Mitochondrial uncoupler SHC517 reverses diet-induced obesity and insulin resistance in mice

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Abstract

Aims/hypothesis Small molecule mitochondrial uncouplers decrease caloric efficiency and have potential therapeutic benefits for the treatment of obesity, diabetes, and related metabolic disorders. We have recently developed and characterized a small molecule mitochondrial uncoupler named SHC517 that is the most potent furazanopyrazine mitochondrial uncoupler discovered to date. Herein we investigate the hypothesis that SHC517 will safely and effectively reverse diet-induced obesity in mice.

Methods Oral bioavailability of SHC517 was assessed in C57BL/6 mice by oral and intravenous administration followed by measurement of plasma SHC517 by LC-MS/MS. SHC517 was then administered to mice as an admixture in western diet. SHC517 was tested in prevention mode where treatment was initiated simultaneous with western diet for 12 days, and in reversal mode where treatment was initiated for the final 7 weeks of an 11 week western diet feeding study. Body composition, glucose tolerance, and blood glucose and insulin levels were assessed as primary readouts of efficacy.

Results In diet-induced obesity prevention mode, mice treated with 0.05% and 0.1% (w/w) SHC517 in western diet were dose-dependently protected from fat gain compared to western diet control. In the obesity reversal study, mice fed 0.05% SHC517 in diet had 50% less body fat than controls fed western diet. In both studies, mice fed SHC517 did not have altered food intake or loss of lean mass. SHC517 treatment markedly improved glucose tolerance to levels comparable with chow-fed control mice.

Conclusions/interpretation These data demonstrate the anti-obesity and insulin-sensitising properties of the potent next-generation furazanopyrazine mitochondrial uncoupler SHC517 in mice.

Keywords: Metabolism; obesity; physiology

Abbreviations:

DNP	2,4-dinitrophenol
ESI	Electrospray Ionisation
mTORC1	Mammalian target of rapamycin complex 1
NAS	Non-alcoholic fatty liver disease activity score
NASH	Non-alcoholic steatohepatitis
NMP	*N*-Methyl-2-pyrrolidone
STAM	Stelic Animal Model
STAT3	Signal transducer and activator of transcription 3
UCP	Uncoupling protein
UNSW	University of New South Wales
WD	Western diet

Research in context

What is already known about this subject?

- The furazanopyrazine mitochondrial uncoupler BAM15 decreases caloric efficiency and results in fat loss and improved insulin sensitivity in mice.
- SHC517 is a more potent derivative of BAM15 that has beneficial effects against non-alcoholic steatohepatitis in the STAM mouse model.
- SHC517 did not cause weight loss in the STAM model; however, STAM mice are relatively small type 1 diabetic animals.

What is the key question?

- Can SHC517 prevent or reverse fat accumulation and insulin resistance in a mouse model of diet-induced obesity?

What are the new findings?

- SHC517 is 100% orally bioavailable.
- SHC517 prevents and reverses diet-induced obesity in mice at half the dose previously shown effective for BAM15.
- SHC517 prevents and reverses glucose intolerance in mice.

How might this impact on clinical practice in the foreseeable future?

- The development of next generation molecules with improved potency is a step forward towards clinical translation.

Introduction

The World Health Organisation classifies normal healthy body weight as a body mass index ($BMI = \frac{Weight\ (kg)}{Height\ (m)^2}$) between 20 and 25. Overweight (BMI 25-30) and obese individuals (BMI >30) account for 39% of the global population [1], a figure which has been increasing for at least 40 years [2]. The majority of overweight or obese patients have excess fat mass that results from a high ratio of energy intake compared to energy expenditure. Overweight individuals have higher risk of developing cardiovascular disease [3, 4], type 2 diabetes [5], non-alcoholic steatohepatitis (NASH) [6], cancer [7] and psychiatric disorders [8-10].Current control measures are not sufficient to control the global obesity epidemic.

Lifestyle modifications such as diet and exercise are most ideal but are rarely successful in the long-term [11]. Bariatric surgery is most effective but is invasive, costly, and may lead to serious complications [12-14]. Currently available pharmacological interventions primarily work by decreasing energy intake or impairing nutrient absorption, but these have had limited large-scale success and sometimes have significant adverse effects [15-17]. For example, in February 2020 the anti-obesity drug Lorcaserin was withdrawn from the market due to potential increased cancer risk. Pharmacotherapies represent the most likely strategy to control obesity on a global level and there is strong need for new drug treatments with a different mechanism of action.

The anti-obesity mechanism of action pursued herein involves decreasing caloric efficiency by mitochondrial uncoupling [18]. Nutrient oxidation is normally coupled to ATP production via a proton cycle across the mitochondrial inner membrane. Nutrient oxidation drives proton pumps that efflux protons from the mitochondrial matrix against a ~10-fold proton gradient. Protons then re-enter the mitochondrial matrix via ATP synthase to produce ATP. Mitochondrial uncoupling occurs when protons are transported into the mitochondrial matrix independent of ATP synthase. This is as a natural process – an estimated 20-25% of nutrient metabolism in the whole body is uncoupled from ATP production, in part due to the action of uncoupling proteins (UCPs) [19]. This study uses chemical mitochondrial uncouplers which are protonated at normal pH but become deprotonated as they enter the alkaline mitochondrial matrix. They cycle across the mitochondrial inner membrane to result in a net transport of protons into the matrix independent of ATP synthase. Mitochondrial uncoupling can result in weight loss because more nutrients are oxidised to produce a given amount of ATP [20]. Lowering the proton gradient also has anti-oxidant effects because mitochondrial superoxide production rate is positively correlated with the strength of the proton gradient [21].

Early human studies in the 1930's with 2,4-dinitrophenol (DNP) demonstrate proof-of-concept for mitochondrial uncoupling as a weight loss strategy. DNP caused significant weight loss [22, 23] but was banned by the FDA in 1938 due to the occurrence of adverse side effects including cataracts, hyperthermia, and sometimes death [24-26]. DNP has a narrow therapeutic range between effective doses and toxic doses [22, 27], possibly due to non-mitochondrial off-target effects including plasma membrane depolarization [28-31].

We previously identified the mitochondrial uncoupler BAM15 ((2-fluorophenyl)(6-[(2-fluorophenyl)amino](1,2,5-oxadiazolo[3,4-e]pyrazin-5-yl))amine) as a mitochondria-specific protonophore uncoupler [32]. BAM15 prevented and reversed diet-induced obesity and

insulin resistance in mice without altering food intake or decreasing lean body mass [33]. In a recent structure-activity relationship study we identified a more potent derivative of BAM15 named SHC517 (N^5-(2-fluorophenyl)-N^6-(3-fluorophenyl)-[1,2,5]oxadiazolo-[3,4-*b*]pyrazine-5,6-diamine) [34] that prevents non-alcoholic steatohepatitis in the streptozotocin-induced diabetes Stelic animal model (STAM) [34]. STAM mice are lean diabetic animals and SHC517 did not alter body composition in this model.

To better understand whether SHC517 has beneficial effects on obesity-related metabolic dysfunction, we investigated SHC517 in a diet-induced obesity model. Herein we fed C57BL/6J mice a physiologically relevant high-fat (45% by kCal) diet that replicates many features of human metabolic syndrome including increased fat mass and insulin resistance. We show that SHC517 prevents and reverses diet-induced obesity and has insulin sensitising effects at a dose 2-fold lower than previously published for BAM15 [33]. Consistent with BAM15, SHC517 lowered body fat mass without altering lean mass or food intake.

Methods

Animal husbandry

All mouse experiments conducted at UNSW were approved by the UNSW Animal Care and Ethics Committee (project approval 17/66B). Age-matched male C57BL/6J mice were purchased from Australian BioResources (Moss Vale, NSW, Australia) and were used in the studies as indicated. Mice were housed in specific pathogen free conditions at 22°C in a light/dark cycle of 12 hours. Mice were housed in groups of 5 prior to treatment, at which point they were single-housed as described below. Mice were monitored as per ethical guidelines, including body weight and composition measurements as described below. Unless otherwise stated, mice were provided with *ad libitum* access to water and standard chow diet (Gordons Specialty Feeds, NSW, Australia).

Animal diets

Western diet (45% fat and 16% sucrose by kCal, based on Research Diets D12451) was prepared in-house as described in [35]. Ingredients were sourced from local suppliers including sucrose (JL Stewart GRAD25B, Glendenning, NSW, Australia), corn starch (JL Stewart CFLR25W, Glendenning, NSW, Australia), wheat bran (JL Stewart BRAN10UF, Glendenning, NSW, Australia), casein (Ross Cottee, Sydney, NSW, Australia), methionine (Sigma M9500, Castle Hill, NSW, Australia), gelatine (JL Stewart GEL2, Glendenning, NSW, Australia), choline bitartrate (Sigma C1629, Castle Hill, NSW, Australia), lard (JL Stewart LARD15, Glendenning, NSW, Australia), safflower oil (Proteco, Kingaroy, QLD, Australia), trace minerals (MP Biomedicals 0296026401, Seven Hills, NSW, Australia), AIN-93M mineral mix (MP Biomedicals 0296040102, Seven Hills, NSW, Australia), and AIN-93-VX vitamin mix (MP Biomedicals 0296040201, Seven Hills, NSW, Australia).

Oral bioavailability study

Pharmacokinetic assessment was performed by GVK Biosciences (Hydrabad, India). Animal studies performed by GVK Biosciences were approved by its Institutional Animal Ethics Committee. Swiss Albino mice were administered 1 mg/kg SHC517 intravenously (in NMP (10%) + 10% Solutol (v/v) in water) or 10 mg/kg by oral gavage (0.5% w/v methylcellulose (93% v/v), 2% v/v Tween-80, and 5% v/v DMSO). Blood samples were collected at time

points indicated in Figure 2, with three replicates (representing individual animals) per time point. Plasma was separated by centrifugation. 50 µL of plasma was precipitated with 200 µL of acetonitrile containing internal standard of Telmisartan at 200 ng/mL. Samples were vortexed for 5 min at 850 rpm and centrifuged at 4000 rpm for 5 min at 4 °C. From this, 110 µL of supernatant was diluted with 150 µL of methanol:water (1:1, v/v). Calibration standards were prepared using 2.0 µL of calibration curve standard added to 48 µL blank matrix, which was processed in the same way as 50 µL of sample plasma. Liquid chromatography tandem mass spectrometry was performed on an API 4000 LC-MS/MS system (SCIEX, Framingham, MA, USA). Chromatographic separation was achieved using an XBridge, C18, 50x4.6 mm, 3.5µ column (Waters, Milford, MA, USA). Mobile phase A consisted of 0.1% v/v formic acid in HPLC grade water. Mobile phase B consisted of 100% methanol. The analyte was eluted with a gradient of 5-95% mobile phase B at a flow rate of 1 mL/minute with 10 µL injection volume. Electrospray Ionisation (ESI) was performed in negative mode. Transitions of *m/z* 339.2 > 157.9 and 1.91-minute retention time was used to identify SHC517, and *m/z* 513.1> 287.1 and 1.72-minute retention time was used to identify Telmisartan. Concentration of test samples was interpolated from a standard curve derived from the intensity values of standards (1-1000 ng/mL).

Fasting insulin measurements

Fasted bloods (5 µL) were collected from a tail nick following a 5-hour fast from 9AM to 2 PM. Whole blood samples were directly added to the Crystal Chem Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc. 90080, Chicago, IL, USA). Insulin analysis was conducted according to manufacturers' instructions except that test samples and standards were incubated overnight at 4°C. As the mouse insulin standard is in ng/mL, this was converted to pmol/L using a molar mass of 5808 Da.

Hepatic lipids

Frozen liver tissue was powdered in liquid nitrogen using a tissue pulverizer (Cellcrusher, Cork, Ireland). Lipids were extracted using a modified version of the Folch method [36]. In brief, 25 mg of frozen powdered tissue was weighed out and vortexed in two parts chloroform (533 µL) and one part methanol (267 µL). Samples were sonicated for 10 minutes then digested on a rocker at room temperature for 1 hour. 400 µL of 0.9% sodium chloride was added and samples were vortexed for 30 seconds. Samples were centrifuged at 3000 rev/min at room temperature and the bottom layer was extracted. The lipid extract was dried under a steady stream of nitrogen in a TurboVap® Evaporator (Biotage, Uppsala, Sweden), then resuspended in 0.4 mL 95% (v/v) ethanol and heated to 37°C prior to lipid assays. Triglyceride levels were measured by a colorimetric assay through reaction with GPO reagent (Pointe Scientific T7532, Canton, MI, USA) according to the manufacturer's protocol, using glycerol standard (Sigma G7793, St Louis, MO, USA). Cholesterol levels were measured by a colorimetric assay using Infinity cholesterol liquid stable reagent (ThermoFisher TR13421) according to the manufacturer's protocol, using cholesterol standard (Pointe Scientific C7509, Canton, MI, USA).

Phenotyping study design

For the obesity-prevention study, 16-week-old male C57BL/6J chow-fed mice were stratified by baseline body composition and glucose tolerance to ensure similar starting parameters.

Glucose tolerance was assessed by intraperitoneal glucose injection of 2 g/kg lean body mass following a 5 hour fast from 9 AM to 2 PM. Mice were then single-housed to ensure accurate monitoring of food intake. Mice were either maintained on chow or switched to WD or WD containing SHC517 at 0.05% and 0.1% (w/w) for 12 days. EchoMRI was used to measure changes in fat and lean mass over 10 days and a glucose tolerance test was repeated on day 11. Mice were euthanised by cervical dislocation and harvested tissues were frozen in liquid nitrogen prior to storage at -80°C.

For the obesity-reversal study, 12-week-old male C57BL/6J mice were fed either chow diet or WD for 4 weeks. All mice were assessed for body composition on a weekly basis by EchoMRI and glucose tolerance testing was performed at the end of the 4 weeks' conditioning period. WD-fed mice were then single-housed and stratified into 2 groups based on body composition and glucose tolerance with 5 male mice per group. One group of mice continued to be fed WD while the other group was fed WD containing 0.05% (w/w) SHC517. Food intake and body weight were measured daily. After 6 weeks of treatment, glucose tolerance testing was repeated. After 7 weeks, mice were anaesthetised with isoflurane and exsanguinated by retroorbital bleeding then euthanised by cervical dislocation.

A surrogate insulin resistance index was calculated based on the method employed in rats in [37]. The product of fasting glucose in mg/dL and fasting insulin in µU/mL was divided by a constant, assuming that baseline 16-week-old male mice from the obesity-prevention study had an average resistance index of 1, which is analogous to HOMA-IR in humans [38]. The equation was as follows: Resistance index= $\frac{\text{Fasting glucose (mg/dL)} \times \text{Fasting insulin (µU/mL)}}{1533.9}$.

Statistical analyses

All data points were collected from discrete biological replicates and are presented as the mean ± SEM. Power calculations were conducted using G*Power (v.3.1.9.7; Heinrich Heine Universität Düsseldorf) designed with 80% power and 5% alpha (95% significance). For glucose tolerance testing, data points were excluded from mice that did not receive a successful i.p. injection of glucose (one mouse in the chow group in the reversal study). Significance was determined to be reached when $p < 0.05$ using Prism (v.8.4.2; GraphPad Software).

For normally distributed data, multiple groups were analysed by One-Way ANOVA with Dunnett's *post-hoc* correction. For non-parametric data, multiple groups were analysed by Kruskal-Wallis test with Dunn's *post-hoc* correction. For multiple groups measured over time, analysis was performed by Two-Way Repeated Measures ANOVA with Dunnett's *post-hoc* correction. Normality was assessed by the Shapiro-Wilk test.

Results

Oral bioavailability and toxicity studies

SHC517 is an isomer of BAM15 that differs by an unsymmetrical fluoro-substituted aniline (Figure 1). To determine the optimal mode of SHC517 delivery to mice, we first investigated oral bioavailability. SHC517 was administered by i.v. and p.o. routes and found to have >100% oral bioavailability based on dose-normalised area under the curve. Oral administration of 10 mg/kg SHC517 resulted in a maximal plasma concentration (C_{max}) of 33.4 µM and a half-life ($T_{1/2}$) of 44 min (Figure 2). In comparison to BAM15, the C_{max} of

SHC517 was 2-fold greater while the $T_{1/2}$ was 103 minutes shorter [33]. Despite the short half-life, SHC517 showed evidence of a second phase exposure bump at 2 hours that may be due to enterohepatic recirculation to account for bioavailability greater than 100%, and bioactive concentrations in circulation for up to 8 hours. In contrast, BAM15 oral bioavailability was 67% and did not show a second phase [33].

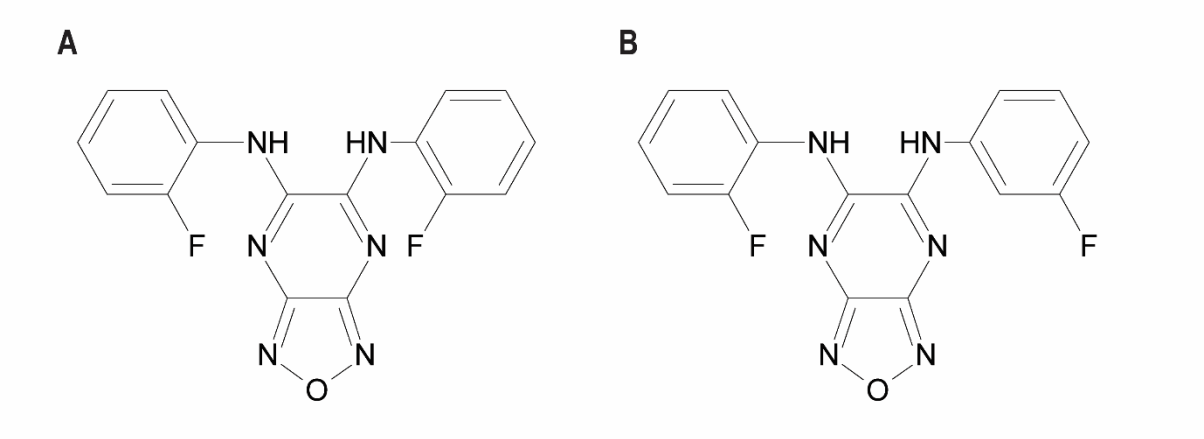

Figure 1. Chemical structure of mitochondrial uncouplers. Illustrations of BAM15 (A) and SHC517 (B).

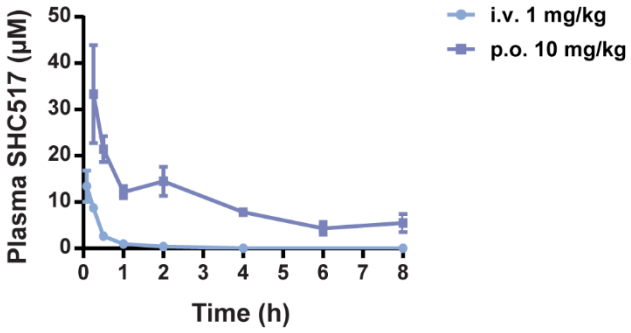

Figure 2. SHC517 is orally bioavailable. Oral bioavailability of SHC517 was assessed by i.v. and p.o. administration in male Swiss Albino mice. Blood plasma was collected at the time points shown and analysed by LC-MS/MS. n=3 male mice per group.

Obesity-prevention study

The anti-obesity effect of SHC517 was investigated by providing SHC517 to mice fed a moderate western diet (WD) containing physiologically relevant concentrations of fat (45%

by kCal), sucrose (16% by kCal, with the remainder of carbohydrate as corn starch), and protein (20% by kCal). SHC517 was delivered to mice as an admixture to WD at concentrations of 0.05% or 0.1% (w/w). One control group was fed WD without drug. Another group was fed normal chow diet and represented a benchmark for normal physiology. Mice were single-housed to enable accurate measurement of food intake.

Over 12 days of treatment, EchoMRI body composition analysis showed that mice fed WD gained an average of 2.5 g fat mass, mice fed WD containing 0.05% SHC517 gained an average of 0.5 g fat mass, and mice fed WD containing 0.1% SHC517 lost an average of 0.4 g fat mass (Figure 3a). At the end of this study period, the WD control mice had nearly 2-fold more body fat mass than mice fed WD containing 0.1% SHC517 (18.7% vs 9.9% body fat, respectively) (Figure 3b).

Fat pad masses were analysed after euthanasia on treatment day 12. Mice fed either concentration of SHC517 had fat pad masses that were statistically similar to chow-fed mice, and mice fed WD containing 0.1% SHC517 had significantly smaller gonadal and retroperitoneal fat depot masses than WD controls (Figure 3c). Changes in fat-free mass were not statistically different in mice fed 0.05% or 0.1% SHC517 in WD, indicating that these mice maintained normal lean body composition compared to chow-fed controls (Figures 3d-e). Mice fed WD containing SHC517 did not have altered food intake compared to WD control mice (Figure 3f). Mice fed WD containing 0.1% SHC517 had 53% less liver triglyceride content than WD controls ($p=0.030$) (Figure 3g); however, SHC517 treatment did not alter liver cholesterol levels (Figure 3h).

We next investigated the effects of SHC517 treatment on glucose homeostasis. WD-fed mice were significantly less glucose tolerant than chow controls, while SHC517 treatment resulted in a dose-dependent improvement in glucose tolerance and fasting blood glucose compared to WD-fed mice (Figures 4a-d). No significant differences in fasting blood insulin concentrations were observed, although there was a general trend towards decreased insulin in SHC517-treated mice (Figure 4e). A surrogate insulin resistance index was calculated as the product of fasting glucose and fasting insulin divided by a constant (1533.9), assuming that the average baseline index equals 1. This index, which incorporates both glucose and insulin levels, was necessary to assess insulin resistance in individual mice while avoiding misrepresentation of animals with normal glucose tolerance but considerable hyperinsulinaemia or vice versa. The insulin resistance index was increased in WD-fed mice compared to chow controls, but was improved by SHC517 in a dose-dependent manner (Figure 4f).

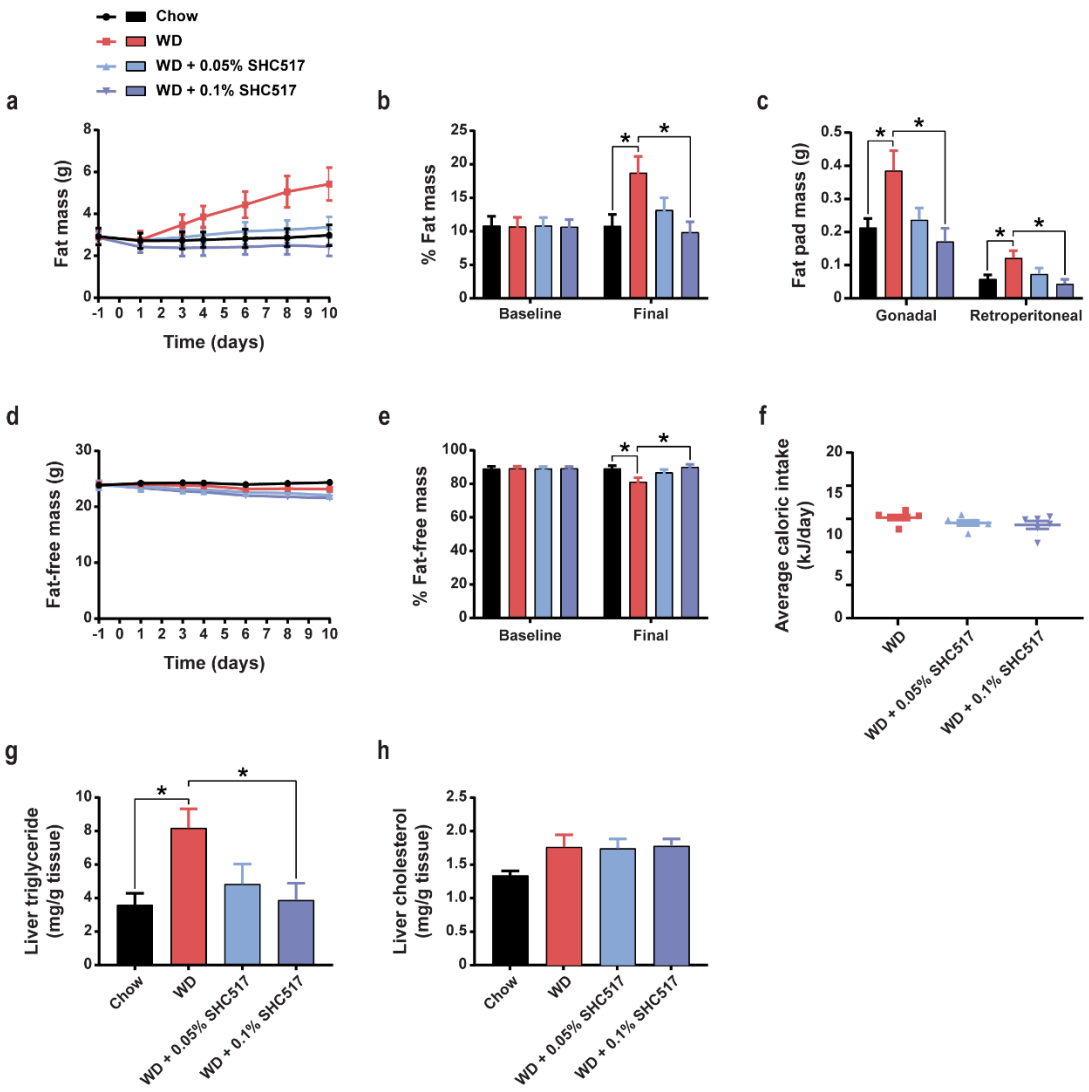

Figure 3. SHC517 prevents diet-induced obesity and fatty liver. Mice were fed WD only or WD containing SHC517 at 0.05% or 0.1% (w/w) for 12 days. Body composition was assessed by EchoMRI analysis of fat mass (a) and percent body fat (b). Fat pad wet weights were recorded after euthanasia (c). EchoMRI-based fat-free mass (d) and percent fat-free mass over total body mass (e). Average energy intake over the course of the study (f). Liver triglyceride content (g) and liver cholesterol content (h) derived from liver tissue after euthanasia. * indicates $p<0.05$ compared to WD, assessed by One-Way ANOVA (c, f, h) or Kruskal-Wallis test for non-parametric data (g). For multiple time points, Two-Way Repeated Measures ANOVA was employed (b, e). n=6 male mice per group.

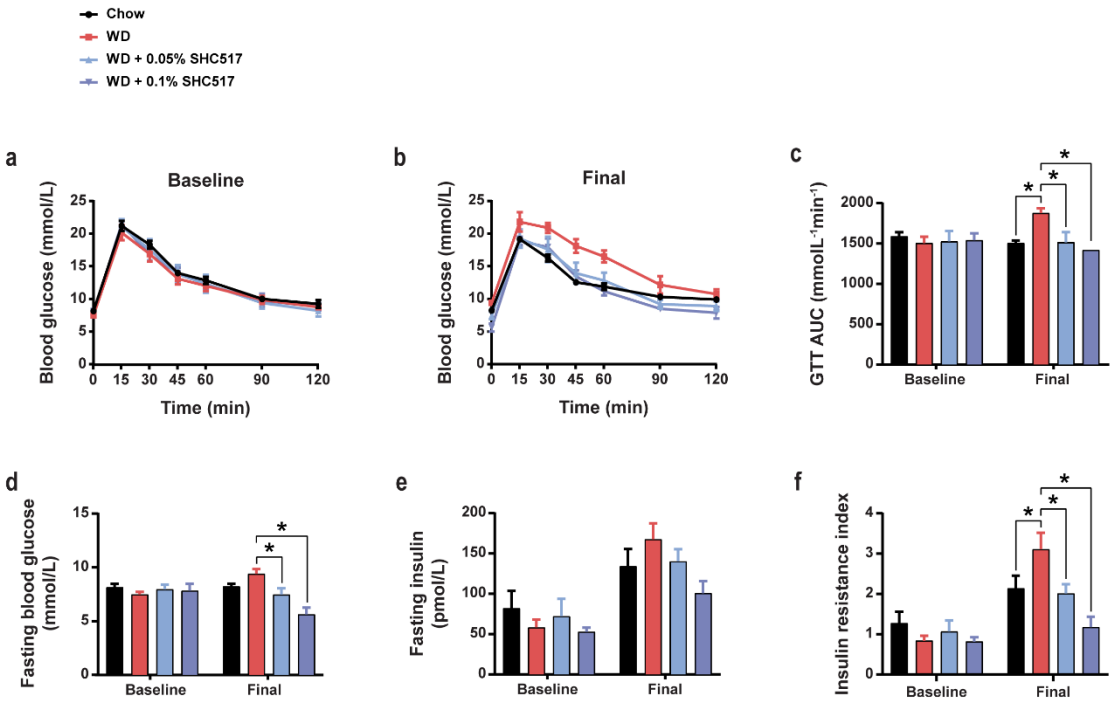

Figure 4. SHC517 prevents diet-induced glucose intolerance. Glucose tolerance testing for the obesity-prevention study was conducted prior to treatment (baseline) and at day 11 (final), shown as glucose curves (a-b) and area under the curve (c). Fasting blood glucose and insulin levels were recorded prior to treatment and at day 11 (d-e). The insulin resistance index was calculated based on fasting glucose and insulin levels (f). * indicates $p<0.05$ compared to WD, assessed by Two-Way Repeated Measures ANOVA. n=6 male mice per group.

Obesity-reversal study

We next investigated whether SHC517 could reverse diet-induced obesity. The lower dose of 0.05% SHC517 in WD was selected for this experiment because it was effective in the prevention study and 2-fold lower than the dose of BAM15 previously published to reverse obesity [33]. Male C57BL/6J mice were single-housed and conditioned by WD feeding for 4 weeks before being stratified into two equal groups that were either maintained on WD or switched to WD containing 0.05% (w/w) SHC517. A third group of age-matched mice were fed chow diet as a control for normal physiology. By 7 weeks of treatment (11 weeks of WD feeding), EchoMRI measurements of body composition showed that WD control mice gained 3.9 grams of fat while mice fed WD containing 0.05% SHC517 lost 1.9 grams of fat mass (Figure 5a), a net difference of 5.8 grams. Mice fed WD containing 0.05% SHC517 had 17.5% total body fat, while WD controls averaged 30.7% body fat (75% more body fat in the WD group, p-value = 0.0012) (Figure 5b). Fat depots measured at necropsy showed that SHC517 decreased the masses of gonadal and retroperitoneal fat pads by over 50% compared to WD control (Figure 5c). Importantly, fat-free mass was not decreased by SHC517 treatment (Figure 5d-e) and caloric intake was also unchanged in mice fed WD containing 0.05% SHC517 compared to WD controls (Figure 5f). Mice fed SHC517 had liver

triglyceride levels statistically similar to chow-fed mice (Figure 5g) while WD-control mice had a 5.6-fold increase in liver triglyceride levels compared to chow. Liver cholesterol content was higher in the WD group compared to the chow group, while liver cholesterol in the SHC517-fed group was not statistically different from either group (Figure 5h).

Glucose tolerance tests were performed prior to the start of SHC517 treatment (pre-treatment) and at the conclusion of the study (post-treatment). SHC517 treatment completely restored glucose tolerance and fasting glucose to levels comparable to chow-treated controls, while WD-fed mice remained glucose intolerant with elevated fasting glucose levels (Figure 6a-d). There were no significant changes in fasting blood insulin (Figure 6e), but the insulin resistance index was decreased in SHC517-treated mice compared to WD controls (Figure 6f).

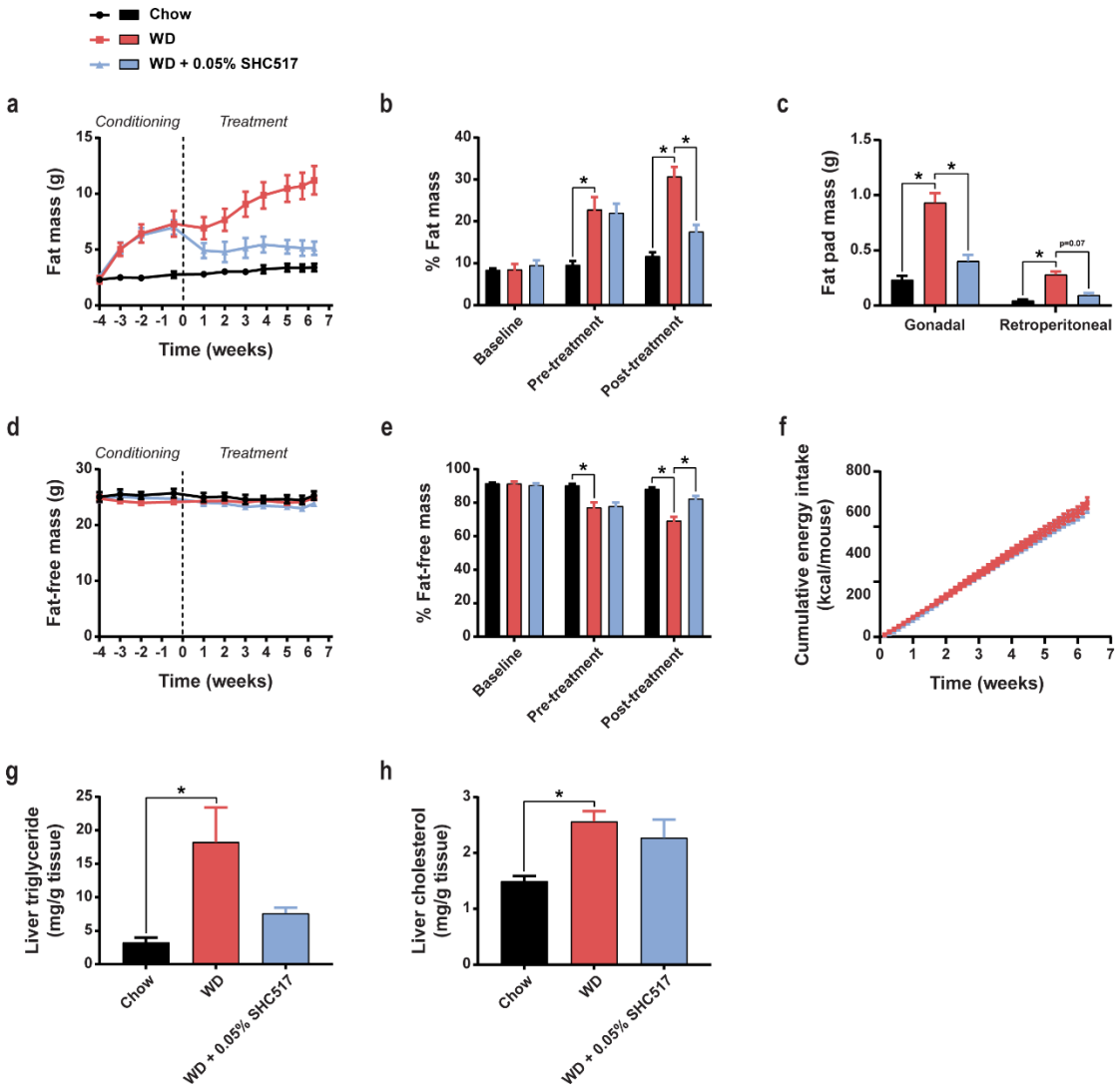

Figure 5. SHC517 reverses diet-induced adiposity. Mice were conditioned on WD for 4 weeks prior to administering SHC517 at 0.05% admixed in WD for an additional 7 weeks. Adiposity was assessed by EchoMRI body composition of fat mass (a) and percent body fat mass (b), and fat pad masses were measured at termination (c). Body composition for fat-free

mass (d) and percent fat-free mass over total body mass (e). Daily energy intake was recorded during the 7 weeks of intervention (f). Liver triglyceride content (g) and liver cholesterol content (h) were measured at termination. * indicates $p<0.05$ compared to WD, assessed by One-Way ANOVA (c, h) or Kruskal-Wallis test for non-parametric data (g). For multiple time points, Two-Way Repeated Measures ANOVA was employed (b, e, f). n=5 male mice per group.

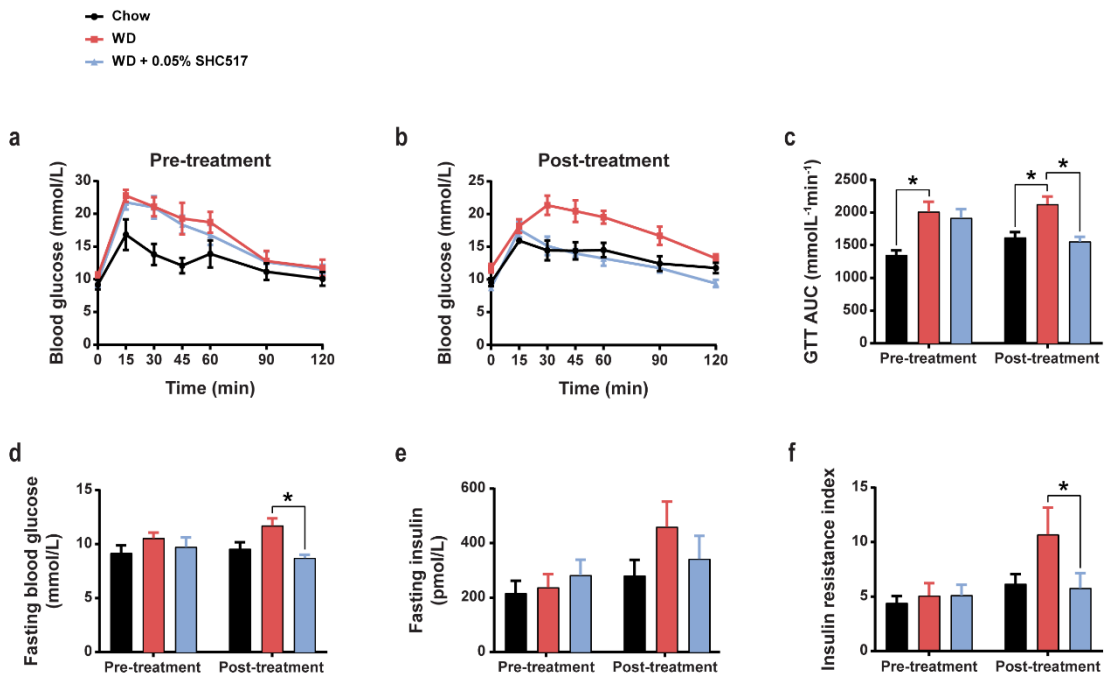

Figure 6. SHC517 reverses glucose intolerance in obese mice. Glucose tolerance testing was conducted prior to treatment (pre-treatment) and at 6 weeks post-treatment, shown as glucose curves (a-b) and area under the curve (c). Fasting blood glucose (d) and insulin (e) were determined prior to the start of treatment and at 6 weeks of treatment. The insulin resistance index was calculated based on fasting glucose and insulin levels (f). * indicates $p<0.05$ compared to WD, assessed by Two-Way Repeated Measures ANOVA. n=5 male mice per group.

Discussion

Mitochondrial uncouplers decrease caloric efficiency and therefore represent a potential therapeutic approach to reverse obesity and obesity-related metabolic disorders. It is well-established that mitochondrial uncouplers improve insulin sensitivity, but not all mitochondrial uncouplers also reverse obesity [18, 39, 40]. Considering that excess body fat accounts for up to 85% of the risk underlying diseases like type 2 diabetes [41], it is important to simultaneously target insulin sensitivity and its underlying driver of excess body fat.

We have previously published that mitochondrial uncoupler BAM15 reverses obesity and insulin resistance in mice fed high fat diet [33]; however, it remains unclear whether the BAM15 derivative SHC517 has similar efficacy. SHC517 differs from BAM15 in its 2-fold greater potency *in vitro* [34], but it also has approximately 2-fold shorter half-life. SHC517 and BAM15 have similar primary tissue distribution to liver and kidney, but SHC517 has greater exposure in white adipose tissue and less exposure to muscle and brain [33, 34].

Due to its liver localization, SHC517 was first tested in the STAM model of fatty liver disease at a dose to achieve 25 mg/kg/d (approximately 0.014 % w/w in diet). SHC517 resulted in a 0.7 point decrease in non-alcoholic fatty liver disease activity score (NAS), a change driven mostly by a decrease in inflammation [34]. SHC517 also lowered fibrosis score and plasma ALT levels in the STAM model [34]. Acute oral gavage of SHC517 did not increase body temperature when tested at doses up to 100 mg/kg [34], which is a dose greater than mice could receive in this study by eating 0.1% SHC517 in WD. The lack of increase in body temperature is an important safety signal that differentiates SHC517 from other mitochondrial uncouplers that are known to cause malignant hyperthermia with overdose, including 2,4-DNP [24]. Mitochondrial uncouplers are expected to decrease body fat mass; however, SHC517 treatment did not affect body fat composition of the lean diabetic STAM mice at the dose and duration tested.

To better understand the effects of SHC517 on obesity and insulin sensitivity in non-diabetic mice, we tested SHC517 in the same model of diet-induced obesity that we used to assess BAM15 [33]. In the obesity-prevention model, mice fed both concentrations of SHC517 were entirely protected from gaining fat mass and had adiposity that was statistically similar to chow-fed control mice. However, mice fed the higher dose of 0.1% SHC517 trended to decrease adiposity below that of chow fed mice, while mice fed 0.05% SHC517 trended to have slightly more body fat mass than chow controls. While neither dose resulted in a statistically significant change in food intake or fat-free lean mass, a slight trend towards less food intake was observed for the higher dose. Therefore, we chose to test SHC517 in the obesity reversal study at 0.05% in diet to decrease any potential for altered food intake over the 7 weeks of treatment in the reversal model.

In the reversal study, mice fed 0.05% SHC517 had similar food intake compared to western diet control animals over the course of the 7 week treatment period, but lost an average of 1.8 grams of fat mass while the mice fed western diet gained 3.9 grams of fat mass. Overall, mice fed 0.05% SHC517 had approximately half of the fat mass of western diet controls despite consuming the same quantity and type of diet. Mice fed SHC517 ended the study with statistically similar body fat and fat-free lean mass as chow-fed animals.

Comparing the effects of SHC517 with BAM15 in this model, SHC517 had similar efficacy at half the concentration [33]. Specifically, BAM15 was tested in reversal mode for 5 weeks where 0.1% BAM15 decreased fat mass by 1.98 ± 0.87 g, while we observed in this study that 0.05% SHC517 decreased fat mass by 1.73 ± 0.20 over the first 5 weeks [33]. Improved potency is beneficial in a clinical context as it would allow a patient to consume less drug while achieving the same level of effect, thus potentially lowering the chances of drug-drug interactions or off-target effects.

Glucose homeostasis was markedly improved by SHC517 in both the obesity-prevention and obesity-reversal studies where 0.05% (w/w) SHC517 was sufficient to restore glucose

tolerance and fasting glucose to normal levels observed in chow-fed mice, despite eating a very poor diet. Furthermore, SHC517 improved the insulin resistance index, which takes into account fasting glucose and insulin levels, to demonstrate that SHC517 is most likely improving insulin sensitivity rather than causing hyperinsulinaemia to compensate for insulin resistance. This is consistent with hyperinsulinaemic-euglycaemic clamp studies with BAM15 showing improved insulin sensitivity in peripheral tissues [33].

In recent years, the prospect of employing mitochondrial uncouplers as a pharmacotherapy for obesity, insulin resistance and fatty liver disease has garnered increasing confidence as a number of new molecules have demonstrated both efficacy and safety in rodent models [33, 34, 39, 40, 42-45]. Even DNP, despite its known toxicity at high doses, has been approved by the FDA to enable a clinical trial in Huntington's Disease patients [46]. Preclinical studies have also investigated liver-targeted and controlled-release versions of DNP, which showed promising effects on glucose homeostasis and liver triglyceride content but without efficacy on adiposity [43, 44].

Other structurally distinct uncouplers have also demonstrated promising effects against metabolic disease; however, limitations remain such as non-specific targets and lack of anti-obesity efficacy. For example, the FDA-approved anti-helminthic drug niclosamide was found to have mitochondrial uncoupling properties resulting in bodyweight loss in high fat fed-C57BL/6J mice, but it increased body mass in *db/db* mice [45]. Furthermore, niclosamide is not a specific mitochondrial uncoupler as it also targets the Wnt/β-catenin, mTORC1, STAT3, NF-κB, AKT, and Notch signalling pathways [47]. The novel mitochondrial uncoupler OPC-163493 had favourable effects on glucose homeostasis and hepatic lipids in multiple animal models, but did not reverse obesity [39]. Another novel compound '6j' increased oxygen consumption *in vitro* and improved glucose homeostasis in *db/db* mice, but did not decrease adiposity, has at least one additional target (pyruvate dehydrogenase) and its mechanism of uncoupling has not been fully characterised [40]. Among these new uncouplers, BAM15, and now SHC517, represent the rare few that demonstrate strong efficacy against diet-induced obesity while improving glucose homeostasis.

The translational potential of SHC517, like BAM15, is limited by its short half-life. However, the strong efficacy of SHC517 provides rationale for future slow-release formulation development or structural modifications to improve pharmacokinetics [42]. Collectively, the current study provides additional evidence supporting the therapeutic potential of furazanopyrazine mitochondrial uncouplers as weight loss drugs.

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Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Authors' relationships and activities

The authors declare the following competing financial interest(s): W.L.S., S.P.T., and K.L.H. have a commercial interest in mitochondrial uncoupling through Continuum Biosciences, Inc.

Contribution Statement

S-Y.C., M.B., S.J.A. and K.L.H. designed the study. S-Y.C., M.B., S.J.A., D.P.S., E.M.O. and S.R.H. performed experiments and analysed data. E.S.C. and J.M.S. contributed reagents, including synthesis of SHC517 and BAM15, and contributed to interpretation of data. A.R., C.C. and T.R. provided advice and intellectual discussion regarding the chemical nature of the compounds. S.P.T., W.L.S. and K.L.H. provided intellectual guidance and supervised the project. All authors critically reviewed the manuscript for important intellectual content and approved the final version to be published. S-Y.C. and K.L.H. are the guarantors of this work.

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Figure Legends

Figure 1. Chemical structure of mitochondrial uncouplers. Illustrations of BAM15 (A) and SHC517 (B).

Figure 2. SHC517 is orally bioavailable. Oral bioavailability of SHC517 was assessed by i.v. and p.o. administration in male Swiss Albino mice. Blood plasma was collected at the time points shown and analysed by LC-MS/MS. n=3 male mice per group.

Figure 3. SHC517 prevents diet-induced obesity and fatty liver. Mice were fed WD only or WD containing SHC517 at 0.05% or 0.1% (w/w) for 12 days. Body composition was assessed by EchoMRI analysis of fat mass (a) and percent body fat (b). Fat pad wet weights were recorded after euthanasia (c). EchoMRI-based fat-free mass (d) and percent fat-free mass over total body mass (e). Average energy intake over the course of the study (f). Liver triglyceride content (g) and liver cholesterol content (h) derived from liver tissue after euthanasia. * indicates $p<0.05$ compared to WD, assessed by One-Way ANOVA (c, f, h) or Kruskal-Wallis test for non-parametric data (g). For multiple time points, Two-Way Repeated Measures ANOVA was employed (b, e). n=6 male mice per group.

Figure 4. SHC517 prevents diet-induced glucose intolerance. Glucose tolerance testing for the obesity-prevention study was conducted prior to treatment (baseline) and at day 11 (final), shown as glucose curves (a-b) and area under the curve (c). Fasting blood glucose and insulin levels were recorded prior to treatment and at day 11 (d-e). The insulin resistance index was calculated based on fasting glucose and insulin levels (f). * indicates $p<0.05$ compared to WD, assessed by Two-Way Repeated Measures ANOVA. n=6 male mice per group.

Figure 5. SHC517 reverses diet-induced adiposity. Mice were conditioned on WD for 4 weeks prior to administering SHC517 at 0.05% admixed in WD for an additional 7 weeks. Adiposity was assessed by EchoMRI body composition of fat mass (a) and percent body fat mass (b), and fat pad masses were measured at termination (c). Body composition for fat-free mass (d) and percent fat-free mass over total body mass (e). Daily energy intake was recorded during the 7 weeks of intervention (f). Liver triglyceride content (g) and liver cholesterol content (h) were measured at termination. * indicates $p<0.05$ compared to WD, assessed by One-Way ANOVA (c, h) or Kruskal-Wallis test for non-parametric data (g). For multiple time points, Two-Way Repeated Measures ANOVA was employed (b, e, f). n=5 male mice per group.

Figure 6. SHC517 reverses glucose intolerance in obese mice. Glucose tolerance testing was conducted prior to treatment (pre-treatment) and at 6 weeks post-treatment, shown as glucose curves (a-b) and area under the curve (c). Fasting blood glucose (d) and insulin (e) were determined prior to the start of treatment and at 6 weeks of treatment. The insulin resistance index was calculated based on fasting glucose and insulin levels (f). * indicates $p<0.05$ compared to WD, assessed by Two-Way Repeated Measures ANOVA. n=5 male mice per group.