Liver-Selective Imidazolopyrazine Mitochondrial Uncoupler SHD865 Reverses Adiposity and Glucose Intolerance in Mice

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Diabetes 2024;73:374–384 | https://doi.org/10.2337/db23-0233

Excess body fat is a risk factor for metabolic diseases and is a leading preventable cause of morbidity and mortality worldwide. There is a strong need to find new treatments that decrease the burden of obesity and lower the risk of obesity-related comorbidities, including cardiovascular disease and type 2 diabetes. Pharmacologic mitochondrial uncouplers represent a potential treatment for obesity through their ability to increase nutrient oxidation. Herein, we report the in vitro and in vivo characterization of compound SHD865, the first compound to be studied in vivo in a newly discovered class of imidazolopyrazine mitochondrial uncouplers. SHD865 is a derivative of the furazanopyrazine uncoupler BAM15. SHD865 is a milder mitochondrial uncoupler than BAM15 that results in a lower maximal respiration rate. In a mouse model of diet-induced adiposity, 6-week treatment with SHD865 completely restored normal body composition and glucose tolerance to levels like those of chow-fed controls, without altering food intake. SHD865 treatment also corrected liver steatosis and plasma hyperlipidemia to normal levels comparable with chow-fed controls. SHD865 has maximal oral bioavailability in rats and slow clearance in human microsomes and hepatocytes. Collectively, these data identify the potential of imidazolopyrazine mitochondrial uncouplers as drug candidates for the treatment of obesity-related disorders.

ARTICLE HIGHLIGHTS

- A growing body of evidence suggests that targeting negative energy balance with mitochondrial uncouplers has potential for the treatment of metabolic disease.
- We report the discovery and characterization of compound SHD865 as a new class imidazolopyrazine mitochondrial uncoupler.
- SHD865 is liver selective, safely reverses diet-induced adiposity, and improves glucose homeostasis in mice without altering food intake or decreasing lean mass.
- SHD865 and related compounds have translational potential for the treatment of obesity, diabetes, and related metabolic disorders.

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Received 26 March 2023 and accepted 4 October 2023

This article contains supplementary material online at https://doi.org/10.2337/figshare.24347104.

See accompanying article, p. 357.

Excess adiposity is a major risk factor for type 2 diabetes, cardiovascular diseases, and some types of cancer (1–3). The fundamental cause of excess adiposity is an energy imbalance between calories consumed and expended. Adiposity can be decreased by strategies that create negative energy balance, including bariatric surgery, lifestyle interventions (e.g., diet and exercise), and pharmacotherapy. Bariatric surgery is one of the most effective options, but it is not a universal solution due to procedure costs, risk of surgical complications, and because 20% of patients experience weight regain with recurrence of comorbidities (4,5). Current U.S. Food and Drug Administration-approved antiobesity drugs target nutrient absorption or food intake (6), but there are no approved pharmacotherapies that primarily target energy expenditure. Mitochondrial uncouplers represent one class of drugs that increase energy expenditure without affecting food intake and have shown antiobesity effectiveness in preclinical animal studies and humans (7).

Mitochondrial uncouplers exert their antiobesity effect by increasing the amount of nutrient oxidation needed to generate a given amount of ATP (8,9). The best-known mitochondrial uncouplers include carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) and 2,4-dinitrophenol (DNP). FCCP is a potent mitochondrial uncoupler most commonly used as a tool molecule to study mitochondrial function in cultured cells, but it has a narrow concentration range to increase mitochondrial respiration before toxic doses are reached that result in mitochondrial inhibition. DNP has a wider concentration range between efficacy and toxicity and was once used as an antiobesity treatment in humans; but ultimately, its therapeutic window is too small, and the U.S. Food and Drug Administration banned its use in 1938 (10). FCCP and DNP both have unwanted off-target effects, including depolarization of the plasma membrane (7,11,12).

Next-generation mitochondrial uncouplers are being developed to overcome the limitations and off-target effects of FCCP and DNP (7). Our group previously identified one such molecule, named BAM15, a mitochondria-specific protonophore uncoupler that does not cause off-target plasma membrane depolarization at doses that drive maximal mitochondrial uncoupling (12). We recently demonstrated that BAM15 has a wide therapeutic window in mice and is effective in both preventing and reversing adiposity and insulin resistance (13,14). However, BAM15 has a short half-life and poor solubility at high doses; therefore, new structural derivatives are sought that can overcome these barriers.

BAM15 was identified from a chemical library screen and characterized as the first furazanopyrazine mitochondrial uncoupler. Structure-activity relationship studies in the furazanopyrazine scaffold identified additional molecules with improved potency or half-life (15–18). Recently, we reported the discovery of a new imidazolopyrazine class of mitochondrial uncouplers and partially characterized the activity of select molecules in vitro but not in vivo (19). Herein, we report on the therapeutic potential of the first imidazolopyrazine mitochondrial uncoupler SHD865 (*N*-[2-fluoro-4-[trifluoromethoxy]phenyl]-5-methoxy-2-[trifluoromethyl]-1*H*-imidazo[4,5-b]pyrazine-6-amine) in a mouse model of diet-induced adiposity. Specifically, we show that SHD865 safely reverses adiposity and glucose intolerance in mice without altering food intake or decreasing lean mass.

RESEARCH DESIGN AND METHODS

Oxygen Consumption Rate Measurements

Cellular oxygen consumption rate (OCR) was measured using a Seahorse XFe96 Flux Analyzer (Agilent Technologies, Santa Clara, CA) as previously published (12). High-resolution respirometry was conducted in C57BL/6J mouse tissue as per Alexopoulos et al. (13) by using the OROBOROS Oxygraph-O2K (Oroboros Instruments, Corp., Innsbruck, Austria).

Western Diet Studies

All mouse experiments for this study were approved by the University of New South Wales (UNSW) Animal Care and Ethics Committee (animal ethics approvals 17/66B and 18/29A). C57BL/6J mice were purchased from Australian BioResources (Moss Vale, New South Wales, Australia) and housed at 22°C, in a light-dark cycle of 12 h. Unless otherwise stated, mice were provided with ad libitum access to water and a normal chow diet (Gordons Specialty Feeds, Yanderra, New South Wales, Australia).

A Western diet (WD; 45% fat and 16% sucrose by kcal, based on Research Diets D12451) was prepared in-house, as previously described in studies describing the metabolic effects of mitochondrial uncouplers BAM15 and SHC517 in mice fed a WD. Diet ingredients were purchased from local suppliers (Supplementary Table 1). For the WD studies, 12-week-old male C57BL/6J mice were fed chow or the WD for 4 weeks. The mice fed the WD were then randomized and stratified by body weight to two similar groups, where one remained being fed the WD and the other was fed the WD containing 0.04% w/w SHD865 for additional 6 weeks. All mice were single housed during the last 6 weeks to enable accurate measurement of food intake and prevent variability related to fighting or huddling. Glucose tolerance tests (GTTs) were performed using 2 g of glucose per kg of lean mass injected intraperitoneally in the form of a 33.3% glucose solution prepared in sterile saline. The first cohort was performed at the same time as a study with compound SHC517 using the same chow and WD control groups (15).

Mass Spectrometry

Rat pharmacokinetics studies were performed by GVK Biosciences (Hyderabad, India) as described in the Supplementary Material. Mouse samples were processed at UNSW and performed as previously published (13), with the exception that standards were prepared by spiking known concentrations of SHD865 into untreated plasma, and electrospray ionization was performed in negative mode

using transitions of m/z 411.1 > 306 and 411.1 > 374.8 with a 5-min retention time to identify SHD865.

Lipid and Biochemical Analysis

Tissue samples and serum were snap frozen in liquid nitrogen immediately after collection and stored at −80°C until analysis of insulin, cholesterol, or triglyceride content using methods previously described (13).

Statistical Analysis

Statistical testing was done using GraphPad Prism 8.4.1 (GraphPad Software), where the threshold for significance was determined to be reached when $P < 0.05$ compared with WD controls, unless otherwise stated.

Data and Resource Availability

The data sets and resources generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

Mitochondrial Uncoupling Properties of SHD865 In Vitro

SHD865 is a structural derivative of the furazanopyrazine class of mitochondrial uncouplers BAM15 (12), SHC517 (15), and SHS4121705 (17), where the furazan ring was replaced with a 2-trifluoromethylimidazole core (Fig. 1*A*). SHD865 also features both a methoxy and a 2-fluoro-4-trifluoromethoxy group on the pyrazine ring (Fig. 1*A*). To determine the mitochondrial uncoupling characteristics of SHD865, we first assessed cellular OCR in vitro in rat L6 myoblasts. L6 cells have a robust threefold spare respiratory capacity and were used for the chemical library screen that first identified BAM15; therefore, this cell line is used for consistency to benchmark new compounds. In Fig. 1*B*, compounds SHD865, BAM15, and FCCP were compared across a dose response ranging from 0.4 to 50 μmol/L. This assay informs compound potency and ability to drive mitochondrial respiration without causing mitochondrial fatigue or failure. SHD865 increased the cellular OCR by up to ∼90% of the maximal OCR induced by BAM15 and FCCP (Fig. 1*B*). SHD865 was less potent than BAM15 and FCCP in the context of half-maximal effective concentration (EC_{50}), with EC_{50} average values of 3.8 μmol/L for SHD865, 0.9 μmol/L for BAM15, and 1.2 μmol/L for FCCP; however, SHD865 maintained uncoupled respiration at a stable rate across a large concentration range as indicated by the plateau-shaped curve (Fig. 1*C*), similar to BAM15. A decline in respiration rate with dose escalation is a sign of mitochondrial inhibition, which is evidenced by FCCP (Fig. 1*B*).

We next compared SHD865 with BAM15 in a mitochondrial stress test. These data showed that both SHD865 and BAM15 could increase mitochondrial respiration in the presence of the ATP synthase inhibitor oligomycin (Fig. 1*D*). The spare respiratory capacity of SHD865-treated cells was ∼90% of BAM15-treated cells (Fig. 1*E*). The spare respiratory capacity and maximal OCR induced by both SHD865 and BAM15 were not altered by the presence of oligomycin, demonstrating that their activities do not require ATP synthase activity. SHD865 had similar bioactivity compared with BAM15 in NMuLi normal murine liver cells as it did in rat L6 myoblasts (Supplementary Fig. 1), with absolute activity shown for comparison between cell lines.

FCCP is known to depolarize the plasma membrane (11); therefore, we next investigated whether SHD865 causes plasma membrane depolarization. Whole-cell current clamp recordings at the resting membrane potential of L6 myoblasts were performed in the presence or absence of SHD865 and FCCP. As illustrated in the representative recording and grouped data (Fig. 1*F*), 25 μmol/L SHD865 caused a slight depolarization that was significantly smaller than that observed in the same cells by 10 μmol/L FCCP (1.4 ± 0.4 mV vs. 4.9 ± 1.0 mV). SHD865 was tested at 25 μmol/L, which is in the middle of the plateau range, for avoidance of doubt that we assessed effects at concentrations where SHD865 has maximal activity. Thus, SHD865 evokes very low levels of plasma membrane depolarization, less than those observed with classical mitochondrial uncouplers.

SHD865 Bioavailability and Tolerability

We next determined the suitability of SHD865 for use in vivo by assessing its pharmacokinetic profile. SHD865 oral bioavailability was assessed in mice and rats by comparing serum drug exposure profiles when delivered per oral (p.o.) or by intravenous (i.v.) tail vein injection. When administered to C57BL/6J mice, the dose-normalized area under the curve calculation showed that SHD865 was 21% orally bioavailable, with 10 mg/kg p.o. delivery resulting in an average maximum plasma concentration (C_{max}) of 9.1 μmol/L with a 1.7-h half-life ($t_{1/2}$) (Fig. 2*A*). In contrast, SHD865 was 100% orally bioavailable in Sprague-Dawley rats, while also exhibiting a 1.7-h half-life (Fig. 2*B*). As a proxy for human drug metabolism, SHD865 was tested in human liver microsomes and hepatocytes with intrinsic clearance results <115.5 and 8.2 μL/min/mg, respectively (Fig. 2*C* and *D*), predicting low first-pass metabolism and high stability in humans.

We next performed a dose escalation study to determine tolerability. Mice were given SHD865 by oral gavage at 300 and 1,000 mg/kg doses and observed over a 24-h period where no behavioral signs of distress or ill thrift were observed, and no effect on body temperature was detected (Fig. 2*E*).

SHD865 Bioactivity Is Liver Selective

We next investigated SHD865 in vivo bioactivity by assessing respiratory rates in mouse tissue preparations 2 h after an oral gavage of 30 mg/kg SHD865. High-resolution respirometry of liver, muscle, heart, and white and brown fat showed that only the liver had significantly increased mitochondrial activity (Fig. 3*A*–*C*), with a threefold increase in the maximal capacity of the electron transport chain compared with the vehicle control (Fig. 3*C*).

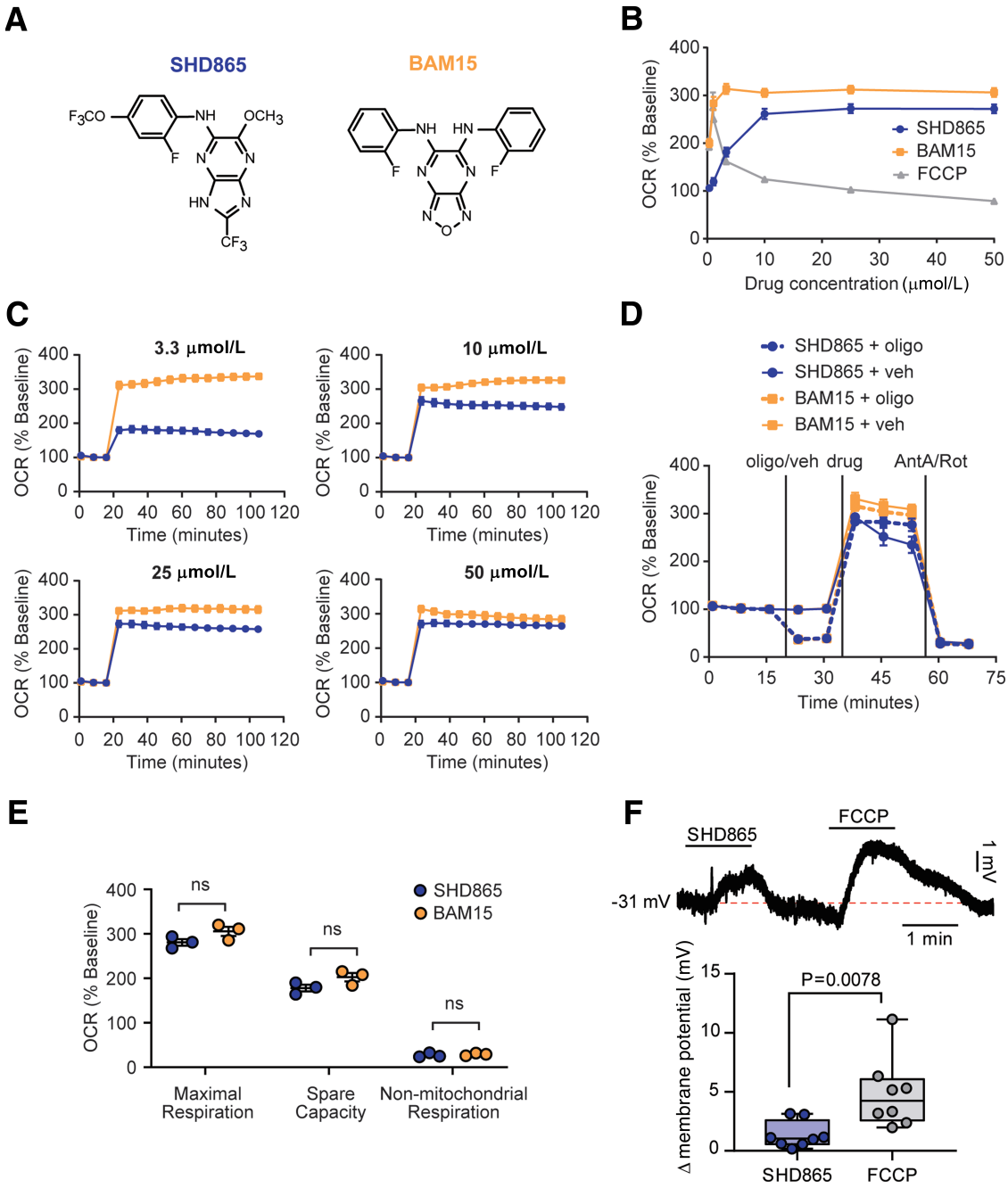

Figure 1—SHD865 mitochondrial uncoupling properties in vitro. *A*: Chemical structures of SHD865 and BAM15. *B* and *C*: SHD865- and BAM15-stimulated OCR in L6 myoblasts. Values are graphed as dose response (*B*) and time course (*C*). For SHD865 and BAM15, $n = 2$ wells per condition from eight separate experiments; for FCCP, $n = 2$–4 wells per condition from three separate experiments. *D*: Mitochondrial stress test, in which L6 cells were sequentially treated with oligomycin (oligo, 1 μmol/L), SHD865 or BAM15 (drug, 50 μmol/L), and antimycin A (AntA, 10 μmol/L) plus rotenone (Rot, 1 μmol/L) at the indicated times ($n = 3$ wells per condition). *E*: Maximal respiration, spare capacity, and nonmitochondrial respiration were quantified from dotted lines in *D*. *F*: Whole-cell recording of membrane potential in a representative L6 myoblast during application of SHD865 (25 μmol/L) and FCCP (10 μmol/L), and grouped data showing the change in membrane potential for SHD865 compared with FCCP ($n = 8$). Insets show current at −31 mV. Values are represented as mean ± SEM for *B*–*D*. The data presented in *F* are box and whiskers format where the median bisects a box bounded by the 25th percentile and 75th percentile, with whiskers depicting the range. ns, not significant; $P = 0.0078$ by Wilcoxon matched-pairs signed rank test.

Diet-Induced Adiposity Is Reversed by SHD865 Treatment

We next investigated whether SHD865 could reverse diet-induced adiposity and its related metabolic disorders caused by 10 weeks of a physiologically relevant WD containing 45% kcal lard fat and 16% kcal sugar. Mice were fed the WD for 4 weeks prior to stratification and randomization to treatment groups of WD +/− SHD865 for additional 6 weeks.

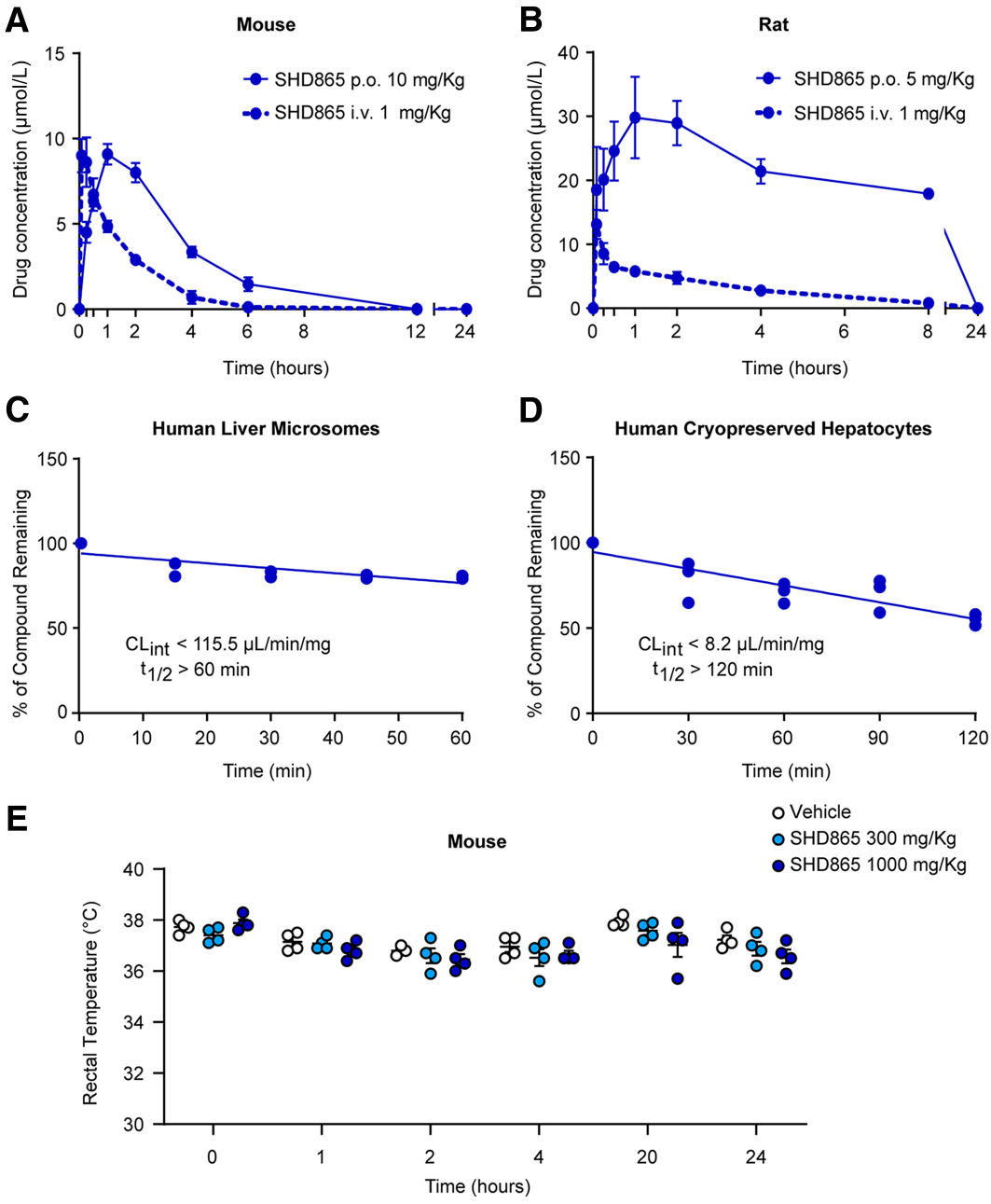

Figure 2—Compound SHD865 is orally bioavailable in rodents, with low metabolic clearance in human hepatocytes and no effect on body temperature. *A*: Pharmacokinetics and bioavailability of SHD865 administered to C57BL/6J mice by i.v. injection at 1 mg/kg (n = 4) and oral gavage at 10 mg/kg (n = 3). *B*: Pharmacokinetic and bioavailability of SHD865 administered to Sprague-Dawley rats by i.v. injection at 1 mg/kg and oral gavage at 5 mg/kg (n = 3). SHD865 intrinsic clearance (CLint) determined in human liver microsomal preparations (*C*) and in cryopreserved hepatocytes following exposure to 1 µmol/L SHD865 (*D*). *E*: Rectal temperature measured over a 24-h period following oral gavage with vehicle or SHD865 at indicated doses (n = 3). Values are represented as mean ± SEM.

Prediet (Supplementary Fig. 2) and pretreatment stratification (Supplementary Fig. 3) showed equal distribution of body weight and fat composition across the groups. SHD865 has a $t_{1/2}$ of 1.7 h in mice; therefore, it was administered by admixture to the diet to avoid stress caused by multiple oral gavage doses per day and to avoid the need for an excipient carrier. Based on pilot studies, a dose of 0.04% w/w in the WD was selected for a 6-week treatment period, resulting in ∼38 mg/kg SHD865 intake per day. To assess the effect of SHD865 on energy expenditure, we assessed whole-body OCR by indirect calorimetry. Compared with mice given the vehicle, mice given 40 mg/kg SHD865 by oral gavage increased whole-body OCR by up to 26% during the first hour after treatment, with respiration returning to normal over 4 h (Supplementary Fig. 4). This bioactivity profile of SHD865 is consistent with its 1.7-h half-life.

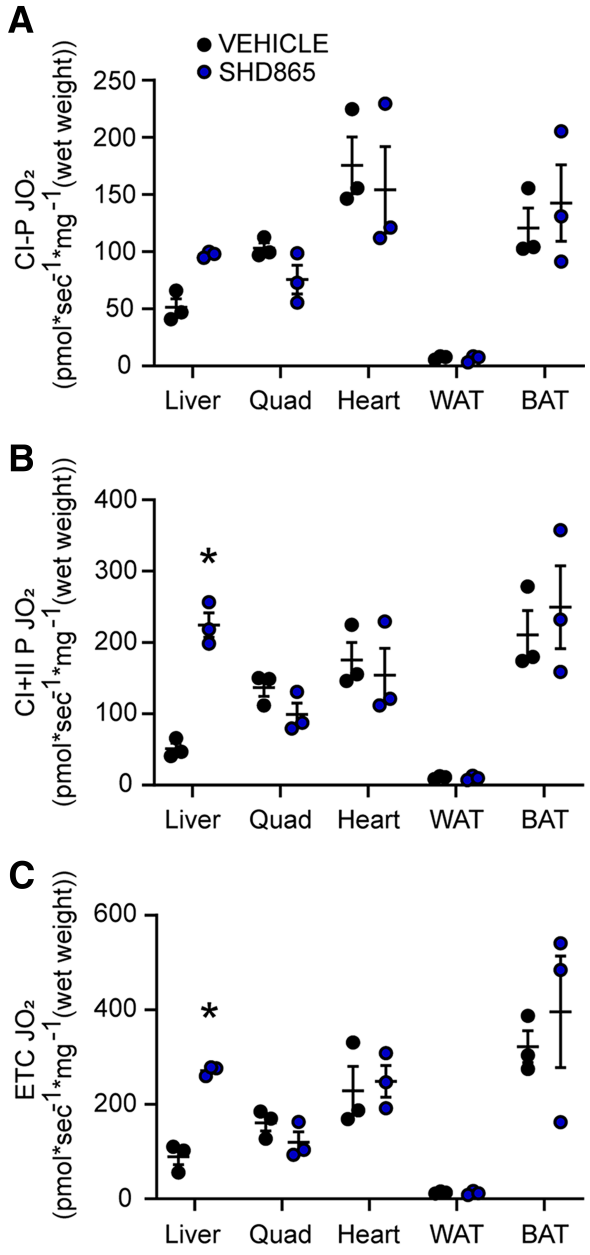

Figure 3—Liver-specific mitochondrial respiration in response to SHD865. Tissue-specific oxygen consumption measured by OROBOROS assay, 2-h after oral gavage of 30 mg/kg SHD865 or vehicle. High-resolution respirometry is represented for liver, quadriceps (Quad), heart, white adipose tissue (WAT), and brown adipose tissue (BAT) normalized on tissue wet weight. Respiration supported via ADP-stimulated respiration complexes I and II (CI-P) (*A*), CI+IIP (*B*), and the maximal capacity of the electron transport chain (ETC) is represented (*C*). Values are represented as mean ± SEM (n = 3). JO_2, rate of oxygen consumption. *$P < 0.05$ compared with vehicle group, determined by two-way repeated-measures ANOVA, followed by the Šidák multiple comparison post hoc test.

Four weeks of WD feeding resulted in a 16% gain in body weight and more than doubling of body fat mass as detected by EchoMRI (Fig. 4*A* and *C*). Within the first 2 weeks of SHD865 treatment, mice recovered normal body weight and body fat mass composition similar to the lean chow-fed control mice (Fig. 4*A* and *C*). From weeks 2–6 of the intervention period, SHD865-treated mice experienced a plateau in fat loss and maintained body composition that was similar to normal chow-fed control animals, despite their continued intake of the WD and no change in SHD865 concentration in the food. Importantly, SHD865-induced fat loss occurred without a decrease in food intake (Fig. 4*B*) or loss of lean mass body composition (Fig. 4*D*). EchoMRI data and terminal weight of gonadal fat pad mass showed that SHD865 treatment completely reversed the WD-induced fat gain (Fig. 4*E* and *F*). Weight loss was not due to lipid malabsorption as SHD865 treatment did not alter fecal triglyceride levels compared with the WD-fed mice (Supplementary Fig. 5).

Glucose Tolerance and Insulin Resistance Are Improved by SHD865

We next investigated the effect of SHD865 treatment on glucose tolerance and insulin sensitivity. A GTT was performed at weeks 4 (before SHD865 treatment) and 9 (after 5 weeks of SHD865 treatment). The GTT at week 4 showed that WD feeding significantly impaired glucose tolerance compared with chow-fed control mice (Fig. 5*A*), as expected. The second GTT at week 9 showed that SHD865 treatment completely reversed glucose intolerance compared with pretreatment and WD-fed control mice (Fig. 5*B*). SHD865-treated mice had a similar glucose tolerance area under the curve as chow-fed control mice (Fig. 5*C*). The improved glucose tolerance observed in SHD865-treated mice was associated with a decrease in random-fed blood glucose (Fig. 5*D*) and insulin levels (Fig. 5*E*) compared with WD-fed controls. Insulin levels were not measured during the glucose tolerance test in this first cohort of mice, so we repeated the study in a second cohort of mice, where SHD865 again improved glucose tolerance (Supplementary Fig. 6*A* and *B*), and insulin levels remained 20–30% lower in SHD865-treated mice compared with WD-fed controls (Supplementary Fig. 6*C*). These data suggest that SHD865 improved insulin sensitivity rather than increased β-cell function.

SHD865 Ameliorates Diet-Induced Lipid Accumulation

Postprandial hyperlipidemia is a phenotype of excess adiposity associated with insulin resistance (20). Therefore, we next evaluated plasma triglyceride and cholesterol levels to assess potential effects of mitochondrial uncoupling on mouse lipid profile following WD feeding. Importantly, SHD865-induced mitochondrial uncoupling significantly lowered postprandial plasma triglyceride and cholesterol compared with pretreatment measurements (Fig. 6*A* and *B*). Given that the liver is a key source of endogenous triglyceride and cholesterol, we next examined liver cholesterol and triglyceride content. Mice treated with SHD865 for 6 weeks had an intermediate phenotype where liver triglyceride levels were between those of chow-fed and WD-fed mice without being statistically different from either group, while

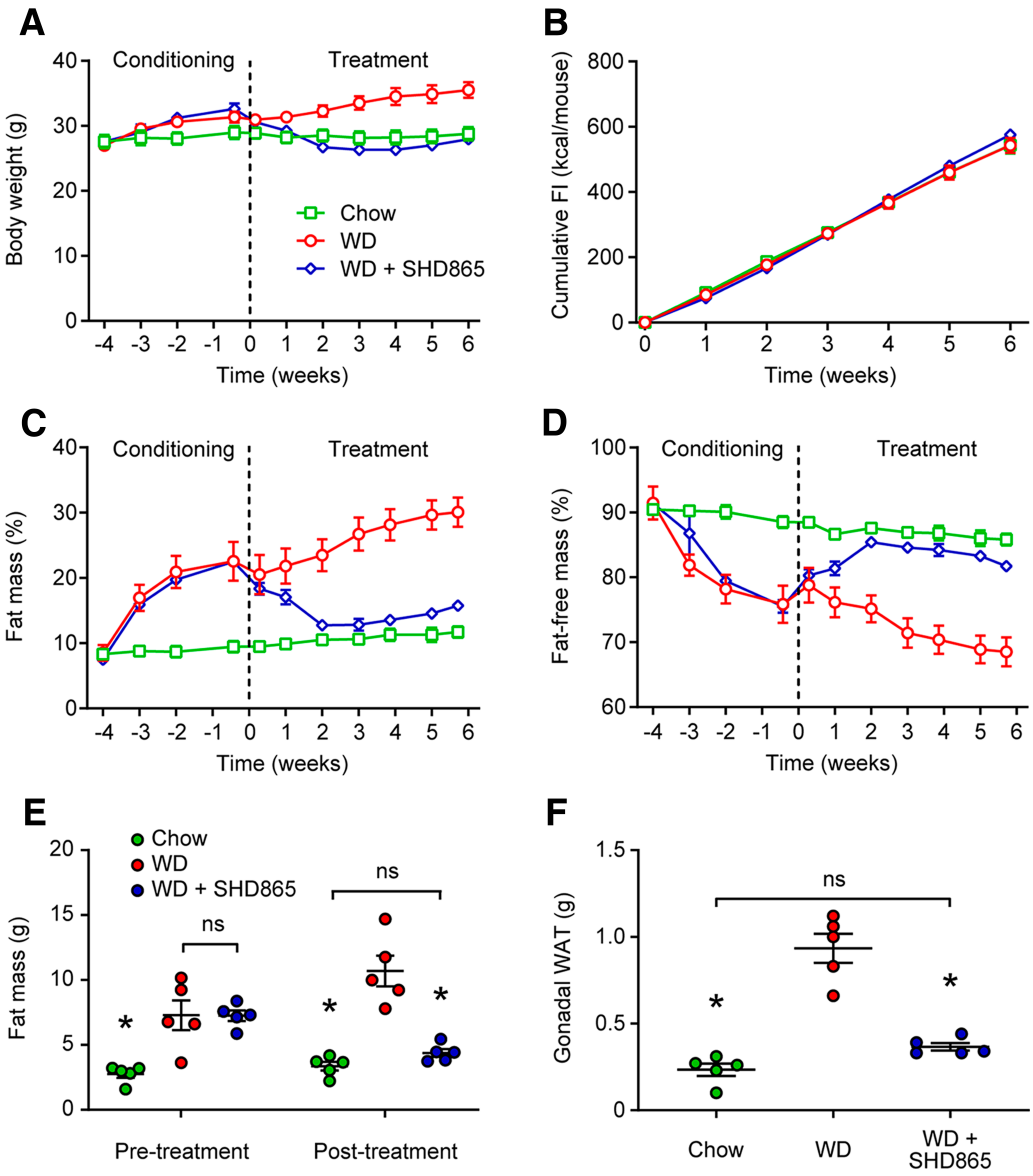

Figure 4—Compound SHD865 reverses diet-induced obesity without affecting food intake. Body weight (*A*) and cumulative food intake (FI) (*B*) measured over time. Measurements of fat (*C*) and lean mass (*D*) over time indicated as a percentage of body weight. *E*: Fat mass grams represented as before and after treatment. *$P < 0.05$ compared with WD, determined by two-way repeated measures ANOVA, followed by the Tukey multiple comparison post hoc test. *F*: Ex vivo gonadal white adipose tissue (WAT) fat pads evaluated at euthanasia. Values are represented as mean ± SEM ($n = 5$). *$P < 0.05$ compared with WD, determined by one-way ANOVA, followed by the Dunnett multiple comparison post hoc test.

mice fed SHD865 had liver cholesterol concentrations that were lower than WD-fed control mice and similar to chow-fed mice (Fig. 6*C* and *D*). Hematoxylin and eosin staining of liver tissue also showed evidence of decreased lipid droplet accumulation in liver tissue, as visualized by clear cytoplasmic spaces that were occupied by lipid prior to sectioning and staining (Fig. 6*E*). WD feeding also resulted in a twofold increase in skeletal muscle triglyceride content that was completely normalized in mice fed SHD865 (Supplementary Fig. 7).

We investigated the postprandial serum triglyceride-lowering effect of SHD865 in the second cohort of mice by assessing triglyceride clearance by oral triglyceride tolerance test (OTTT) and hepatic triglyceride production using poloxamer 407. However, neither experiment showed a phenotype for mice fed SHD865 in WD-fed versus WD-fed controls (Supplementary Fig. 8). However, the data revealed that 4–5 h fasting prior to the OTTT or poloxamer 407 treatments normalized circulating triglycerides to demonstrate that elevated triglyceride levels in WD-fed control mice are only present in the fed state.

We further investigated the mechanism for SHD865 effects on liver triglyceride content by assessing protein expression and regulatory phosphorylation of rate-limiting

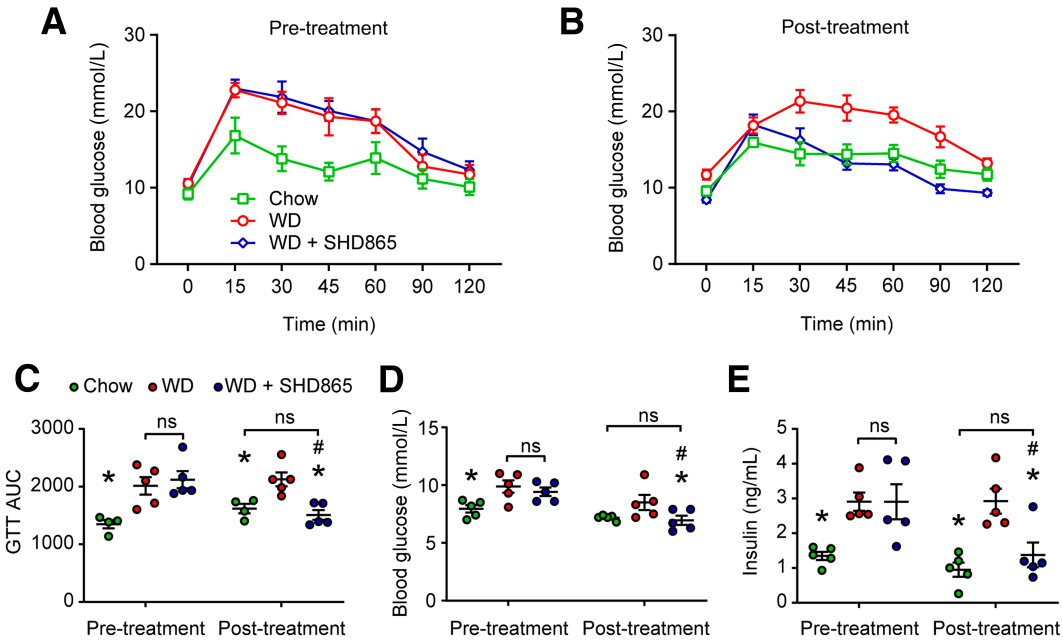

Figure 5—Compound SHD865 normalizes glucose tolerance and insulin sensitivity in mice fed the WD. GTT was performed pre- (*A*) and posttreatment (*B*). *C*: Total area under the curve (AUC) pre- and posttreatment. Random-fed (RF) blood glucose (*D*) and plasma insulin (*E*) measurements pre- and posttreatment. Values are represented as mean ± SEM ($n = 5$). ns, not significant; *$P < 0.05$ compared with WD, determined by two-way repeated-measures ANOVA, followed by the Dunnett multiple comparison post hoc test; #$P < 0.05$ compared with pretreatment measurements, determined by two-way repeated-measures ANOVA, followed by the Šidák multiple comparison post hoc test.

enzymes for de novo lipogenesis, including acetyl-CoA carboxylase (ACC), inhibitory pSer79-ACC, fatty acid synthase (FAS), and total and activated (pThr172) AMPK, which is the upstream kinase for pSer79-ACC. These data revealed that mice fed SHD865 in the WD had lower FAS and ACC expression than WD-fed control mice, with a trend for increased Ser79 phosphorylation of ACC that is consistent with increased phosphorylation of AMPK-Thr172, the major kinase responsible for Ser79-ACC phosphorylation (Supplementary Fig. 9*A*–*E*). Together, these data suggest that mice fed SHD865 in the WD have lower capacity for lipogenesis compared with control mice fed the WD. We also investigated whether SHD865 altered fatty acid oxidation capacity by assessing enzyme activity of β-hydroxy-acyl-CoA dehydrogenase (HAD) and medium-chain acyl-CoA dehydrogenase (MCAD). However, no treatment had any effect on MCAD activity, while HAD activity was decreased by 12% versus WD-fed control (Supplementary Fig. 9*F* and *G*).

Blood ALT is as a marker of liver damage; therefore, we assessed ALT levels in both cohorts of mice treated with SHD865. We found that 10 weeks of the WD had little effect on ALT levels compared with chow-fed mice and that drug treatment with SHD865 for 6 weeks did not raise ALT levels (Supplementary Fig. 10). Finally, we determined SHD865 concentrations in tissues collected at study termination to better understand how the observed phenotypes correlated with steady-state tissue distribution. SHD865 was primarily localized to adipose and liver tissue depots, with lesser exposure in quadriceps muscle, heart, and brain (Supplementary Fig. 11). These data suggest that tissue distribution is not directly correlated with bioactivity in adipose tissue, potentially due to sequestration in lipid droplets; thus, liver-selective bioactivity is not due exclusively to distribution.

DISCUSSION

In this work, we characterized the pharmacokinetics and demonstrated the in vivo efficacy of mitochondrial uncoupler SHD865 to reverse diet-induced adiposity and glucose intolerance in mice without affecting energy intake or lean mass. SHD865 is a derivative of BAM15, a mitochondrial protonophore uncoupler that we have recently shown to prevent and reverse excess adiposity in mouse models (13,21). However, compared with BAM15, SHD865 is a milder mitochondrial uncoupler in vitro that increases mitochondrial respiration to ∼90% of maximal capacity while maintaining high levels of mitochondrial respiration over a broad drug concentration dosing range without causing mitochondrial failure. Furthermore, SHD865 had significantly less impact on plasma membrane potential than FCCP, indicating that it confers better on-target action compared with FCCP and previously reported uncouplers (11). Together, these in vitro data provided evidence that SHD865 may have a good safety profile in vivo and justified further testing in mice.

Oral administration of SHD865 in C57BL/6J mice resulted in improved tolerability compared with BAM15 as we observed no adverse effects of SHD865 after an acute

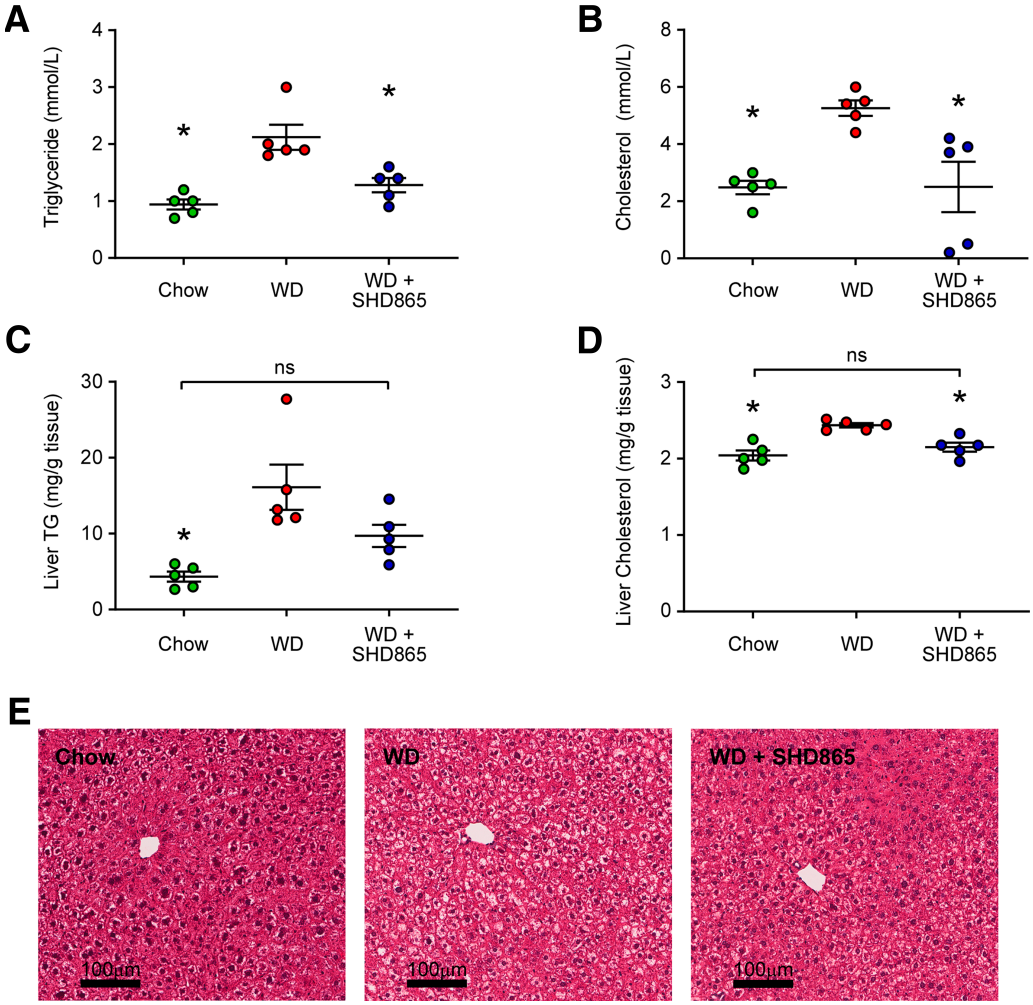

Figure 6—SHD865 improves plasma markers of hyperlipidemia and normalizes liver lipids to levels similar to chow-fed controls. Plasma triglyceride (TG) (*A*) and cholesterol (*B*) content at the end of treatment. Liver TG (*C*) and cholesterol lipid levels (*D*) at the end of the study. Values are represented as mean ± SEM ($n = 5$). ns, not significant; *$P < 0.05$ compared with WD, assessed by one-way ANOVA, followed by the Dunnett multiple comparison test or Kruskal-Wallis test for nonparametric data. *E*: Representative images of hematoxylin and eosin staining. Scale bar = 100 μm.

oral bolus of 1,000 mg/kg while the maximum amount of BAM15 that could be tested was 200 mg/kg due to poor solubility (13). Similar to BAM15, we found that SHD865 reversed adiposity in mice and restored glucose tolerance to levels comparable with lean healthy controls without affecting food intake or lean body mass. The dose of SHD865 tested in the study was 0.04% w/w admixed in the diet that resulted in average daily consumption of ∼38 mg/kg, thus SHD865 had strong efficacy to improve glucose tolerance and reverse adiposity at doses 27-fold lower than the dose level that had no observed adverse effect. SHD865 also restored blood, muscle, and liver lipid profiles toward normal levels observed in lean chow-fed mice and normalized blood glucose and insulin levels.

BAM15 was tested in a very similar protocol to the one performed herein with SHD865 (13), including 4 weeks of a WD conditioning, followed by 5 weeks of treatment with BAM15 admixed in the WD; therefore, comparisons of general drug efficacy can be made with SHD865. Mice consuming the 0.1% BAM15 diet had an average daily consumption of ∼100 mg/kg, which is 2.5-fold more BAM15 than SHD865, despite nearly identical pharmacokinetics in mice. However, a limitation of the comparison is that we do not know the approximate steady-state concentration of both compounds in circulation/tissues during the treatment period. Nevertheless, 0.04% SHD865 and 0.1% BAM15 treatments had remarkably similar phenotypes, including normalization of glucose tolerance and fat pad masses to levels comparable with chow-fed controls without altering food intake or lean body mass. Both compounds had similar effects in lowering fed insulin, liver triglyceride, and fed circulating triglyceride levels.

The current study with SHD865 has gone further than previous studies with BAM15 to investigate the effect of mitochondrial uncoupling to lower liver triglyceride levels and circulating fed triglyceride levels. For example, we show

herein that mice fed SHD865 in the WD had lower expression of ACC and FAS, enzymes considered rate-limiting for lipogenesis. We also performed an OTTT and treated mice with poloxamer 407 to show that SHD865 treatment did not affect triglyceride clearance or hepatic triglyceride production. We also found that 4- to 5-h fasting was sufficient to normalize triglyceride levels so that there was no significant difference between the WD-fed and WD-fed plus SHD865-treated mice. The mechanism whereby mice fed SHD865 had decreased postprandial triglyceride levels remains unclear but is likely due to a combination of normalization of body composition, lower triglyceride content in liver, muscle, and adipose tissues, and improved insulin sensitivity.

Tissue distribution studies showed that SHD865 concentration in the liver after 6 weeks of treatment was 14.5 μg/g, ~34 μmol/L (Supplementary Fig. 11), which is in the bioactive concentration range of >10 μmol/L, as determined in vitro (Fig. 1*B*). In contrast, less compound was present in quadriceps, heart, and brain. Adipose tissues had the greatest concentrations of SHD865, with gonadal fat having 27 μg/g SHD865 and brown fat having 18 μg/g in the tissue. The lack of bioactivity in tissue adipose samples suggests that SHD865 could be sequestered by fat droplets where it is unable to act on mitochondria in those tissues. In summary, SHD865 represents a new class of small molecule mitochondrial uncoupler that has powerful effects to normalize glucose tolerance and adiposity in mice fed a WD without altering food intake or decreasing lean body mass. SHD865 has similar in vivo bioactivity as BAM15 but with improvements, including 2.5-fold lower dosage, a higher level of no observed adverse effects, and superior stability in human hepatocytes.

This is the first study to characterize the in vivo bioactivity of SHD865, so there remain some limitations and scope for further work. For example, SHD865 restored total plasma and liver cholesterol levels, but we have not assessed lipoprotein-bound fractions or assessed cholesterol biosynthesis and metabolism. In this study, we investigated WD feeding over a period of 10 weeks, but further work should investigate SHD865 efficacy in other mouse models of excess adiposity, including long-term WD and genetic obesity models including *db/db* or *ob/ob* mice. Understanding these unknowns and progressing SHD865 into more advanced toxicology testing will be necessary to determine whether SHD865 represents a drug candidate for human testing.

Acknowledgments. The authors thank Joan Mannick, Jerry McLaughlin, and Sharon Rosenzweig-Lipson (Life Biosciences) for advice and discussions concerning SHD865. This work was also possible through the help of animal facility services staff for weekend monitoring of mice and maintenance of equipment in the Biological Resources Facility and Imaging Laboratory, the Bioanalytical Mass Spectrometry Facility, the Katharina Gaus Light Microscopy Facility, and the Mark Wainwright Analytical Centre of UNSW.

Funding. This work was supported in part by the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Disease Center for Scientific Review (R01DK128612), and the Australian National Health and Medical Research Council (GNT2014079). D.P.S., S.-Y.C., and S.J.A. were supported by UNSW Scientia PhD Scholarships.

Duality of Interest. This study received support from Life Biosciences. K.L.H., W.L.S., J.M.C., and M.W. declare a financial interest in Life Biosciences. K.L.H. and W.L.S. declare a financial interest in Uncoupler Biosciences. S.P.T. declares a financial interest in Firebrick Pharma and Life Biosciences. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. M.B. coordinated and led the experiments. M.B., E.M.O., C.S.V., D.P.S., S.-Y.C., S.J.A., and C.L. performed all animal experiments conducted at UNSW. N.T. provided key reagents for the fatty acid oxidation assays. M.B., F.L.B., and K.L.H. wrote the manuscript, with editing by N.T., Y.D. and J.A.S.-R. performed synthesis of SHD865. A.M.P. and A.P. performed OROBOROS experiments. B.L. performed tail vein injection. S.R.H. performed OCR measurements in vitro in the laboratory of T.E.H. S.R.H. and T.E.H. validated and interpreted the OCR measurements. K.L. conducted the whole-cell current clamp experiments in the laboratory of D.A.B. K.L. and D.A.B. validated and interpreted the whole-cell current clamp experiments. M.W. and W.L.S. designed and directed synthesis of SHD865. J.M.C., S.P.T., W.L.S., and K.L.H. conceived and directed the project. All authors reviewed and gave approval to the final version of the manuscript. W.L.S. and K.L.H. are the guarantors of this work and, as such, had full access to all the respective data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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