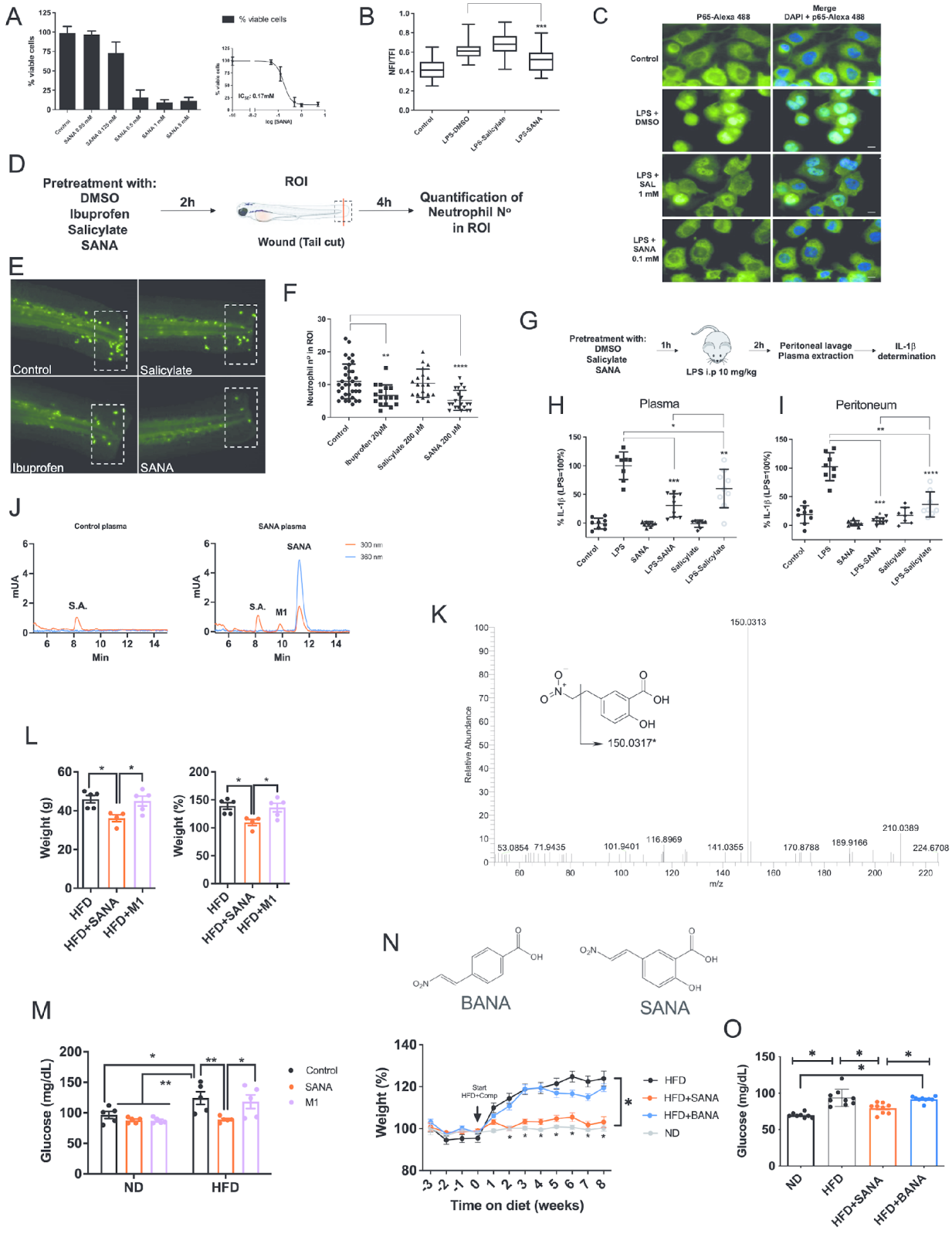
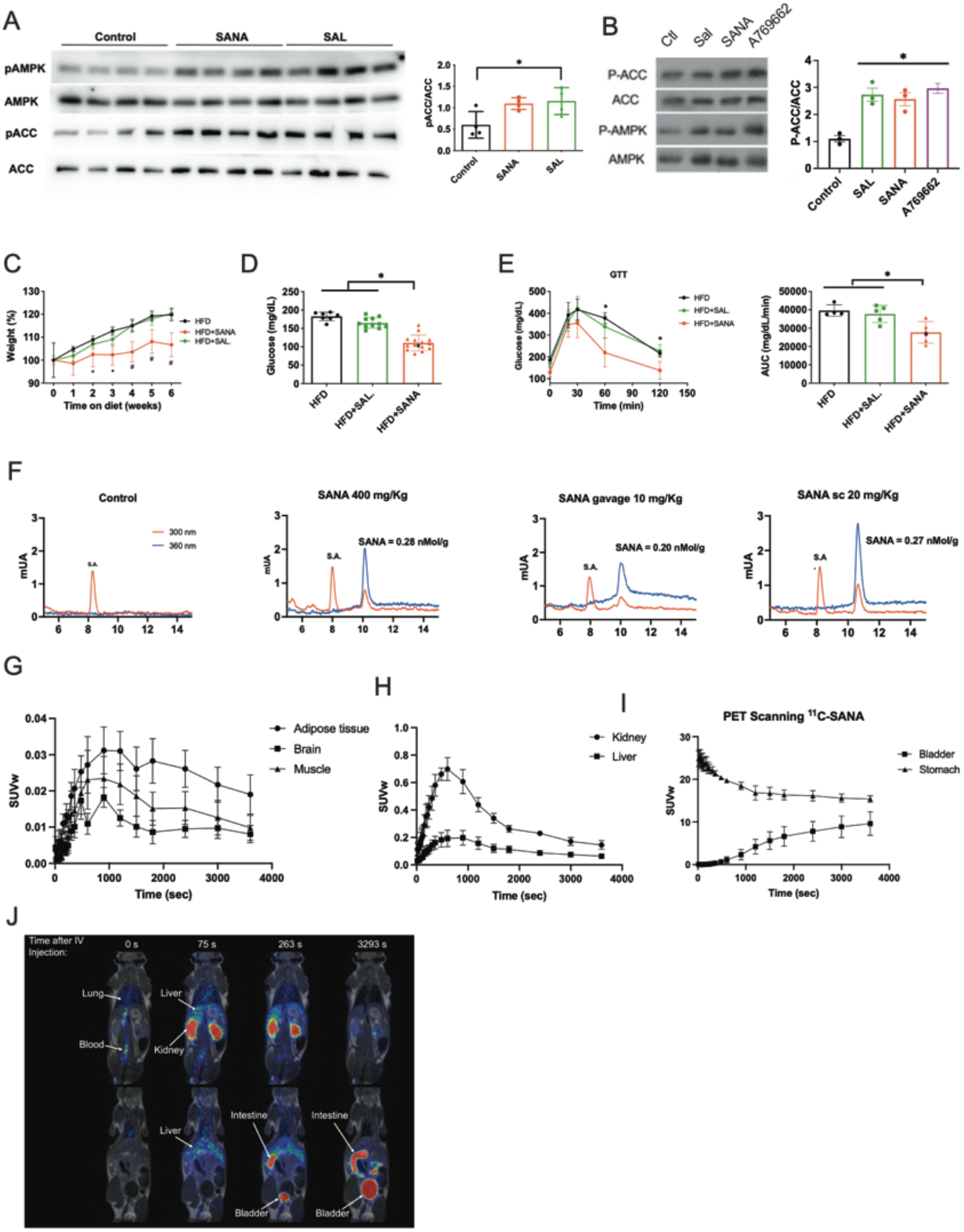
Supplementary information

Supplementary Figure 1



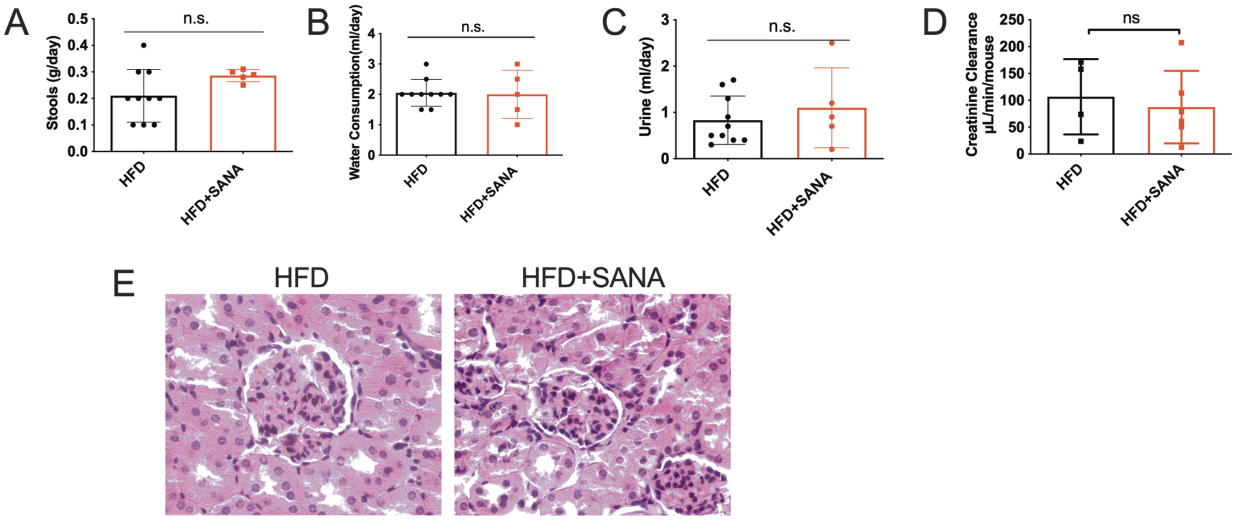
Supplementary Figure 1. Physicochemical and biological characterization of SANA and *in vivo* anti-inflammatory effects. A) Cytotoxicity of SANA in THP-1 human macrophages. THP-1 cells were treated with SANA (0.05-5 mM for 24 hours) and cytotoxicity was measured by MTT assay. Insert shows the dose response curve fit. Results are expressed as IC_{50} (compound concentration that reduced 50% control absorbance at 570 nm). IC_{50} = 0.17mM. **B-C)** Effect of SANA on the subcellular localization of NF-κB/p65 subunit in THP-1 macrophages analyzed by immunofluorescence microscopy. **B)** Binary quantification of nuclear fluorescence intensity (NFI, nuclear translocation of p65) versus total fluorescence intensity (TFI). **C)** Representative pictures used for quantification. Differentiated THP-1 cells were treated with SANA (100 μM), salicylate (SAL, 100 μM) or vehicle for 2 h and then activated with LPS (1 μg/mL) for 30 minutes. In the negative control (control), cells were not treated with LPS. Scale bar = 10 μm. **D-F)** SANA inhibits neutrophil recruitment in Zebrafish. **D)** Diagram of acute inflammation assay. Zebrafish larvae at 3dpf were pre-treated with DMSO, ibuprofen 20μM, SAL 200μM or SANA 200μM for 2 h. Wounding of the tail fins was performed with a scalpel, and larvae were incubated again in the presence of drugs. Neutrophils at the site of injury were imaged and counted at 4 h post transection. **E)** Representative pictures used for quantification. Dotted rectangles indicate the wounded region of interest (ROI) where neutrophils were counted. **F)** Quantification of recruited neutrophils after 4 h of treatment. Larvae numbers were: DMSO, ibuprofen 20 μM, SAL 200 μM, SANA 200 μM. **G-I)** SANA inhibits IL-1β release in an *in vivo* LPS-induced inflammatory model. **G)** Diagram of acute inflammation assay. C57BL/6 mice were injected intraperitoneally (i.p) with SANA, SAL (100 mg/kg) or vehicle (DMSO) for 1 h before i.p. injection of 10 mg/kg LPS or PBS. Mice were euthanized after 2 h and levels of IL-1β in serum and peritoneum were measured by ELISA. **H)** Quantification of IL-1β in the plasma. **I)** Quantification of IL-1β in the peritoneum. Data represents three different experiments. **J)** Organic extraction chromatograms of plasma from control mice (left) and mice treated with SANA (10 mg/kg gavage)(right). Salicylic acid (S.A.) was used as internal standard. **K)** Plasma detection of SANA main metabolite 5-(2-nitroethyl)salicylic acid (M1) by MS. **L)** Weight gain and percent of initial weight (at day 0) of mice fed with HFD and treated with SANA (20 mg/kg/day SC) or M1 (20 mg/kg/day SC)for 8 weeks. **M)** Basal glucose levels in normal chow (ND) or after 8 weeks of HFD. **N-O)** Comparison of the effect of SANA and BANA (300 mg/kg/day) on DIO **(N)** plasma glucose levels after 8 weeks of treatment. Mice kept under normal chow diet (ND) were used as reference.

Supplementary Figure 2



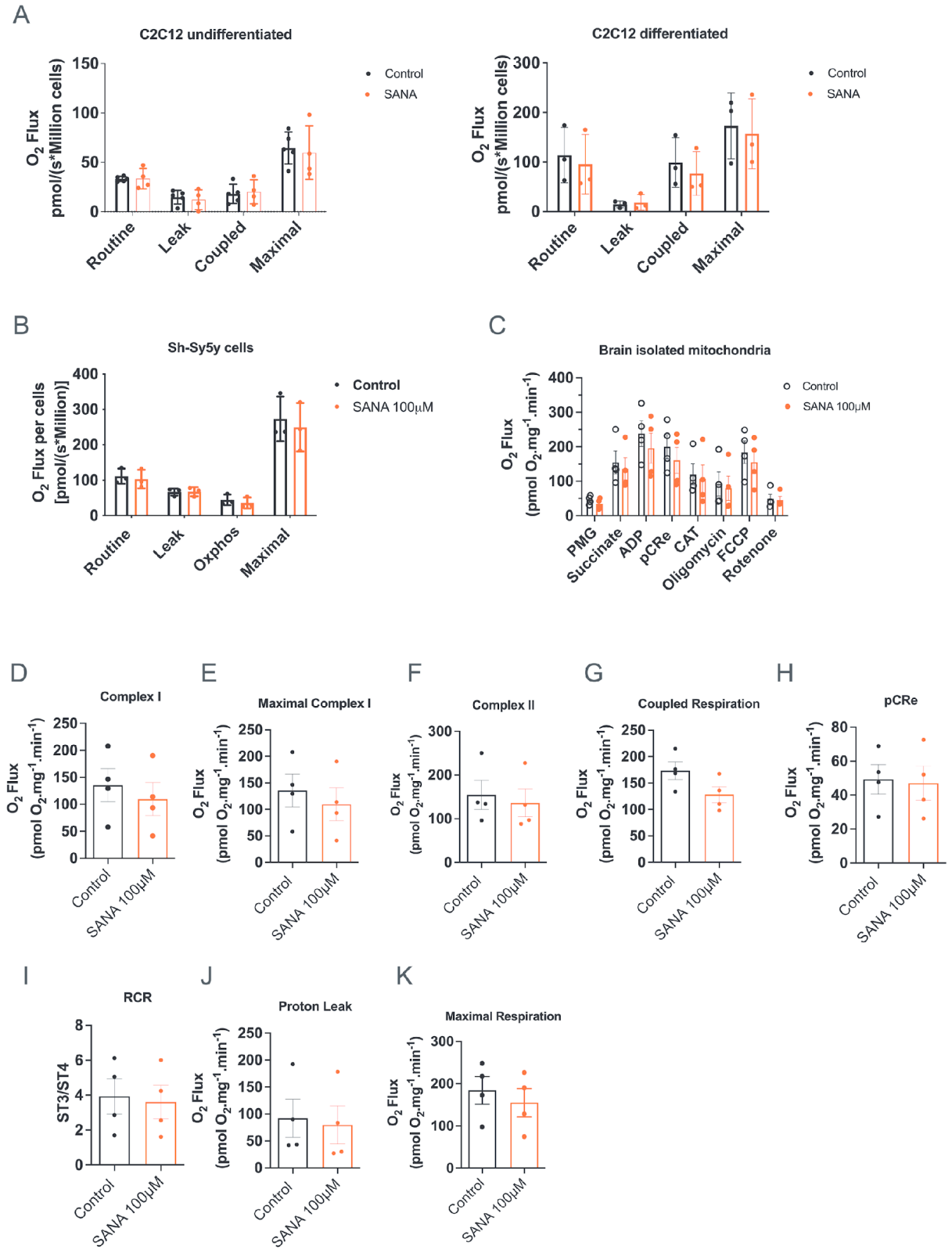
Supplementary Figure 2. Characterization of *in vitro* and *in vivo* AMPK activation by salicylate (SAL) and SANA. A-B) Acute AMPK activation by SANA and SAL *in vivo* and *in vitro.* **A)** Mice were acutely treated with vehicle (Phosphate Buffer 100mM, pH 6.5), SANA or SAL (200 mg/kg, IP, 1 h). AMPK activation was determined in the liver by AMPK phosphorylation (Thr172) and ACC phosphorylation (S80). **B)** AMPK activation was also measured *in vitro* in 3T3L1 differentiated adipocytes. SAL and SANA were used at 100 μM in DMSO. A769662 (10 μM) was used as positive control. . **C-F)** SANA (or SAL) were delivered subcutaneously (by injection) at 20mg/kg/day from the onset of the experiment. Control mice (HFD) were administered with vehicle (PBS:PEG, 50:50 v/v). **C)** Percent of initial weight (at day 0) **D)** Basal glucose levels and **E)** GTT measured at week 6. **F)** Steady state levels of SANA in iWAT following different concentrations and administration routes (400 mg/kg/day powder mixed with food (PO), 10 mg/kg/day, gavage and 20 mg/kg/day, SC). **G-H)** Biodistribution analysis performed by ^{11}C-SANA followed by PET-MRI scan revealed detectable SANA in different tissues. **G)** Inguinal white adipose tissue, brain and muscle, **H)** kidney and liver, and **I)** balder and stomach. **J)** Representative picture of mice injected with ^{11}C-SANA and followed by PET-MRI scan over time that were used to perform the analysis.

Supplementary Figure 3



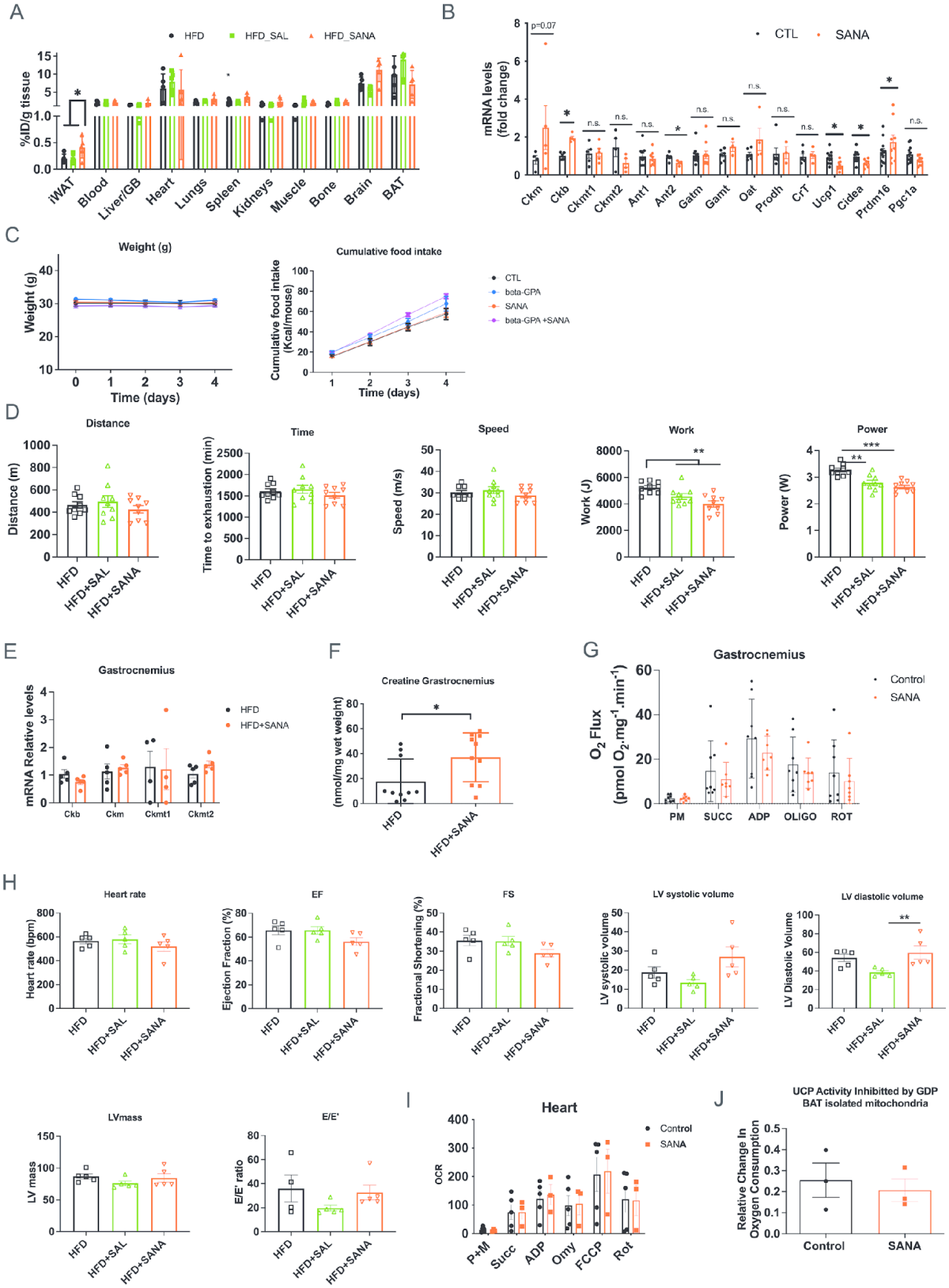
Supplementary Figure 3. Comprehensive toxicity evaluation of mice treated with SANA. A) Quantification of stool, **B)** water consumption and **C)** urine production of mice fed with HFD, HFD+SANA at 200 mg/kg/day, PO. **D)** Analysis of renal function by measuring creatinine clearance and **E)** the glomerular histology. H&E kidney staining from mice fed with HFD or HFD+SANA at 200 mg/kg/day PO.

Supplementary Figure 4



Supplementary Figure 4. Assessment of mitochondrial respiration *in vitro* in non-adipose cells and isolated mitochondria treated with SANA. A) Undifferentiated and differentiated C2C12 myoblasts and **B)** Sh-sy5y cells mitochondrial respiration treated with SANA**. C)** Brain Isolated mitochondrial respiration treated with SANA. **D-K)** Mitochondrial parameters obtained from brain mitochondria: **D)** complex I = (PMG) **E)** Maximal Complex I = (FCCP – Rot)
F) Complex II = (SUCC) **G)** Coupled Respiration = (ADP-OLIGO) . **H)** pCRe**. I)** RCR= (ADP) /(OLIGO).**J)** Proton leak=Oligo. **K)** Maximal Respiration = FCCP

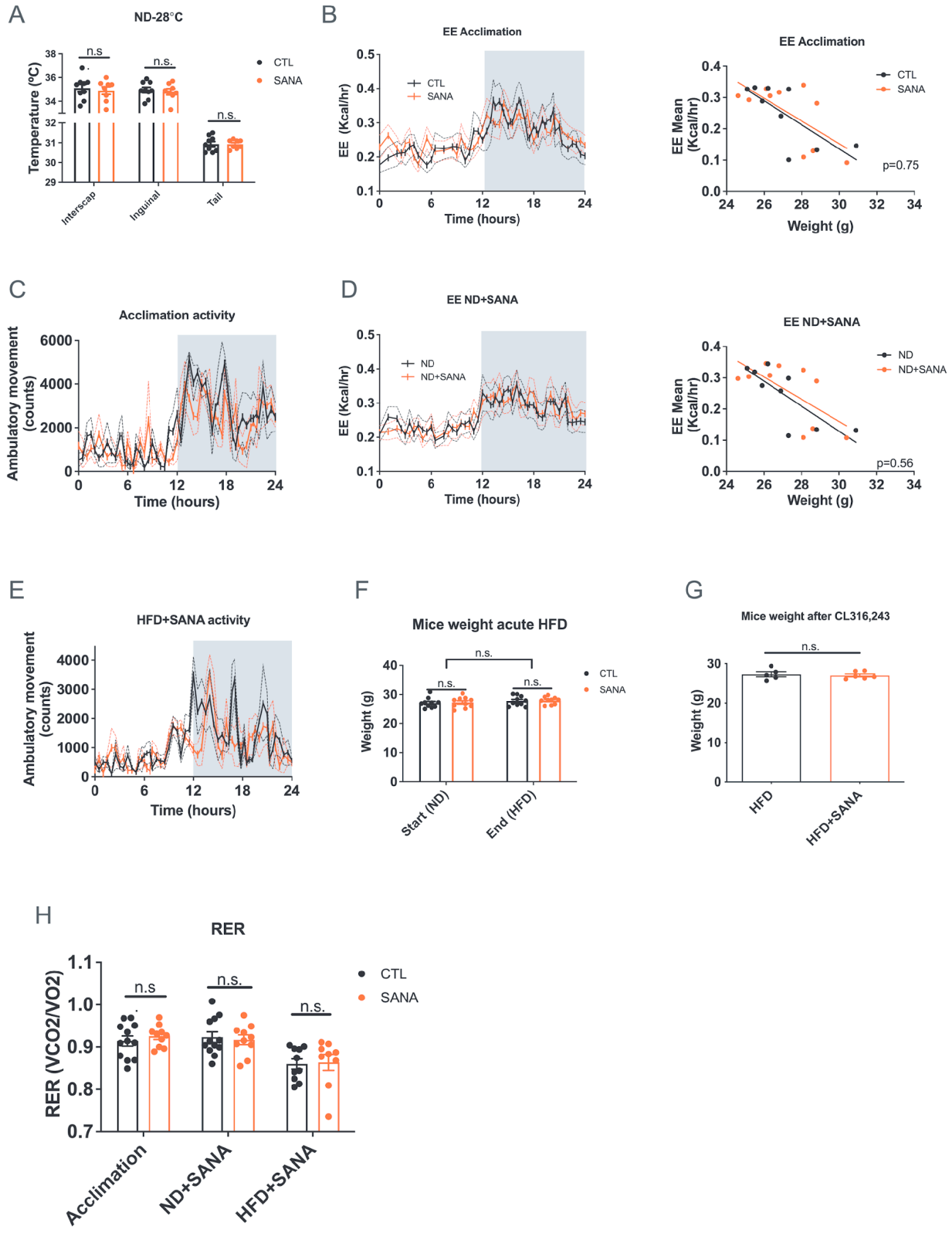
Supplementary Figure 5



Supplementary figure 5. Effect of SANA in non-adipose tissues mitochondrial function and physiology

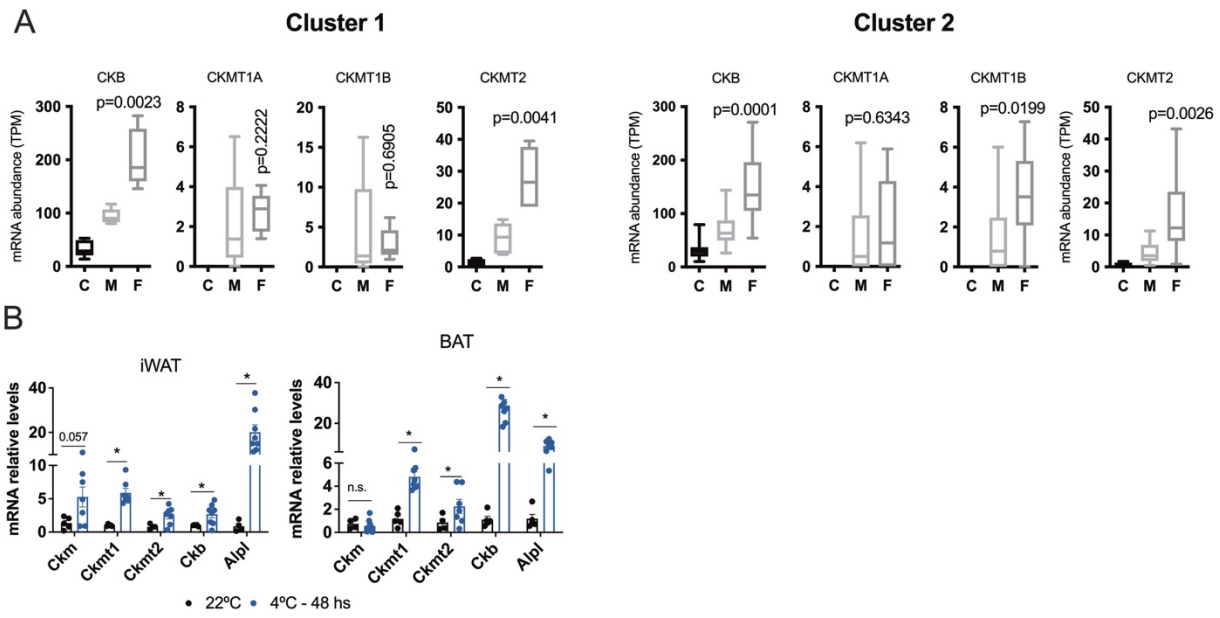
A) Glucose *in vivo* uptake measured by ^{18}FDG uptake in different tissues of mice treated with SANA (400 mg/kg/day, PO). **B)** qPCR analysis of gene expression of thermogenic markers in iWAT from mice treated with SANA (20 mg/kg/day, SC) after cold challenge exposure. **C)** Weight gain and cumulative food intake of mice daily treated with SANA (20 mg/kg/day, SC) or β-GPA (0.4 mg/kg/day, IP) or both, for 5 consecutive days before performing the cold challenge. **D)** Aerobic muscle capacity was measured in the treadmill at the end of treatment. Mice were fed during 22 weeks with HFD and HFD+SANA (or SAL) at 400 mg/kg/day, PO. **E)** qPCR analysis of creatine kinases expression in gastrocnemius muscle and **F)** Creatine levels measured by NMR of mice treated with HFD or HFD+SANA (400 mg/kg/day, PO).**G)** Respiration of gastrocnemius permeabilized fibers from mice treated or not with SANA (20mg/Kg/day, SC). **H)** Echo Cardiac function assessment in mice in HFD or HFD+SANA (or SAL) at 400 mg/kg/day, PO after 22 weeks of treatment. **I)** Respiration of permeabilized fibers from heart muscle coming from mice treated with SANA (20 mg/kg/day, SC). **J)** Effect of direct incubation of isolated mitochondria from BAT with SANA (100 µM) on UCP1 activity inhibited by GDP.

Supplementary Figure 6



Supplementary Figure 6. Supporting additional information to CLAMS data of mice under acute HFD and SANA treatment under thermoneutral conditions. A) Surface temperature quantification from thermal images of mice fed with normal chow diet and treated with SANA under thermoneutral conditions (day 9). **B)** Left, EE measurements over a 24-hour period at the end of the acclimation period at thermoneutrality. Right, Regression plot of these data. **C)** Total activity over a 24-hour period during acclimation **D)** Left, **EE** Measurements over a 24-hour period at the end of SANA treatment under normal chow feeding (day 9). Right, Regression plot of these data. **E)** Total activity over a 24 h period at the end of SANA treatment under HFD. **F)** Mice weight at the beginning and end of the acute HFD treatment. **G)** Mice weight after acute single treatment with CL316,243 (1mg/kg). **H)** Respiratory exchange ratio (RER) from mice under acute HFD treatment at the end of acclimation period (day 7), at the end of ND+SANA (day 9) and at the end of HFD+SANA (day 12).

Supplementary Figure 7



Supplementary figure 7. Gene expression of creatine kinases during different thermogenic stimuli. A) RNAseq expression analysis of all creatine kinases in two clusters of human adipocytes progenitor cells (Min et al., 2019). Parametric (t-test) or non-parametric (Mann-Whitney test) statistical analysis of the mRNA abundance (transcript per million, TPM) was performed after evaluating normality distribution. Respective statistical comparisons were set between vehicle-treated differentiated adipocytes (M), and forskolin-treated differentiated adipocytes (F). C corresponding to adipocytes maintained in a non-differentiated state (n=5 per group in cluster 1- Left, Lipogenic adipocytes and n=14 per group in cluster 2-Right, Thermogenic adipocytes). **B)** Analysis of creatine kinases and the phosphatase Alpl expression by qPCR in iWAT and BAT from mice fed with chow diet and maintained at RT or after a prolonged cold challenge (48 hours at 4 degrees).

Supplementary Methods

Reagents and resources

The key reagents and resources are listed in Supplementary Table VIII.

Protein extraction

Total adipose tissue and mitochondrial pellets were homogenized in RIPA buffer (25 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS), containing 5 mM NaF, 5 mM nicotinamide, 1 μM trichostatin A (TSA), 50 mM β-glycerophosphate and SIGMAFAST™ Protease Inhibitor Cocktail (Sigma) according to the supplier's instructions. Sample homogenization was conducted in cold weather using a bullet blender (Next Advance) or by ultrasonication. Homogenates were centrifuged at 10000 g for 10 min, 4 °C, and the supernatant's protein concentrations were measured by Bradford assay (PanReac AppliChem) using a BSA (Capricorn Scientific) standard.

Western blot (WB)

Proteins were separated by SDS-PAGE and later transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5 % non-fat milk in TBS-T buffer solution (5 mM Tris pH 8.0, 15 mM NaCl, 0.2% Tween 20) and incubated overnight at 4 °C with the following primary antibodies for Ckmt1, Ckmt2, Ucp1, ATP5A, salicylate, P-ACC, ACC, P-AMPK and AMPK. Finally, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and the bands were detected using chemiluminescence. The densitometry of the bands was performed using Image J software.

RNA isolation and Real-Time qRT-PCR

Total RNA was isolated from cells or tissues using TRIzol Reagent TMand the Direct-zol RNA MiniPrep kit (Zymo Research) according to the manufacturer's instructions. Reverse transcription of 1 µg of RNA was done using the SuperScript II reverse transcriptase (Invitrogen) and 20 ng of resultant cDNA was analyzed by Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (Real-Time qRT-PCR) using the KAPA SYBR Fast Universal kit (Kapa Biosystems). Relative mRNA expression levels were calculated using the comparative CT method (Kenneth J. Livak, Thomas D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method and normalized to β-actin mRNA. All primers were synthesized by Integrated DNA Technology (IDT), the complete list can be found in Tables I and II.

Table I: List of mouse primers used for Real-Time qRT-PCR.

Target gene reference	Target gene name UniProt	Forward (5′to 3′)	Reverse (5′to 3′)
Actb	Actb	AGCCATGTACGTAGCCATCC	GCTGTGGTGGTGAAGCTGTA
Ckm	Ckm	TTCCTGTTTGACAAGCCCGT	CTCGTTCACCCACACAAGGA
Ckb	Ckb	TGATGAGCACAAGACCGACC	CAGACAGGTCGCCATCTAGG
Ckmt1	Ckmt1	TGAGGAGACCTATGAGGTATTTGC	TCATCAAAGTAGCCAGAACGGA

Ckmt2	**Ckmt2**	**GCATGGTGGCTGGTGATGAG**	**AAACTGCCCGTGAGTAATCTTG**
Ckmt1 (knockdown)	**Ckmt1**	**TGTCTTCAAGAGTCAGAACTGGC**	**AGCATCCACCACAACACGTT**
Ckmt2 (knockdown)	**Ckmt2**	**CACGCAGCAGGATCTGGATA**	**GCATTTCGGCCAGTTAGCAA**
Alpl	**Alpl**	**GCACCTGCCTTACCAACTCT**	**GTGGAGACGCCCATACCATC**
Ant1	**Slc25a4**	**CTCTGTCCAGGGCATCATCA**	**TTCCTCCAGCAGTCAAGTGTC**
Ant2	**Slc25a5**	**TCAACATGACAGATGCCGCT**	**CACGCAGTCTATGATGCCCT**
Gatm	**Gatm**	**GACCTGGTCTTGTGCTCTCC**	**GGGATGACTGGTGTTGGAGG**
Gamt	**Gamt**	**GCAGCCACATAAGGTTGTTCC**	**CTCTTCAGACAGCGGGTACG**
Oat	**Oat**	**CCATTGCGGCTCTTGAGGTTT**	**AGGCACACCTTCCAAGCATC**
Prodh	**Prodh**	**ATGCTATGTACCACAGGTGCC**	**CAGCAGGATGCAGGCCTATC**
Crt	**Slc6a8**	**TGCATATCTCCAAGGTGGCAG**	**CTACAAACTGGCTGTCCAGA**
Ucp1	**Ucp1**	**CTGCCAGGACAGTACCCAAG**	**TCAGCTGTTCAAAGCACACA**
Cidea	**Cidea**	**ATCACAACTGGCCTGGTTACG**	**TACTACCCGGTGTCCATTTCT**
Prdm16	**Prdm16**	**CAGCACGGTGAAGCCATTC**	**GCGTGCATCCGCTTGTG**

Pgc1a	Ppargc1a	GGAGCTCCAAGACTCTAGACA	CCAAAGTCTCTCTCAGGTAGC

Table II: List of Human primers used for Real-Time qRT-PCR.

Target gene reference	Target gene name	Forward (5′to 3′)	Reverse (5′to 3′)
hActb	hActb	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
hUCP1	hUCP1	GTG TGC CCA ACT GTG CAA	CCA GGA TCC AAG TCG CAA GA
hPGC1a	hPGC1a	CTG TGT CAC CAC CCA AAT CCT TAT	TGT GTC GAG AAA AGG ACC TTG A
hGATM	hGATM	AAGCCCACAATGGCTGATGA	TGCCTAGGTAGTTTGTAACCTGG
hCrt	hCrt	GGACTTCATCATGTCGTGCG	GCCTTTGAACAGGGGACAGA
hCKMT1	hCKMT1	TGA GGA GAC CTA TGA GGT ATT TGC	CTC ATC AAA GTA GCC AGA ACG G
hCKMT2	hCKMT2	AGG AGT CCT ATG AGG TGT TTG	CGT AAT GCT CGT CGA ACT GC
hCKB	hCKB	CCG ATA CTA CGC GCT CAA GA	ACA CCT CCT TCA TGT TGC CC
hANT1	hANT1	AACGTCTCTGTCCAAGGCATC	CCAGCAGTCAACTGTCCCC
hANT2	hANT2	TTCAACATGACAGATGCCGC	GCTCCTTGGGAATACGGACC
hALPL1	hALPL1	GACCTCGTTGACACCTGGAA	CTGGCTCGAAGAGACCCAATA
hALPL2	hALPL2	AACCGAGATACAAGCACTCCC	TCCTGTTCAGCTCGTACTGC
hTBP	hTBP	CAC GAA CCA CGG CAC TGA TT	TTT TCT TGC TGC CAG TCT GGA C

Analysis of biological properties of SANA in macrophages.

Cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). THP-1 cells (ATCC TIB-202) were grown with 10% FBS/RPMI media and differentiated to macrophages with PMA 200 nM for 48 h at 37°C in 5% CO_2.

Cell viability.

Differentiated THP-1 cells were treated with increasing doses of SANA (0,05-5mM) for 24h at 37°C. A standard MTT assay was performed by measuring the mitochondrial-dependent reduction of MTT added to cells at a 0.5 mg/mL final concentration. Formazan crystals were dissolved in isopropanol and absorbance was read at 570 nm using a microplate spectrophotometer. IC_{50} were calculated using GraphPad Prism and refer to SANA dose at which cell viability is reduced by half. 100% viability corresponds to DMSO control. IC_{50} is the average of 3 independent determinations.

Immunocytochemistry for NF-kB

Differentiated THP-1 cells were incubated with salicylate (100 µM), SANA (100 µM) or vehicle for 2h. Cells were later activated with LPS from *Escherichia coli* serotype 0127:B8 (1 µg/mL; Sigma Aldrich) for 30 min and then fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with 2% BSA - Triton 0.3% in PBS for 30 min, and blocked with 3% BSA in PBS. NF-kB labeling was performed overnight at 4 °C using rabbit anti-p65 antibody (Cell Signaling) and then Alexa Fluor 488-conjugated secondary antibody against rabbit was used. Nucleus were stained using DAPI. Images were acquired using an Olympus IX81 epifluorescence microscope equipped with an Orca-Hamamatsu CCD camera. Images were processed using Image J 1.51h (Wayne Rasband, National Institutes of Health USA) for binary quantification of nuclear

fluorescence intensity (NFI, nuclear translocation of p65) normalized by the total fluorescence intensity (TFI).

Zebrafish husbandry and inflammation assay.

Zebrafish were raised and maintained according to standard protocols. The neutrophil-specific zebrafish line Tg (mpx:GFP)i114, referred to as Tg(mpx:GFP), was used for all inflammation assays, and neutrophil tracking assays at 3 days post fertilization (dpf) as previously described (53). In all inflammation assays, larvae were preincubated for 2 h with compounds at the doses indicated in each figure. Tail fins were transected with a sterile scalpel at the region indicated in Supplementary Fig. 1 and larvae were incubated in the presence of compounds for 4 h, and fixed in 4% paraformaldehyde overnight. To assess neutrophil number, whole mount immunohistochemistry was performed using rabbit polyclonal anti-GFP (Invitrogen A-11122). Images were captured in an inverted fluorescence microscope (Olympus) at 10x magnification, and neutrophils in the region posterior to the circulatory loop were counted.

LPS challenge

For the LPS challenging experiment in mice, C57BL/6 mice were injected intraperitoneally (i.p) with 100 mg/kg of salicylate, 100 mg/kg of SANA or vehicle (DMSO) 1 h before i.p. injection of 10 mg/kg LPS *Escherichia coli* 0127:B8 (Sigma-Aldrich) or PBS. After 2 h mice were killed and levels of IL-1β in serum and peritoneum were measured by ELISA (BD 559603, BD OptEIA).

Preparation of [^{11}C]SANA

^{11}C-SANA was prepared by the radiosynthesis as illustrated in Fig. 1 and described in detail below. Cyclotron-produced [^{11}C]CO_2 or [^{11}C]CH_4 was initially converted to [11C]CH_3I. This intermediate was passed through a column of $AgNO_2$ heated to 80 degrees to give [^{11}C]CH_3NO_2 which was directed to the reaction vial containing the precursor 5-formylsalycilic acid in dimethylsulfoxide with ammonium acetate as a supporting base. After heating the reaction mixture for 5-7 min at 120 degrees, the crude product is diluted with 1 mL water and purified by reverse phase HPLC on a Luna C18(2) (10 µm, 10x250 mm) (Phenomenex) column with 80% aqueous NaH_2PO_4 (70 mM):20% acetonitrile as mobile phase (flow 6 mL/min; λ = 254 nm). The fraction from the chromatographic separation corresponding to ^{11}C-SANA was collected (from ca. 5.5 - 6.5 min), diluted with 40 mL sterile water and further purified on a C18 Sep-pak. The product was washed with 10 mL water and then eluted off with 1 mL ethanol followed by 9 mL saline. Finally, the product was passed over a sterile filter (Cathivex-GS 0,22 µm (Millipore)) into the final sterile product vial.

The radiochemical purity of the synthesized ^{11}C-SANA was determined by analytical HPLC using an Ultimate® 3000 system (Dionex) (λ= 280 nm) connected to a GabiStar radio-detector (Nuclear Interface). The chromatographic column was a Luna 5µ C18(2) 100A (150 x 4.6 mm) (Phenomenex) with a mixture of aqueous NaH_2PO_4 (70 mM) and acetonitrile (80:20) as eluent (isocratic, 2 mL/min). The chromatographic data were analyzed using Chromeleon software (Dionex) (version 6.80). The identity of ^{11}C-SANA (retention time: 3.4 min) was confirmed by co-injection of reference material.

The one-step radiosynthesis provided 0.1-0.6 GBq of ^{11}C-SANA within 40 min with a radiochemical yield of 15 - 20% and a radiochemical purity greater than 95%.

MicroPET/MRI

Mice underwent anatomical magnetic resonance imaging (MRI) 1T and dynamic positron emission tomography (PET) (Mediso Medical Imaging Systems, Budapest, Hungary). A bolus of ^{11}C-SANA (9 MBq/animal) was given orally and the mice were quickly anesthetized with isoflurane initiated in a acrylic glass chamber and maintained with respiration in a mask during the scan or injected in an anesthetized mouse via a tail vein catheter (25 MBq/animal) in the scanner. PET-scanning was performed the first 60 minutes after injection with frame structure: 8x15s, 4x30s, 2x60s, 2x120s, 4x300s, and 3x600s. PET images were reconstructed with a three-dimensional ordered subset expectation algorithm (Tera-Tomo 3D; Mediso Medical Imaging Systems) with four iterations and six subsets and a voxel size of 0.6 x 0.6 x 0.6 mm3. Data was corrected for dead-time, decay, and randoms using delayed coincidence window without corrections for attenuation and scatter and presented as standardized uptake value (SUV).

The PET-scans were followed by a 25 minutes T1 weighted MRI-scan to define anatomical regions of interest. Body temperature and respiration frequency were monitored during anesthesia. The animal study was approved by Dyreforsøgstilsynet under the Danish Ministry for Veterinary and Food Administration (permission#: 2017-15-0201-01242).

Treadmill exhaustion test

Exercise capacity was assessed by measuring running time, distance, maximal running speed until mice achieved exhaustion, using a motorized treadmill (Columbus Instruments). The mice were acclimated to the treadmill for 3 consecutive days, 5 minutes per day, at a speed of 5m/min for 2 minutes, 7m/min for 2 minutes and 9m/min for 1 minute, at a grade of 5%. After a day of rest, animals ran on the treadmill at an initial speed of 5 m/min and 5% grade for 2 minutes, after which the speed was increased by 2 m/min every subsequent 2 minutes until the mice were exhausted. Exhaustion was defined as the inability of the mouse to remain on the treadmill despite an electrical shock stimulus. The electric stimuli are pulses (200 msec/pulse) of electric current

with adjustable pulse repetition rate (1 time per second, Hz) and intensity (3 Hz, 1.22 mA; respectively).

Running time was recorded from a timer, running distance and maximal speed were recorded from the software (Columbus Instruments), and work (the product of body weight [kg], gravity [9.81 m/s^2], vertical speed [m/s × angle], and time [s]) and power (the product of work [J] and time [s]) were calculated (1).

Four limbs Inverted Hanging Grip

Balance, coordination, and muscle condition were assessed by hanging test. This test is based on the knowledge that mice are eager to remain hanging on a wire or grid till exhaustion. The four limbs hanging Grip test uses a wire grid system to noninvasively measure the ability of mice to exhibit sustained limb tension to oppose their gravitational force. The procedure measures the 4-limb hanging time in seconds as well as the minimal Holding impulse (Body mass x Hanging Time) that is used to oppose the gravitational force.

Each mouse was placed on top of the wire grid and was accommodated to this environment for 3 to 5 seconds before the grip was inverted and held at least 35 cm over a cage containing 5 to 7 cm of soft bedding. If the mouse fell right after the timer was started, the test was repeated up to 3 times until the mouse maintained a hold for >2 seconds. Each hanging period must begin with all four paws of the mouse grasping the wire grid. The hanging time (s) was recorded using a stopwatch and was defined as the amount of time the mouse stayed in the wire grid after it was inverted. This procedure was repeated 3 times (3 TRIALS) for each mouse with a rest interval between the hanging attempts. The Holding impulse (Strength) associated with the holding test equals the hanging time multiplied by the body weight (grams x sec or Newtons x sec; conversion factor - 9.806 x 10-3 Newtons/gm).

Echocardiography

Echocardiography was performed as previously described (53). Briefly, mice were sedated with isoflurane 1%. Once the animal was sedated, it was quickly positioned on a pre-heated platform (37°C) in a supine position, making sure that the forefeet and hind feet lie on the ECG sensors of the platform in order to obtain an ECG signal and therefore a gated echocardiographic recording. The anterior chest was shaved, and warmed gel was applied, to improve acoustic interface. Images were obtained in real-time using a 30 MHz linear-array transducer, coupled to the VisualSonics Vevo 2100 Imaging Platform (FUJIFILM). Two-dimensional images were acquired at ~200 sec-1 in parasternal long- and short-axis views and stored off-line for subsequent analysis. Pulsewave Doppler interrogation of mitral inflow was used, as well as Pulsewave Tissue Doppler to obtain the mitral annulus e´ wave (and therefore calculate the E/e´ ratio). Images were analyzed in a blinded fashion. Traced endo- and epicardial borders were electronically determined at end-systole and end-diastole using a software created for this purpose (Vevo lab -VisualSonics - FUJIFILM). Left ventricular end-diastolic volume, end-systolic volume, stroke volume, ejection fraction, fractional shortening and mass were calculated using the bi-plane area-length method, previously validated.

SANA detection in iWAT by HPLC

Mice were treated with SANA in three different ways: orally (400 mg/kg), gavage (10 mg/kg) and subcutaneous (20 mg/kg) during 7 days. After that mice were killed by using an overdose of ketamine/xylazine, and 100-200 mg of iWAT were extracted with acetonitrile. The presence of SANA was analyzed by RP-HPLC using salicylic acid (S.A.) as internal standard (MACHEREY-NAGEL C18, 5 µM, 250 × 4.6 mm I.D. column; 1 mL/min gradient method, 40-45% of B in 10 min, Phase A: H2O 0.1 % FA, Phase B: ACN 0.1 % FA; detection at 300 and 360 nm; Agilent 1200 HPLC system).

SANA detection in plasma by HPLC

Mice were treated via gavage with SANA 10 mg/kg. After that mice were killed by using an overdose of ketamine/xylazine, and 100uL of plasma was extracted with acetonitrile. The presence of SANA was analyzed by RP-HPLC using salicylic acid (S.A.) as internal standard (MACHEREY-NAGEL C18, 5 µM, 250 × 4.6 mm I.D. column; 1 mL/min gradient method, 40-45% of B in 10 min, Phase A: H2O 0.1 % FA, Phase B: ACN 0.1 % FA; detection at 300 and 360 nm; Agilent 1200 HPLC system).

Brain and brown adipose tissue mitochondrial respirometry

Brown mitochondria was isolated as described in methods. To isolate brain mitochondria, the brain was dissected without the cerebellum in a petri plate with the isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 10 mM HEPES and 0,1% BSA, pH 7.4). It was then minced and disrupted in an ice-cold glass/Teflon Dounce homogenizer with the isolation buffer, and centrifuged at 1300 g for 5 min at 4°C. The supernatant was collected and centrifuged at 10000 g for 10 min at 4°C. The supernatant was discarded, and pellet was resuspended with 10 mL of isolation buffer and 0.01% digitonin, and centrifuged at 10000 g for 10 min at 4°C. The supernatant was discarded, and the pellet resuspended with the isolation buffer containing the isolated mitochondria. Brain isolated mitochondria (BIM) and brown isolated mitochondria were incubated for 15 min at 37 °C with 100 µM SANA or vehicle (DMSO). The oxygen consumption was accessed by high resolution respirometry (Oroboros O2K, Innsbruck, Austria) at 37 °C. Brain and brown isolated mitochondria were transferred to the Oroboros chamber with 2.1 mL of MIR05 buffer and experiment was performed following the SUIT protocol {Gnaiger, 2020 #154}. Respiration was induced by addition of substrates for mitochondria electron transport system complexes (5 mM pyruvate, 2,5 mM malate, 5 mM glutamate, 10 mM succinate and 0.1 mM ADP to induce respiratory control), inhibitors of the electron transport system (5 µM

carboxyatractyloside, 0,5 µg/mL oligomycin, 0,5 µM FCCP, 1 µM rotenone and 2,5 µM antimycin A) and phosphocreatine 1 mM and carboxyatractiloside 5 µM (CAT).

Muscle fibers permeabilization

Gastrocnemius muscle and heart were dissected from mice and placed in BIOPS (2,7 mM CaK2EGTA, 7,23 mM K2EGTA, 5,77 mM Na2ATP, 6,56 mM MgCl2, 20 mM taurine, 15 mM Na2Phosphocreatine, 20mM imidazole, 0,5mM DTT, 50mM MES) and the fibers were torn apart with tweezer on a petri plate with ice cold BIOPS. The fibers were permeabilized with 0,5% of saponin for 30 min and washed with respiration buffer MIR05 (0,5 mM EGTA, 3 mM MgCL2, 60 mM MES, 20 mM taurine, 10 mM KH2PO4, 10 mM HEPES, 110 mM sucrose, 1 mg/mL BSA fatty acid free, pH 7.4) for 10 min; 1,5 - 2 mg were weighted after drying on filter paper.

Muscle oxygraphy

Oroboros O2K was used to perform a high resolution respirometry and measurement of UCP activity. The Oroboros was calibrated with MIR05. 1.5 - 2 mg of permeabilized muscle fibers were transferred to the chamber and the assay was performed by adding 5 mM pyruvate, 2,5 mM malate, 5 mM succinate, 1 mM ADP, 10 µM cytochrome C, 0,7 µg/mL oligomycin, 0,5 µM FCCP, 2 µM rotenone and 5 µM antimycin A.