The Chemical Uncoupler 2,4-Dinitrophenol (DNP) Protects Against Diet-induced Obesity and Improves Energy Homeostasis in Mice at Thermoneutrality*

Margalit Goldgof†1, Cuiying Xiao‡1, Tatyana Chanturiya§1, William Jou§, Oksana Gavrilova§, and Marc L. Reitman‡2

From the ‡Diabetes, Endocrinology, and Obesity Branch, and §Mouse Metabolism Core, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Running title: Chemical Uncouplers in Weight Loss Therapy

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1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Marc Reitman, Building 10-CRC, Room 5-5940, 10 Center Drive, Bethesda, MD, USA 20892-1453, (301) 496-6442, E-mail: marc.reitman@nih.gov

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Background: The chemical uncoupler 2,4-dinitrophenol was widely used as treatment for obesity in the past.

Results: In mice, 2,4-dinitrophenol generates heat and turns off brown fat heat production. It reduces weight gain at thermoneutrality, but not at cooler ambient temperatures.

Conclusion: Environmental temperature should be considered when assessing anti-obesity drugs in mice.

Significance: Chemical uncouplers deserve further investigation for the treatment of obesity.

ABSTRACT

The chemical uncoupler 2,4-dinitrophenol (DNP) was an effective and widely used weight loss drug in the early 1930s. However, DNP’s physiology has not been studied in detail as toxicity, including hyperthermia and death, reduced interest in the clinical use of chemical uncouplers. To investigate DNP action, mice fed a high fat diet and housed at 30°C (to minimize facultative thermogenesis) were treated with 800 mg/l DNP in drinking water. DNP treatment increased energy expenditure by ~17%, but did not change food intake. DNP-treated mice weighed 26% less than controls after 2 months of treatment due to decreased fat mass, without a change in lean mass. DNP improved glucose tolerance and reduced hepatic steatosis without observed toxicity. DNP treatment also reduced circulating T3 and T4 levels, Ucp1 expression, and brown adipose tissue activity, demonstrating that DNP-mediated heat generation substituted for brown adipose tissue thermogenesis. At 22°C, a typical vivarium temperature that is below thermoneutrality, DNP treatment had no effect on body weight, adiposity, or glucose homeostasis. Thus, environmental temperature should be considered when assessing an anti-obesity drug in mice, particularly agents acting on energy expenditure. Furthermore, the beneficial effects of DNP suggest that chemical uncouplers deserve further investigation for the treatment of obesity and its comorbidities.

Obesity prevalence is ~35% in the United States and ~20% in Europe, and is increasing worldwide (1,2). Obesity contributes to serious comorbidities, including type 2 diabetes and cardiovascular disease. Despite the epidemic’s
magnitude, few effective therapeutic options exist. Current treatments with diet, exercise, and behavior modification work well in the short-term, but have a low long-term success rate (3). Bariatric surgery is effective, but is invasive and supported by limited long term (decades) safety and efficacy data. Pharmacotherapies for obesity are also meager. One modestly effective drug, orlistat, was approved by the US Food and Drug Administration in 1999 and two others (lorcaserin and phentermine/topiramate) were approved in 2012.

The effect of 2,4-dinitrophenol (DNP) to increase metabolic rate has been known since the late 1800s and was studied during World War I (4). In the early 1930’s, DNP was widely used as a weight loss drug (5); however, enthusiasm for DNP therapy waned due to its low therapeutic index and serious toxicities, including hyperthermia, skin reactions, cataracts, and death (6,7). In 1948, DNP’s mechanism of action was discovered, revealing that it is a protonophore, able to move protons through lipid bilayers and thus to chemically uncouple substrate oxidation from ATP production (8). Mammals naturally use an uncoupling protein in brown adipose tissue (BAT) and beige/brite adipose tissue to generate heat to maintain core body temperature (Tb). Thus, BAT and beige/brite adipose tissue activation is of interest as a component of drug therapy for obesity (9,10). Facultative thermogenesis persists in Ucp1 null mice, which lack BAT function, suggesting a role for other tissues, such as muscle (11-13). However, while facultative thermogenesis is extremely important in small mammals, both thermogenic need and capacity in adult humans are much lower than in the mouse. Therefore, using a chemical uncoupler that is presumably active in all tissues might be a complementary approach to obesity pharmacotherapy, avoiding the constraint that there is limited facultative thermogenesis in adult humans.

Mice are a useful model system to study human obesity physiology and treatment due to the conserved biology, plethora of genetic tools, and modest resources (compounds, housing) required. However, the profound differences in thermal biology (14) have not typically been considered when studying obesity physiology and treatment (15). Given the need for improved obesity pharmacotherapy and the dearth of data on chemical uncouplers, we investigated the physiological effects of chemical uncoupling in mice, using DNP as a model compound. While doing so, we also examined the effect of ambient temperature (comparing 30°C, which is approximately thermoneutral for mice, to 22°C, which is below thermoneutrality (16,17)) on drug effectiveness.

**EXPERIMENTAL PROCEDURES**

Animals and DNP: Female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were housed 4/cage, unless otherwise indicated, at 22°C or 30°C with *ad libitum* access to water and high fat diet (D12492, 60% kcal fat, 5.24 metabolizable kcal/g; Research Diets, New Brunswick, NJ) starting at 6 weeks of age with a 12:12-h dark-light cycle (lights on at 0600h). Female mice can be housed together and were used due to limited capacity for thermoneutral housing. Body weight, food intake, and water intake were measured twice weekly. DNP (800 mg/l, Sigma-Aldrich, St. Louis, MO) treatment began when mice were ~6-8 weeks old. DNP was neutralized with NaOH, protected from light, administered in drinking water, and changed twice weekly. DNP was kept moist and skin/mucous membrane contact was avoided. DNP concentrations were measured assuming a molar extinction coefficient of 10,356 M⁻¹cm⁻¹ at 400 nm in 50 mM sodium phosphate, pH 7.0. Tap water adjusted to pH 7.0 was vehicle. Protocols were approved by the NIDDK Institutional Animal Care and Use Committee. Male DIO C57BL/6J mice were also tested at 30°C with DNP in drinking water and were housed 2/cage.

Indirect Calorimetry: Indirect calorimetry was performed with access to food and water using a 12-chamber Environment Controlled CLAMS (Columbus Instruments, Columbus, OH). Female mice were treated with DNP or vehicle at 22°C and 30°C for 6 weeks. Treatment was continued in the chambers, and the chamber temperature was 30°C for all groups. To minimize thermal deconditioning of the 22°C groups while providing sufficient time for familiarization to the chambers, mice were placed into the indirect calorimetry chambers at 1700 and baseline EE was measured.
Testing: Body composition was measured by time domain Echo MRI 3-in-1 (Echo Medical Systems, Houston, TX). Rectal temperatures were measured using a digital thermometer (YSI 455, Measurement Specialties, Shrewsbury, MA). Intraperitoneal glucose (2g/kg, after a 16 hour fast, with AUC calculated from 0 mg/dl) and insulin tolerance (0.75U/kg, ad lib fed) tests were performed at 0930. Glucose was measured with a Glucometer Contour (Bayer, Mishawaka, IN). Liver triglyceride was measured as glycerol after NaOH hydrolysis (Cayman Chemical, #10010755).

Gene expression analysis by quantitative RT-PCR: RNA was extracted (Qiagen RNeasy Plus Mini Kit, Germantown, MD), converted to cDNA (Roche Transcriptor High Fidelity cDNA Synthesis Kit, Indianapolis, IN), and quantified by real-time polymerase chain reaction (qRT-PCR, Applied Biosystems 7900HT, Foster City, CA) using SYBR green. Data are normalized to 18S RNA (18S DNA for mitochondrial DNA) in the same sample. Primer sequences are shown in Table 1.

Western blot analysis: Protein from BAT was prepared using RIPA buffer and quantified (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL). Protein (10 μg/lane) was separated in 4-12% SDS-PAGE gels, transferred to PVDF membrane and probed with anti-UCP1 antibody (1:5000, U6382, Sigma-Aldrich), or anti-α-Tubulin (1:5000, T6074, Sigma-Aldrich). Signals were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), using exposure times appropriate to signal intensity.

Histology: Tissues were fixed in 10% formalin, blocked in paraffin wax, and sectioned. Sections were stained with hematoxylin and eosin and examined by light microscopy.

Data analysis: Data were analyzed using unpaired t-tests or ANOVA with Tukey HSD post-hoc testing using Sigmaplot 12.5 (Systat Software Inc). Statistical significance was defined as p<0.05.

RESULTS

Preliminary dose-ranging experiments at thermoneutrality demonstrated that a dose of 800 mg/l DNP in drinking water (~89 mg/kg/day) reduced body weight without affecting food or water intake or causing noticeable changes in behavior. This dose was used for all experiments as higher DNP doses reduced water and food intake, possibly an aversive effect, while lower doses did not affect body weight (Figure 1).

**DNP treatment reduces weight gain and improves glucose homeostasis**--We treated female C57BL/6J mice with DNP for 8 weeks at 30°C on a high fat diet. DNP-treated mice weighed 18% (p<0.05) less than vehicle-treated mice, with no effect on food intake (Figure 2A,B). DNP treatment reduced fat mass with no change in lean mass (Figure 2C). Free-living total energy expenditure (TEE), calculated by energy balance, from the caloric intake, change in body weight, and change in body composition (18), was increased by 13% (p<0.05) (Figure 2D). DNP treatment lowered fed and fasting glucose levels, HOMA-IR, and improved glucose tolerance, with no obvious effect on insulin tolerance (Figure 2E-K). Treatment with DNP in male DIO mice showed similar effects on body weight and composition, food intake, and TEE over 2 weeks (Figure 3).

**DNP-treated mice weigh less at 30°C but not at 22°C**--We next examined the interaction between environmental temperature (Ta) and DNP treatment, comparing thermoneutrality (30°C)
with a typical vivarium Ta (22°C). Vehicle-treated mice gained more weight at 30°C than at 22°C (Figure 4A), consistent with prior reports (19-21). At 30°C, DNP-treated mice weighed 23% less than vehicle-treated controls, due to reduced fat mass with little change in lean mass (Figure 4C,D). In contrast, at 22°C DNP had no effect on body weight or fat mass. Mice at 22°C ate 23% more than mice housed at 30°C, with no DNP effect on food (Figure 4B) or water intake (not shown). DNP treatment increases metabolic rate at thermoneutrality—We used the energy balance technique to measure free-living TEE (18). Over 5 weeks of treatment, the free-living TEE of the vehicle groups at 22°C was 32% greater than at 30°C. At 30°C, DNP treatment increased TEE by 16%, while at 22°C DNP treatment had no effect on free-living TEE (Figure 4E).

Next, we used indirect calorimetry to measure DNP’s effect on metabolic rate and thermogenic capacity. These experiments were performed at thermoneutrality to remove the contribution of facultative thermogenesis. To minimize thermal deconditioning of the 22°C groups while providing sufficient acclimatization time, mice were placed in the metabolic chambers 13 hours before baseline measurements. DNP treatment increased baseline TEE measured at thermoneutrality (Table 2). In the 30°C groups, the DNP-mediated metabolic rate increase, compared to vehicle-treated controls, was 6% per mouse (0.209/0.196, p=ns), 37% per g body weight (8.82/9.46, p=0.009), 28% per (g body weight)^(0.75) (19.4/15.1, p=0.02), or 11-13% using a statistical model. Comparing the 22°C groups (where the DNP-treated and vehicle groups have the same body weight), the mean DNP-mediated increase was 17% (0.237/0.202, p=0.05) above the light phase thermoneutral metabolic rate. There were no differences in RER (respiratory exchange ratio, the ratio of CO₂ produced to O₂ consumed, which indicates metabolic fuel type) or physical activity.

CL316243, a selective β3-adrenoceptor agonist, was used in thermoneutral conditions to maximally stimulate BAT thermogenesis and WAT lipolysis, assaying the capacity for facultative thermogenesis (22-25) (Figure 5, Table 2). In all groups, CL316243 caused a drop in RER to ~0.71, indicating that fatty acids were the predominant energy source. In vehicle-treated mice, TEE stabilized after CL316243 treatment at increases of 79 ±7% and 109 ±10% over baseline in the mice that were chronically maintained at 30°C and 22°C, respectively (p=0.03, 30°C vs. 22°C). The smaller TEE increase with chronic 30°C exposure is likely due to the reduced capacity for BAT activation. Surprisingly, in DNP-treated mice acute CL316243 treatment had a qualitatively different effect, a large acute increase in TEE (due to fat oxidation, since the RER is ~0.71), which then diminished gradually. By ~5 hours after drug dosing, the 30°C DNP TEE was lower than the 30°C vehicle controls.

DNP treatment increases metabolic rate measured at thermoneutrality in both the 22°C and 30°C Ta groups. Our interpretation is that at 22°C, the DNP-mediated increase in metabolic rate is quantitatively offset by a reduction in facultative thermogenesis, so there is no net change in TEE. With an unchanged TEE and food intake, there was no effect on body weight. In contrast, upon chronic adaptation to 30°C, there is little ongoing BAT thermogenesis to suppress, so the DNP-mediated increase in metabolic rate increases the TEE. At 30°C, the TEE increase caused by DNP was not compensated by increased food intake, thus weight loss ensued.

DNP reduces BAT activation—The BAT phenotype was investigated to see if it supports the above interpretations. In vehicle-treated mice, BAT had larger lipid droplets at 30°C, consistent with the greater adiposity of the mice (Figure 6A). BAT at 22°C had higher Ucp1 mRNA and protein levels compared to the 30°C vehicle group, indicating greater activation (Figure 6B,C). However, other mRNA markers of BAT activation (Prdm16, Cidea, Dio2, Fgf21) or mitochondrial function/mass (CytoC, Cox8b mRNAs; mitochondrial DNA) were not significantly increased, consistent with a relatively modest activation of BAT (Figure 6C,D).

DNP treatment at 30°C reduced Ucp1, Prdm16, Cidea, Dio2, and Fgf21 mRNA levels. Other mitochondrial markers were also reduced, either significantly (Cox8b mRNA) or non-significantly (CytoC mRNA and mitochondrial DNA). At 22°C, DNP tended to reduce BAT activation markers, but not to the level seen at 30°C (Figure 6A-D). No consistent changes in BAT mitochondrial appearance were seen with electron microscopy (data not shown).
WAT and Endocrine effects—Leptin mRNA in WAT and serum levels reflected the underlying adiposity, being reduced by DNP vs. vehicle at 30°C and were proportional to each other and body weight/fat mass (Figure 7, and data not shown). No changes in WAT mRNA markers of browning were detected. Adiponectin levels did not differ between the groups (Figure 7). Igf-1 levels were higher at 22°C vs. 30°C (p=0.001), with no effect of DNP treatment. Fgf21 concentrations were variable, but appeared to be reduced by DNP. DNP treatment decreased serum T3 and T4 levels at both temperatures (p<0.005) (Figure 7).

Effect of DNP on liver and lipid metabolism—At 30°C, DNP treatment reduced liver weight by 13% (p=0.058), due to reduced hepatic steatosis with a 49% reduction in triglyceride amount (p=0.02) (Figure 8A-C). DNP-treated livers showed no histological evidence of injury. Indeed, at 30°C, DNP treatment actually reduced alanine aminotransferase levels by 32% (p=0.002, Figure 8D).

Although DNP treatment might be predicted to induce mitochondrial function to compensate for less efficient ATP production, we observed that DNP had no effect on liver mRNA levels of Pgc1α or Pgc1β, transcription factors that stimulate mitochondrial biogenesis, Cox5b (Figure 8E), or mitochondrial DNA levels (data not shown). Liver mRNA levels of metabolic proteins were similar among the four treatment groups; only Fgf21 and Mcad mRNA levels were higher in 22°C vs. 30°C in vehicle-treated mice. DNP treatment reduced Scd1 mRNA at both temperatures.

Serum β-hydroxybutyrate, acetoacetate, and triglyceride levels were increased in DNP-treated mice maintained at 30°C, while no change was observed in free fatty acid, lactate, or cholesterol levels (Figure 8F,G). Additionally, there was no change in the β-hydroxybutyrate to acetoacetate ratio suggesting that the liver mitochondrial redox state was not significantly altered by DNP treatment.

Thermal biology—No difference in rectal body temperature (Tb) was observed in mice fed a HFD at 22°C. At 30°C, results were inconsistent, with DNP treatment sometimes increasing Tb, possibly more so during handling. Rectal Tb was more systematically monitored in singly-housed, chow-fed male C57BL/6J mice at 30°C treated with DNP or vehicle (n=8/group). Light phase Tb was measured on 12 of 21 days and the global mean Tb was 0.22°C higher in the DNP-treated mice (36.53 ±0.06°C vs. 36.31 ±0.06°C; p=0.006). These results suggest that DNP treatment can slightly increase Tb at 30°C.

DISCUSSION

We studied the effect of chronic DNP treatment in mice housed below (22°C) and at (30°C) thermoneutrality. Below thermoneutrality, a DNP dose that increased thermoneutral metabolic rate by ~17% had no effect on TEE, body weight, or adiposity. Our results suggest that in mice maintained below thermoneutrality, the increase in energy expenditure generated by DNP-mediated uncoupling can be compensated by reduced BAT thermogenesis. At thermoneutrality, this compensatory mechanism is absent due to the fact that BAT and other sources of facultative thermogenesis are virtually inactive in thermoneutral conditions. Therefore at thermoneutrality DNP treatment increases TEE, leading to reduced body weight, adiposity, and hepatic steatosis, and improved glucose tolerance. No attenuation in efficacy or clear toxic effects of DNP treatment were observed. Recently, a DNP prodrug that is activated in the liver was reported, which also improved hepatic steatosis and glucose homeostasis, and did so with an improved therapeutic index (26). Its effects at thermoneutrality were not investigated.

Chronic DNP treatment reduced circulating thyroid hormone levels, the principal regulator of basal metabolic rate (27). The lower thyroid tone reduces BAT activation (28), likely via reduced action in the hypothalamus (29). Lower leptin levels are probably not a major cause of the reduced thyroid tone (30) by DNP, since reduced thyroid hormone levels occurred in the 22°C DNP group in which neither adiposity nor leptin levels were affected.

As DNP increased TEE while reducing BAT activity, fuel oxidation in non-BAT tissues must be increasing. The observed increase in β-hydroxybutyrate levels suggests that there is a contribution from fatty acid oxidation by liver. The greater increase in TEE immediately after β3-agonist administration in DNP-treated mice
suggests that there is increased capacity for fatty acid oxidation—notably seen in the 22°C groups that are matched for body weight and adiposity and thus triglyceride supply. Substrate flux studies will be required to quantify the individual contributions of specific tissues to the increased oxidation.

DNP treatment improved glucose homeostasis, as evidenced by improved glucose tolerance, decreased fasting and fed blood glucose levels, and lower insulin levels in response to glucose challenge. Since there was no clear improvement in insulin tolerance, the lower fed serum insulin levels and HOMA-IR suggest either a modest improvement in insulin sensitivity and/or an increase in non-insulin-regulated (eg, DNP-stimulated) glucose disposal. The current studies are not sufficiently quantitative to determine if the beneficial effects of DNP on glucose are at the expected level for the degree of weight reduction. It is not known if BAT inactivation is deleterious to glucose or lipid metabolism. Since BAT clears triglyceride-rich lipoproteins (31), DNP-induced BAT inactivation may contribute to the observed increase in circulating triglyceride levels.

In DNP-treated mice at thermoneutrality, why did caloric intake not increase sufficiently to prevent weight reduction? This is not due to physical limitations on ingestion, since the amount of food required is less than what was eaten at 22°C. The biology may be analogous to exercise’s effect on food intake and body weight: In the sedentary human or rat, low levels of exercise slightly reduce body weight and food intake, while with further increases in exercise level food intake rises and the weight remains stable (32,33). In mice at thermoneutrality, increasing cold exposure may have a similar response (initial weight loss, then increasing food intake to maintain body weight) (19-21,34). If the analogy to exercise and BAT activation holds, the DNP dose used was likely too low to increase food intake. An alternate explanation is that increased thermogenesis and increased food intake are intrinsically coupled in the coordinated response to a cold environment, and DNP treatment bypasses this homeostatic regulation.

DNP treatment did not affect Tb at 22°C, but slightly increased Tb at 30°C. The small degree of hyperthermia with DNP treatment in mice is consistent with the intrinsically high rate of heat loss in small mammals, which is protective against hyperthermia. In humans, death caused by DNP is typically attributed to hyperthermia. However, if hyperthermia is the principal toxicity, DNP should have increased toxicity in small mammals housed at thermoneutrality, which is not the case (35). It is plausible that the proximate cause of death is actually energy depletion, with the hyperthermia being secondary to the increased metabolism in the attempt to maintain ATP levels.

A previous long-term mouse DNP study used a 1 mg/l dose and reported increased longevity and reduced body weight, presumably at ambient temperatures (36). We found that an 800-fold larger dose caused mild weight reduction over 1-2 weeks at thermoneutrality, while lower doses did not. The 800 mg/l mouse dose is, after scaling, comparable to the human dose (the clinical dose, 1.2-4.3 mg/kg/d (37), scales to 15-54 mg/kg/d in mice (38)). This dose is also consistent with the reported narrow therapeutic index: in young mice 25% mortality was observed with DNP at ~325 mg/kg/d in the diet for 1 week (37). Single doses of DNP are more toxic (LD$_{50}$ of 35 to 72 mg/kg) (35,39), likely due to higher peak exposures. Compared with gavage or parenteral dosing, dosing in drinking water or food is expected to produce lower peak concentrations and more even coverage over the day. A DNP prodrug (26) may also achieve some of its improved therapeutic index by reducing the peak concentration of the active species.

Thermal biology is fundamentally different between small and large mammals due to the different surface area:volume ratios (14,40,41). Large mammals have developed physiologic mechanisms for heat dissipation, while thermal physiology in small mammals is oriented to heat generation, with a major contribution from BAT (9). Large mammals defend Tb and let body shell and surface temperature drop, thereby increasing insulation, while mice have little insulation and adapt by regulated changes in Tb (42). In humans, the broad thermoneutral zone and low level of facultative thermogenesis (increases of only a few percent (43-45)) need to be considered. Since BAT function in adult humans is modest and reduces with age, drugs that increase energy expenditure by BAT-independent mechanisms deserve further investigation.
The theoretical implications of the mouse/human differences in thermal biology for drug development have been noted (46-48), but we are unaware of pharmacotherapy studies actually evaluating this theory. We chose DNP treatment to investigate the role of Ta because DNP selectively increases energy expenditure. Obesity therapies that target food intake reduction might be less sensitive to Ta. The result that DNP-mediated thermogenesis at 22°C substitutes for BAT thermogenesis with no weight loss contrasts with the significant DNP-induced weight reduction observed at 30°C. This is an experimental confirmation that Ta can affect the measured efficacy of a potential anti-obesity drug and thus suggests that drugs increasing metabolic rate might have greater human efficacy than would be predicted from the typical preclinical studies in mice below thermoneutrality. It is also notable that obese people, the pharmacotherapy target population, have less BAT activity than lean people (49), further strengthening the rationale for studying anti-obesity drugs in mice at thermoneutrality.

While DNP itself has an unacceptable benefit:risk ratio for the treatment of obesity (7), our data support further investigation into uncoupling as an approach to obesity therapy. Improved properties of a chemical uncoupler include a better therapeutic index, which might be achieved by a wider dynamic range (50), saturation absorption pharmacokinetics, or reducing exposure to the most sensitive organs, such as the eye (which can develop cataracts (51)). Recently a DNP prodrug that is activated in the liver showed remarkable properties including improved hepatic steatosis and glucose homeostasis, with an improved therapeutic index (26). Combination therapy with a low dose of an uncoupler and another obesity drug (particularly one that reduces food intake) could also be investigated. Our study aimed to further explore the physiology of DNP in order to inform further consideration of chemical uncouplers in weight loss therapy. Given the demonstrated therapeutic benefits, it is worth considering whether a sufficiently safe chemical uncoupler can be developed for the treatment of obesity.

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http://www.atsdr.cdc.gov/ToxProfiles/TP.asp?id=729&tid=132


**FOOTNOTES**

3 The abbreviations used are: BAT, brown adipose tissue; iBAT, interscapular BAT; DIO, diet-induced obesity; DNP, 2,4-dinitrophenol; HFD, high fat diet; RER, respiratory exchange ratio; Ta, environmental temperature; Tb, core body temperature; TEE, total energy expenditure; WAT, white adipose tissue
FIGURE LEGENDS

FIGURE 1. DNP dose response. A. Body weight, B. food intake, and C. water intake. 6-week old female mice housed 4/cage on a high-fat diet were acclimatized to 30°C for 7 days and then treated with the indicated dose of DNP in the drinking water for 1 week. Body weight change data are mean ±SEM, n=8/group, with *indicating a p<0.05 as compared to 0 mg/ml DNP dose. Intake data are mean of two cages. Data are combined from two independent experiments. Initial body weight was 24.7 ±0.6 g in the first experiment (0-400 mg/l) and 26.4 ±0.8 g in the second experiment (0-2000 mg/l).

FIGURE 2. Effect of DNP treatment at thermoneutrality in C57BL/6J mice. A. Body weight and B. food intake. C. Body composition at 3 weeks and D. energy expenditure over the first 3 weeks of treatment. E-H. Glucose tolerance test, area under the glucose excursion curve (AUC), plasma insulin before and 15 min after glucose administration, and HOMA-IR levels after 5 weeks. I. Insulin tolerance test after 6 weeks. J.K. 4-Hour fasted serum glucose and insulin levels at euthanasia after 11 weeks of treatment. 8-week old female mice on a HFD were acclimatized to housing 4/cage at 30°C for 2.5 weeks and then treated with vehicle or DNP (800 mg/l in the drinking water). *indicates p<0.05.

FIGURE 3. Effects of DNP treatment in male C57BL/6J mice. A. Body weight, B,C. body composition, D. food intake, and E. TEE over 2 weeks of treatment. Eleven-week old male mice on a HFD were acclimatized to housing 2/cage at 30°C for 4 days and then treated with DNP 800 mg/l. Weight, fat mass, and composition are, mean ±SEM, n=8/group, *indicates p<0.05. Food intake and energy expenditure data are based on 4 cages/group, expressed per mouse.

FIGURE 4. Effect of environmental temperature on response to treatment with DNP in C57BL/6J mice. A. Body weight, B. food intake, C. fat mass, D. body composition and E. energy expenditure. 8-week old female mice on a HFD were acclimatized to housing 4/cage at 22°C or 30°C for 2.5 weeks and then treated with DNP 800 mg/l. Fat mass was measured at euthanasia after 9 weeks of treatment. Body composition was measured after 5 weeks and energy expenditure is the average over the first 5 weeks of treatment. Weight, fat mass, and composition are mean ±SEM, n=8/group, *indicates p<0.05. Food intake and energy expenditure data are based on 2 cages/group, expressed per mouse.

FIGURE 5. Effect of chronic DNP and acute CL316243 treatment on A. TEE and B. RER. Mice were studied after 6 weeks of DNP/vehicle treatment at the indicated Ta. CL316243 was injected ip at time 0 and metabolic rate was measured at 30°C for all groups; see Experimental Procedures for details. There is a handling effect during the first half hour, with an increase in TEE, RER, and physical activity. Data are mean ±SEM, n=8/group.

FIGURE 6. DNP effect in BAT. A. Histology, B. Ucp1 protein, C. mRNA levels, and D. mitochondrial DNA content. Scale bar is 100 microns. mRNA and DNA levels are normalized to 30°C vehicle. Mice were studied after 9 weeks of DNP/vehicle treatment at the indicated Ta. Data are mean ±SEM, n=8/ group, *indicates p<0.05.

FIGURE 7. Effect of DNP on hormone levels. A-G. The indicated hormone levels were measured in samples obtained from ad lib fed mice at euthanasia after 9 weeks of DNP/vehicle treatment at the indicated Ta. Data are mean ±SEM, n=8/group, *indicates p<0.05 for vehicle-DNP comparison, within Ta.
FIGURE 8. DNP effects in liver. A. Histology, B. mass, C. triglyceride levels, D. alanine aminotransferase (ALT) activity, E. mRNA levels, and F,G. serum analytes. FFA, free fatty acid; β-HB, β-hydroxybutyrate. Scale bar is 50 microns. Mice were studied after 9 weeks of DNP/vehicle treatment at the indicated Ta. Data are mean ±SEM, n=8/group, *indicates p<0.05.
**Table 1.** PCR Primer sequences

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<td>GAGGCAGAGAGGAGGAGGAG</td>
<td>GACGAGAGAGACCGAGAG</td>
</tr>
<tr>
<td>Scd1</td>
<td>CTTCCCCTTGACTACTCTG</td>
<td>GCCATGCCCTGAGGCTG</td>
</tr>
<tr>
<td>mtCytB</td>
<td>ACCAATCTCCAAAACATCA</td>
<td>GCCATGCCCTGAGGCTG</td>
</tr>
</tbody>
</table>
| 18S*       | CGATCCGAGGGCCTCCTCTG | **Note:** For mitochondrial DNA copy analysis.
Table 2. Effect of CL316243 in DNP-treated mice. ‘Baseline (pre CL316243)’ is the 2 h time period ending 1 h before CL316243 injection. ‘After CL316243’ indicates measurement ~5 h after CL316243 injection. All measurements were performed at thermoneutrality, see Experimental Procedures for details. Last three columns show significance from 2-way ANOVA; no value indicates P>0.05. T x D is temperature - drug interaction. The individual baseline TEE points were also analyzed with a statistical model incorporating temperature, drug, T x D, activity, and body weight (nested within drug and temperature) constructed using JMP (SAS). Drug (p<0.0001), activity (p<0.0001), and body weight (p=0.02) contributed significantly, while temperature and T x D did not (P>0.05). The model has an adjusted $r^2 = 0.60$. As examples, TEE for a ‘mean’ mouse (body weight of 27.43 g; activity of 198 beambreaks/13 min) and ‘median’ mouse (body weight of 27.03 g; activity of 55 beambreaks/13 min) are included.

<table>
<thead>
<tr>
<th></th>
<th>30°C Veh</th>
<th>30°C DNP</th>
<th>22°C Veh</th>
<th>22°C DNP</th>
<th>Temperature</th>
<th>Drug</th>
<th>T x D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline (pre CL316243)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>30.9 ±1.6</td>
<td>23.8 ±0.9</td>
<td>27.5 ±1.2</td>
<td>27.4 ±1.5</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>TEE (kcal/h/mouse)</td>
<td>0.196 ±0.006</td>
<td>0.209 ±0.014</td>
<td>0.202 ±0.010</td>
<td>0.237 ±0.013</td>
<td>0.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEE (kcal/h/bw)</td>
<td>6.46 ±0.36</td>
<td>8.82 ±0.69</td>
<td>7.42 ±0.44</td>
<td>8.86 ±0.75</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEE (kcal/h/bw$^{0.75}$)</td>
<td>15.1 ±0.7</td>
<td>19.4 ±1.4</td>
<td>16.9 ±0.9</td>
<td>20.1 ±1.5</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RER</td>
<td>0.764 ±0.012</td>
<td>0.756 ±0.006</td>
<td>0.758 ±0.005</td>
<td>0.754 ±0.006</td>
<td></td>
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</tr>
<tr>
<td>Activity (beambreaks/13min)</td>
<td>174 ±29</td>
<td>204 ±37</td>
<td>191 ±34</td>
<td>225 ±23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEE (kcal/h/mean mouse)</td>
<td>0.226</td>
<td>0.251</td>
<td>0.230</td>
<td>0.268</td>
<td>&lt;0.0001</td>
<td></td>
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</tr>
<tr>
<td>TEE (kcal/h/median mouse)</td>
<td>0.185</td>
<td>0.208</td>
<td>0.189</td>
<td>0.228</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>After CL316243</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEE (kcal/h/mouse)</td>
<td>0.350 ±0.009</td>
<td>0.286 ±0.017</td>
<td>0.419 ±0.018</td>
<td>0.433 ±0.015</td>
<td>&lt;0.001</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>TEE, ratio to baseline</td>
<td>1.79 ±0.07</td>
<td>1.45 ±0.19</td>
<td>2.09 ±0.10</td>
<td>1.85 ±0.08</td>
<td></td>
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</tr>
<tr>
<td>RER</td>
<td>0.707 ±0.007</td>
<td>0.716 ±0.005</td>
<td>0.702 ±0.003</td>
<td>0.717 ±0.010</td>
<td></td>
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</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
DNP Figure 7
Figure 8
The Chemical Uncoupler 2,4-Dinitrophenol (DNP) Protects Against Diet-induced Obesity and Improves Energy Homeostasis in Mice at Thermoneutrality
Margalit Goldgof, Cuiying Xiao, Tatyana Chanturiya, William Jou, Oksana Gavrilova and Marc L. Reitman

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