weight (Fig. 1C), did not induce steatosis like the full agonist Rosiglitazone, and decreased hepatic triglyceride (TG) content (Fig. 2A–C). Lipid metabolism in the liver is a complex process, in which hepatic steatosis is developed when the input of fat exceeds its oxidation or export, promoting hepatic lipogenesis [49]. This event is not necessarily correlated with liver TG content, since only small amounts of FAs are processed inside the liver, and the majority of TG is exported as TG-VLDL, and taken up by muscle and adipose tissue [50], improving insulin resistance [51]. Our results indicate that reduced TG on the higher dose of AM-879 group could be related to improved insulin sensitivity, while increased hepatic steatosis observed in the Rosiglitazone group may be caused by impaired liver lipid metabolism and PPARγ target genes activation [21,52–55], which is not induced by the non-agonism of AM-879.

In addition, AM-879 did not promote the increase of WAT (Fig. 1C), which is consistent with the fact that AM-879 was reported as non-agonist, and does not activate PPARγ, nor promoting coactivator's recruitment, or adipogenesis. However, in terms of adipocytes size, AM-879 promoted a slight hypertrophy of these cells, whereas Rosiglitazone promoted their hyperplasia (Fig. 3A–C). The WAT hyperplasia promoted by Rosiglitazone was previously described to occur in the first days of treatment and is related to depot-specific regulation of perilipin and adiponectin [52,56]. This causes insulin sensitivity improvement due to the additional lipid depot in adipose tissue, that request the available circulating lipids [57]. In addition, Rosiglitazone treatment was also related to the enlargement of existing adipose cells and to the recruitment of new small adipose cells in T2D patients, improving fat storage and, thus, systemic insulin sensitivity [58]. Both processes, hyperplasia and hypertrophy are linked to insulin sensitivity improvement, and our data show that modulation of adipose tissue promoted by AM-879 is similar to the effects of Rosiglitazone.

In terms of BAT weight, AM-879 behaves in opposite way in comparison to Rosiglitazone, reducing it (Fig. 1E). Increased BAT weight was already described after Rosiglitazone treatment, since it induces lipogenesis, triacylglycerol synthesis, hyperplasia and hypertropia of this tissue [59]. On the other hand, AM-879 effects are consistent with other PPARγ partial agonists, as GQ-16, which promoted reduction on this organ weight, due to an increased expression of *Ucp1,* resulting on BAT activation on cold exposure or thermogenesis [21,60].

Regarding phosphorylation function, AM-879 treatment at higher dose led to a decrease of *Nr1d2, Txnip* and *PPM1A* gene expression, while its lower dose did not change them (Fig. 3D). Rosiglitazone, also described as an inhibitor of PPARγ phosphorylation, did not change *Nr1d2* but reduced *PPM1A* and increased *Txnip* expression. Concerning PPM1A, our results confirm the blocking of Ser273 phosphorylation-dependent signaling is affected by the ligand, since it is related to the control of diabetic gene programs through PPARγ dephosphorylation, and the absence of p-PPARγ significantly decreases its expression [61]. However, for *Nr1d2* and *Txnip*, the expected behavior - an increase on these genes' expression after AM-879 and Rosiglitazone treatments - was partially achieved. These effects might be related to PPARγ Ser273 phosphorylation promoted by HFD, decreasing these gene expression.

However, the found differences for *Nr1d2* and *Txnip* in higher dose of

AM-879 seems to be related to the involvement of these genes on other pathways related to insulin resistance, adipogenesis and eating behavior, and not only to the PPARγ phosphorylation. Some studies show that *Txnip* expression profile in adipocytes did not change after treatment with non-agonist ligands that also blocks Ser273 phosphorylation [62]. The overexpression of *Txnip* is implicated in the pathogenesis of type 2 diabetes, acting as a promoter of ROS-induced insulin resistance [63,64], protecting mice from fasting induced liver steatosis [65], and which is induced by hyperglycemia and blocks glucose uptake into fat and muscle [53]. Additionally, *Nr1d2,* regulator of circadian clock and a dominant regulator of hepatic lipid metabolism, was reported to be an important modulator of lipid metabolism, since its ectopic expression potentiated adipocyte differentiation [66], its super expression induces expression of PPARs, C/EBP-a, aP2 and LPL, affecting Intramuscular fat deposition [67], and HFD-knockout-*Nr1d2* mice are resistant to developing obesity consuming slightly more food [52]. Our results indicate that these pathways are more related to AM-879 and Rosiglitazone treatments, and that Ser 273 phosphorylation effects might be superposed by other systemic effects.

Moreover, G*df3* is another marker related to insulin resistance, since *knockin* animals for Ser273 phosphorylation had a decrease on its expression, mainly on mice macrophages after prolonged HFD exposure [44]. Our results show opposite effects on AM-879 treated animals in terms of *Gdf3* expression, while Rosiglitazone treatment agrees with that (Fig. 3D). In addition, Gdf3$^{-/-}$ mice presented an increased HFD intake than wild-type as a compensatory response to their higher metabolic rate [68], which is in line with our results, since AM-879 treatment increased expression of *Gdf3* together with a decrease on food consumption. The regulation that PPARγ and Gdf3 play on each other, since PPARγ-deficient mice have increased *Gdf3* expression [69] agrees with our results. Moreover, partial agonists, non-agonists and full agonists of PPARγ act through different structural and dynamics mechanisms in the ligand binding cavity of the receptor, which is also regulated by this receptor dynamics and conformational changes during ligand binding [70,71].

In terms of adipogenesis, AM-879 treatment showed differential effects on adipsin (*Cfd*) and leptin (*Lep*) depending on the dose (Fig. 3E). *Cfd* and *Lep* are markers of mature adipocytes released into circulation to maintain homeostasis [72], which is related to the improvement of pancreatic β-cell function in obesity and T2D [73]. Increased expression of *Cfd* at both doses of AM-879 suggests an improvement in β cell function. In addition, the increased *Lep* expression in the lower dose of AM-879 treatment and its decrease in the higher dose of the ligand (Fig. 3E) suggest some activation of CRH pathway in the lower AM-879 dose [26] (PubChem AID 651639 and CID 1209375), while the observed effects in the higher dose of AM-879 seem to have stronger involvement in PPARγ-modulating pathways.

Regarding lipid metabolism, *Fabp4* gene expression was increased by AM-879 (Fig. 3E). Some reports describe *Fabp4* as a mature adipocyte marker involved on obesity, insulin resistance and atherosclerosis development, and in these cases, it is increased in circulating plasma [74–76]. On the other hand, its decreased expression in obese patients and mice induces reduction of fatty acid β-oxidation and an overflow of fatty acid to other tissues [77]. Metabolic and functional differences in *Fabp4* adipose tissue depots show that this marker plays some role in metabolic balance, with an inverse relationship between *FABP4* expression and obesity, presenting a severe downregulation in acute insulin resistance in these tissues [77]. According to this, our data show that AM-879 may improve the conditions of obesity and insulin resistance, as the higher dose of AM-879 decreased *Cd36* (Fig. 3E). *Cd36* is related to lipid accumulation and inflammatory response in the liver and is considered a candidate for regulating apoptosis and inflammation due to its contribution to obesity and insulin resistance development [78,79]. Also, CD36 and FABP4 interact with each other [80], causing lipid accumulation on WAT and liver [81]. However, in the case of AM-879, we observed an inverse correlation, with decreased *Cd36* and increased *Fabp4*, which may be related to lower WAT lipid accumulation, as it was observed in Fig. 1C, and possible effects on reducing fatty acid β-oxidation [77].

Concerning another lipid metabolism marker observed in our study, UCP1, a mitochondrial membrane protein involved on BAT thermogenesis [82,83], related to browning process [84], as well as is negatively correlated with insulin and glucose levels, and positively correlated with adiponectin [85]. We observed that Rosiglitazone did not alter *Ucp1* expression on both adipose tissues (Fig. 3F), which may be a result of the dose and duration of treatment. In contrast, AM-879 decreased its expression in the lower AM-879 dose group and increased its expression in the higher dose in WAT (Fig. 3F). Its reduction suggests some interference on CRH pathway, also increasing leptin expression [86]. The CRH pathway is involved on stress response, and the increased CRH signaling promote the activation of hypothalamic-pituitary-adrenal (HPA) axis, intensifying attentive behaviors, suppressing feeding behaviors and mobilizing energy stores, also influencing on glucose metabolism [28,29].In this way, our results suggest that changes in UCP1 might rise as a beneficial effect of PPARγ pathway activation.

Still in the context of gene expression, we evaluated markers of liver toxicity, as aspartate and alanine aminotransferases (AST and ALT), which are related to hepatocyte integrity; alkaline phosphatase (ALPL) and gamma glutamyl-transpeptidase (GGT1), both related to bile flow; albumin (ALB), and lactate dehydrogenase (LDHA), related to liver function [87]. Increased expression or plasmatic levels of these enzymes are all related to hepatic damage and/or biliary diseases [88]. Our results of AM-879 treatment reveal that, mainly on the higher dose, there is a reduction on the liver expression of all toxicity markers (Fig. 4), which may be considered a positive effect, since increased ALT and AST indicate hepatic damage [89], with an inverse correlation between adiponectin and ALT [90]. Except for decreased of albumin expression, all the other reductions are indicatives that AM-879 treatment may prevent liver damage and hepatobiliary disorders [87,91].

Finally, the observed differences between the two AM-879 doses suggest that at the lower dose, might suggest some effect through CRH pathway in AM-879-treated animals in lower doses (Fig. 4A), altering leptin expression, as well as *Crh* and *Npy*, and decreasing only insulin levels and food intake. These effects are consistent with the increase in leptin (Fig. 3E), which has been reported to inhibit *Npy* and activate *Crh* [26]. However, the latter effects were not observed in this study. The leptin pathway also explains the increase in *Npy* at higher dose of AM-879, as we observed lower expression of *Lep* in these group, thus avoiding the inhibition of neuropeptide Y expression. Meanwhile, this event was not so expressive because no changes in circulating corticosterone hormone were observed, suggesting that AM-879 treatment was not sufficient to promote significant physiological effects related to this pathway. On the other hand, the AM-879 higher dose promotes a predominant action on PPARγ pathway, improving insulin sensitivity, reducing food intake, insulin levels, and increasing *Ucp1*. Based on these results, AM-879 can be considered as a promising molecule for the treatment of obesity and T2D.

In conclusion, we present here a non-agonist ligand for PPARγ, previously described as a protector of S273 phosphorylation [25]. Our data show that this ligand promotes interesting effects *in vivo* on insulin sensitization and food intake reduction, without promoting the weight gain or hepatic steatosis already described for PPARγ full agonists, also reducing the expression of genes related to liver toxicity. Despite its low affinity for PPARγ and its apparent chemical instability, we observed that it is an interesting lead molecule, which might be improved in terms of potency and stability. Moreover, our data show that this molecule may have some influence on the CHR pathway, but without promoting negative effects on this pathway, which should be further investigated (Fig. 6).

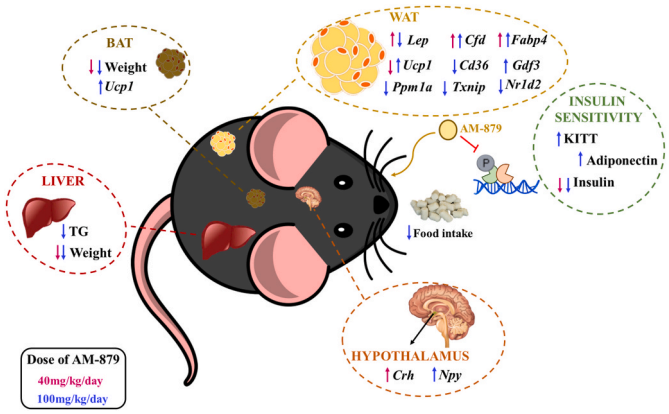

Fig. 6. Summarized effects of AM-879 considering its involvement on PPARγ and CRH pathways. Lower dose predominantly acts through CRH pathway, increasing *Lep* and *Crh* expression, decreasing *Npy* expression, also decreasing liver and BAT weights, and decreasing insulin levels. On the other hand, higher dose acts mainly through PPARγ pathway, improving insulin sensitivity due to Ser273 blockage, decreasing insulin levels and increasing glucose disappearance rate (KITT), also decreasing food intake, liver triglycerides and weight, increasing *Ucp1* expression on both WAT and BAT, and altering the expression of genes involved on lipid metabolism.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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CRediT authorship contribution statement

A.C.M. Figueira: designed the research and article, provided funding, wrote, corrected, and revised the article. M. F. Terra: designed the research and experiments , performed animal experiments, analyzed, and discussed results and wrote the article. M. Garcia-Arevalo: designed the research and experiments, performed animal experiments, and discussed results, and revised the article. T. M. Avelino: performed animal experiments and discussed results and revised the article. K. Y. Degaki: performed animal experiments. C.C.Malospirito: revised the English writing. A. Saito: provided the animals, discussed the results, and revised the article. M. de Carvalho: developed the scripts for histology analysis and revised the article. F. R. Torres: helped with primers development and standardization, and qPCR analysis. All authors contributed to the article and approved the submitted version.

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Appendix A. Supplementary data

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