

Randomized Controlled Trial of a Leucine-Metformin-Sildenafil Combination (NS-0200) on Weight and Metabolic Parameters

Michael B. Zemel¹, Orville Kolterman¹, Mary Rinella², Raj Vuppalachchi³, Omar Flores¹, A. Sidney Barritt IV⁴, Mohammad Siddiqui⁵, and Naga Chalasani³

Objective: Leucine was previously demonstrated to allosterically activate mammalian sirtuin 1 and synergize with other sirtuin 1/AMP-activated protein kinase/nitric oxide pathway activators to modulate energy metabolism. The objective of this study was to evaluate the effects of a triple combination of leucine, metformin, and sildenafil (NS-0200) on body weight and obesity comorbidities in a phase 2 randomized trial.

Methods: A total of 91 subjects with obesity were randomized to placebo, low dose (1.1 g leucine/0.5 g metformin/0.5 mg sildenafil), or high dose (1.1 g leucine/0.5 g metformin/1.0 mg sildenafil) twice daily for 16 weeks. Seventy subjects completed the trial and met all a priori compliance criteria. Hypertensive ($n=35$) and hypertriglyceridemic ($n=22$) subcohorts were also analyzed.

Results: NS-0200 dose-responsively reduced weight; high dose reduced weight by 2.4 and 5.0 kg in the full and high-triglyceride cohorts, respectively ($P<0.0001$). High-dose NS-0200 treatment also decreased blood pressure (-5.5 mm Hg diastolic pressure; $P=0.011$), with greater effects among hypertensive subjects. NS-0200 also significantly reduced triglycerides and hemoglobin A1c. Significant improvement in ≥ 2 comorbidities was exhibited by 54% of subjects in the high-dose arm versus 5% of placebo subjects ($P=0.0009$). Treatment-emergent adverse events did not significantly differ among groups.

Conclusions: These data support further study of NS-0200 as a therapy for obesity and associated comorbidities.

Obesity (2019) **27**, 59–67. doi:10.1002/oby.22346

Introduction

The prevalence of obesity has markedly increased over the past three decades and is a major public health challenge, especially in disproportionately affected population segments. Women have a somewhat higher prevalence than men (40.5% vs. 35.2%), and minority women are disproportionately affected, with a prevalence of 46.6% among Hispanic women and 57.2% among Black women in the United States (1).

Obesity is associated with multiple comorbidities, including cardiovascular disease, hypertension, type 2 diabetes, nonalcoholic fatty liver disease (NAFLD), and some cancers (2,3). These comorbidities are thought to arise as a consequence of obesity. Multiple large studies have demonstrated an association between obesity and reduced

life-span (4–8), with a predicted loss of 9 to 13 years of life for individuals with BMI ≥ 35 (4). The Global BMI Mortality Collaboration recently reported data from 10.6 million participants followed for an average of 14 years over 239 large studies, including 189 studies that included 4 million participants who were never-smokers (8); the data demonstrate a 31% increase in risk of premature death for every 5-BMI-unit increase over 25, as well as an overall increased risk of 45% for stage 1 obesity, 94% for stage 2 obesity, and ~3-fold for stage 3 obesity (8).

Lifestyle interventions focused on reducing caloric intake and increasing caloric expenditure are important first-line interventions, but poor adherence sharply limits sustained success. Accordingly, development of safe and effective pharmacotherapy is an important tool to achieve meaningful, sustained weight loss.

¹ NuSirt Biopharma, Nashville, Tennessee, USA. Correspondence: Michael Zemel (mzemel@nusirt.com) ² Division of Gastroenterology and Hepatology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA ³ Division of Gastroenterology and Hepatology, Indiana University School of Medicine, Indianapolis, Illinois, USA. Correspondence: Naga Chalasani (nchalasa@iu.edu) ⁴ Division of Gastroenterology and Hepatology, UNC School of Medicine, Chapel Hill, North Carolina, USA ⁵ Department of Internal Medicine, School of Medicine, Virginia Commonwealth University, Richmond, Virginia, USA.

Funding agencies: This work was supported by NuSirt Biopharma.

Disclosure: NC and MR are advisors for the NuSirt Biopharma nonalcoholic steatohepatitis (NASH) development program. OK is an advisor for the NuSirt Biopharma diabetes, NASH, and obesity programs. MZ and OF are employees of NuSirt Biopharma. The other authors declared no conflict of interest.

Clinical trial registration: ClinicalTrials.gov identifier NCT02546609.

Additional Supporting Information may be found in the online version of this article.

Received: 6 June 2018; **Accepted:** 20 September 2018; **Published online** 20 December 2018. doi:10.1002/oby.22346

AMP-activated protein kinase (AMPK) and mammalian sirtuin 1 (Sirt1) are well-known regulators of lipid and energy metabolism. These two systems are coordinated via reciprocal activation, with Sirt1 activating AMPK via liver kinase B1 deacetylation and AMPK activating Sirt1 via nicotinamide phosphoribosyltransferase induction (9), and they inhibit lipid storage and stimulate muscle and hepatic mitochondrial biogenesis and fatty acid oxidation (10,11). High-fat diet and excess energy intake decrease Sirt1 and AMPK activity (12-14). This, in turn, may lead to mitochondrial loss or dysfunction, which plays a pivotal role in the development of metabolic diseases, including obesity, diabetes, and cardiovascular disease. In contrast, both Sirt1 and AMPK activation prevent or attenuate lipid accumulation in response to positive energy balance (15-17), thus representing attractive therapeutic targets for obesity.

We have demonstrated that L-leucine is an allosteric activator of Sirt1 that lowers the activation energy for nicotinamide adenine dinucleotide (NAD)⁺ and thereby modulates lipid and energy metabolism and increases insulin sensitivity in mice (10,18-23). Consequently, combining leucine with other sirtuin pathway activators results in synergistic coactivation of sirtuin pathway signaling and sirtuin targets. For example, adding L-leucine to metformin results in a novel synergistic interaction that has enabled significant dose reduction of metformin with no loss of antidiabetic efficacy (24). These effects may be further enhanced via the endothelial nitric oxide synthase (eNOS)/nitric oxide (NO)/cyclic GMP (cGMP) pathway using low doses of sildenafil; although best known for its inhibitory effects on phosphodiesterase 5, low doses of sildenafil activate eNOS (25-28) and increase NO production. NO also stimulates Sirt1, while Sirt1 deacetylates and activates eNOS in a positive feedback loop (27,29-31). We have found leucine to synergize with sildenafil and other eNOS activators to exert amplifying downstream effects of AMPK and Sirt1 activation on glucose and fat metabolism (32), while a triple combination of leucine, metformin, and sildenafil (NS-0200) reduced body weight and adiposity (M Zemel, unpublished data, 2018) and regressed nonalcoholic steatohepatitis (21) in preclinical studies. Accordingly, the present study evaluates the efficacy of NS-0200 in reducing weight and obesity comorbidities in subjects with overweight and obesity.

Methods

Study design

A randomized, placebo-controlled, double-blind, phase 2 multicenter study was conducted to test the effects of two fixed-dose combinations of leucine, metformin, and sildenafil (NS-0200) versus placebo for 16 weeks. The two active treatment arms consisted of capsules containing 1,100 mg of leucine, 500 mg of metformin, and either 0.5 or 1.0 mg of sildenafil, and the placebo utilized matching capsules containing a 99% Avicel-PH302/1% magnesium stearate blend (FMC BioPolymer (Newark, Delaware), all administered orally twice daily. The primary end point of this study was change in hepatic fat as assessed by proton density fat fraction; we recently reported this end point, along with associated hepatic parameters (33). Herein, we report the effects of NS-0200 on body weight and metabolic parameters.

Subjects were screened for eligibility based on medical history, physical examination, and blood analysis 2 weeks prior to enrollment in the study, and those who met basic eligibility criteria returned for assessment of hepatic fat via proton density fat fraction magnetic resonance imaging (MRI-PDFF) approximately 7 days prior to study enrollment.

Those with hepatic fat fraction $\geq 15\%$ via MRI-PDFF were enrolled in the study and returned to the clinic on study day 1 for baseline laboratory evaluation and first dose of study medication, followed by visits on days 7, 28, 56, 84, and 112 (study conclusion). Subjects were contacted via telephone between clinic visits on days 14, 42, 70, and 98 to establish a biweekly pattern of contact to enhance compliance.

Inclusion criteria were to be aged 18 to 75 years at study entry and to have BMI between 25 and 40 kg/m², stable health and body weight for the preceding 12 weeks, MRI-PDFF $\geq 15\%$, and alanine aminotransferase ≥ 30 U/L for men and ≥ 19 U/L for women. Key exclusion criteria were participation in a weight loss program within the preceding 12 weeks, bariatric surgery, evidence of chronic liver disease (other than fatty liver disease), history of significant alcohol consumption (defined as > 7 drinks/week), use of any component of the study medication (leucine, metformin, or sildenafil) or drugs from related classes, or use of medications known to affect body weight.

Subjects were randomized to treatments using a computer-generated randomized block (block 6) schedule prepared by a third party, and sponsor personnel, study staff, and subjects were blinded to treatment assignment. Treatment assignment was performed centrally for all sites, and randomization numbers were allocated in strict chronological order. Study medication was provided to the study centers in a blinded fashion using each subject's randomization number.

This study was approved by the Central Institutional Review Board and, if applicable, the institutional review boards at each participating clinical site, and written informed consent was obtained from all participants prior to study enrollment. Recruiting was initiated November 2015, and the study was concluded in November 2016.

Efficacy outcomes

All outcome assessments were conducted following an overnight fast of at least 10 hours; subjects refrained from taking study medication prior to each clinic visit and instead took their morning dose at the study site following all measurements. Body weight, blood pressure, fasting glucose, insulin, and blood lipids (cholesterol, low-density lipoprotein, high-density lipoprotein [HDL], and triglycerides) were measured at baseline and following 4, 8, 12, and 16 weeks of treatment. Hemoglobin A1c (HbA1c) was measured at baseline and following 12 and 16 weeks of treatment. All laboratory measurements were conducted in a centralized laboratory. Body weight was measured in light clothing with no shoes at each clinical site.

Safety outcomes

Safety assessment included physical examination with vital signs, 12-lead electrocardiogram, clinical chemistry, hematology, and urinalysis. Adverse events were categorized by severity, outcome, and relationship to study drug.

Statistics

The primary analysis population was per protocol (PP) for efficacy end points and intent to treat (ITT) for safety end points. The PP population consisted of all ITT subjects who completed all study visits and who adequately complied with the study protocol without major protocol deviations and received $\geq 80\%$ of provided study medication via pill counts. Secondary analyses were conducted on the ITT population,

and post hoc subgroup analyses were also conducted, consisting of (a) hypertensive subjects, defined as either subjects taking antihypertensive medication or with baseline blood pressure $\geq 140/90$ mm Hg ($n=35$), or (b) participants with baseline fasting triglycerides ≥ 200 mg/dL ($n=22$).

A mixed-model analysis of covariance (ANCOVA) was used to analyze changes in each outcome measure from baseline to week 16, with baseline value serving as a covariate. The least squares means, SEs, and corresponding 95% CIs for the changes from baseline to week 16

were derived from the model for each treatment. Each of the fixed-dose combinations of leucine, metformin, and sildenafil treatment groups (low-dose, treatment group B; and high-dose, treatment group C) was compared with the placebo group (treatment group A), and the least squares mean for the treatment difference (treatment B or C minus treatment A), SEs, associated 95% CIs, and *P* values were computed accordingly. Sample size requirements for the trial were estimated based on the trial primary outcome of change in hepatic fat, as recently reported (33).

TABLE 1 Demographic and baseline characteristics by treatment

	Per-protocol population ($n=70$)			All ($n=70$)
	Placebo (treatment A) ^a ($n=22$)	NS-0200 (treatment B) ^a ($n=24$)	NS-0200 (treatment C) ^a ($n=24$)	
Gender, n (%)				
Male	11 (50.0)	8 (33.3)	12 (50.0)	31 (44.3)
Female	11 (50.0)	16 (66.7)	12 (50.0)	39 (55.7)
Age (y) ^b				
Mean (SD)	46.7 (10.59)	45.7 (11.77)	46.0 (13.55)	46.1 (11.90)
Race, n (%)				
American Indian or Alaska Native	0 (0.0)	0 (0.0)	1 (4.2)	1 (1.4)
Black or African American	2 (9.1)	1 (4.2)	0 (0.0)	3 (4.3)
White	19 (86.4)	22 (91.7)	22 (91.7)	63 (90.0)
White/Asian	1 (4.2)	1 (2.9)	0 (0.0)	2 (2.2)
Not reported	0 (0.0)	1 (4.2)	1 (4.2)	2 (2.9)
Ethnicity, n (%)				
Hispanic or Latino	5 (22.7)	7 (29.2)	7 (29.2)	19 (27.1)
Not Hispanic or Latino	17 (77.3)	17 (70.8)	17 (70.8)	51 (72.9)
Weight (kg)				
Mean (SD)	98.16 (14.98)	94.21 (14.01)	96.70 (17.84)	96.31 (15.57)
Height (cm)				
Mean (SD)	170.5 (9.8)	169.8 (9.4)	168.6 (10.9)	168.9 (9.9)
BMI (kg/m ²)				
Mean (SD)	33.82 (4.60)	32.62 (3.39)	33.62 (3.84)	33.34 (3.93)
Fasting plasma glucose (mg/dL)				
Mean (SD)	109.1 (16.9)	103.0 (14.6)	112.1 (24.8)	108.1 (19.5)
HbA1c (%)				
Mean (SD)	5.62 (0.45)	5.62 (0.57)	5.74 (0.71)	5.66 (0.59)
Systolic pressure (mm Hg)				
Mean (SD)	121.3 (9.9)	124.6 (13.3)	122.9 (12.3)	123 (11.9)
Diastolic pressure (mm Hg)				
Mean (SD)	79.5 (10.1)	80.6 (9.9)	81.9 (10.9)	80.7 (10.2)
Triglycerides (mg/dL)				
Mean (SD)	163.0 (65.9)	202.8 (122.1)	261.4 (381.2)	210.4 (237.5)
Cholesterol (mg/dL)				
Mean (SD)	183.0 (38.0)	207.1 (42.4)	201.3 (45.3)	197.5 (42.8)
HDL (mg/dL)				
Mean (SD)	41.2 (12.9)	45.8 (13.3)	42.0 (13.1)	43.0 (13.1)

^aTreatment A = placebo; treatment B = 1,100 mg leucine + 500 mg metformin + 0.5 mg sildenafil twice daily; treatment C = 1,100 mg leucine + 500 mg metformin + 1.0 mg sildenafil twice daily.

^bAge at consent.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; PDFF, proton density fat fraction; SD, standard deviation.

Results

Enrollment and baseline characteristics

Of 215 subjects screened for participation, 91 met all enrollment criteria and were randomized as follows: 24 to placebo (treatment group A), 35 to low dose (1,100 mg leucine/500 mg metformin/0.5 mg sildenafil; treatment group B), and 32 to high dose (1,100 mg leucine/500 mg metformin/1.0 mg sildenafil; treatment group C) (Supporting Information Figure S1). Of the enrolled participants, 90 received at least one dose of medication and therefore constitute the ITT group. Of these 90, 20 (22%) discontinued early, as follows: 2 (8.3%) from placebo (treatment group A), 11 (31.4%) from treatment group B, and 7 (21.9%) from treatment group C. Of the 71 completing the trial, 1 was excluded from the PP analysis because of nonadherence with a priori compliance criteria, leaving 70 (78% of ITT population) in the

PP analysis group. Baseline characteristics are shown in Table 1. There were no significant baseline differences among the three groups in any of the variables studied.

Weight change

NS-0200 dose-responsively reduced weight in the full PP cohort, with a 2.4-kg weight loss over 16 weeks in the high-dose group ($P < 0.0001$; Figure 1). Weight loss was linear over time in the treatment group, while there was no significant weight change in the placebo group. Of the high-dose treatment group, 58% exhibited > 2 -kg weight reduction versus 23% of placebo ($P = 0.027$), and 42% exhibited > 3 -kg reduction in 16 weeks versus 0% for placebo ($P = 0.003$). The ITT group exhibited comparable effects on weight, albeit of smaller magnitude (-1.9 ± 0.7 kg weight loss; $P < 0.05$).

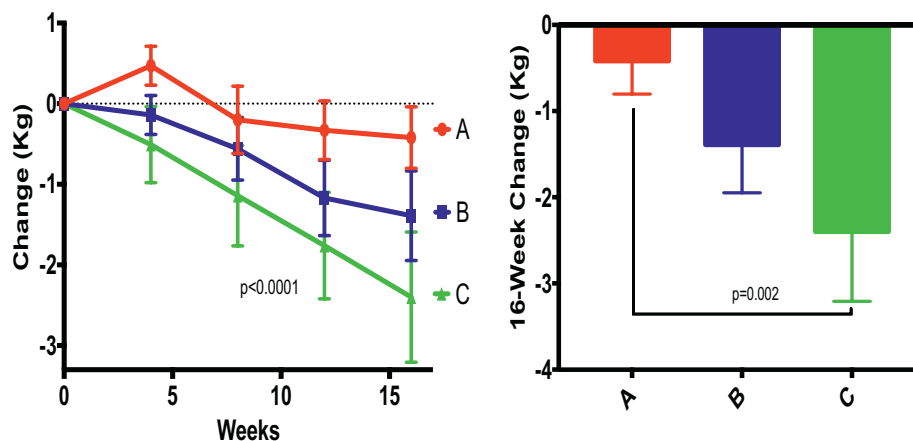


Figure 1 Effects of treatments on body weight change in the full per-protocol cohort. A = placebo; B = 1,100 mg leucine/500 mg metformin/0.5 mg sildenafil twice daily; C = 1,100 mg leucine/500 mg metformin/1.0 mg sildenafil twice daily. Left panel shows time course, and right panel shows the 16-week change. Data presented as means \pm SEM.

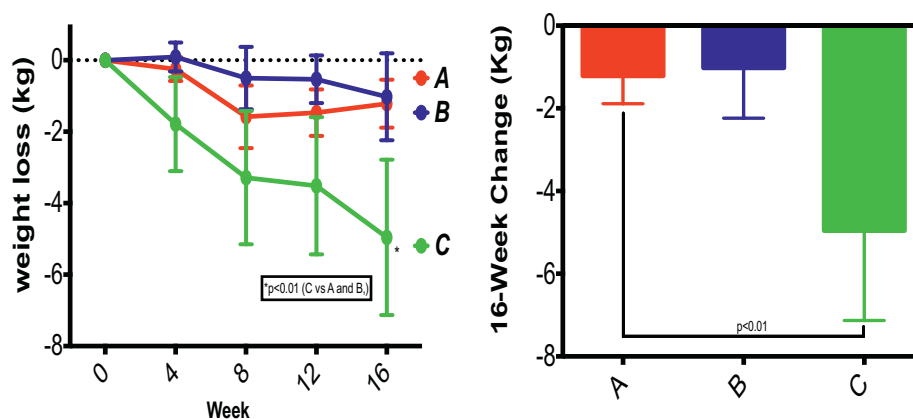


Figure 2 Effects of NS-0200 on body weight change in subjects with elevated triglycerides (≥ 200 mg/dL at baseline) over 16 weeks ($n = 22$). A = placebo; B = 1,100 mg leucine/500 mg metformin/0.5 mg sildenafil twice daily; C = 1,100 mg leucine/500 mg metformin/1.0 mg sildenafil twice daily. Left panel shows time course, and right panel shows the 16-week change. Data presented as means \pm SEM.

The subcohort of individuals with elevated triglycerides (≥ 200 mg/dL) exhibited weight loss of approximately twice the magnitude of the full cohort in response to the higher dose of NS-0200 (5 kg; $P < 0.01$; Figure 2).

Blood pressure

High-dose NS-0200 treatment also resulted in decreased blood pressure (-5.5 mm Hg diastolic pressure; $P=0.011$; Figure 3) in the PP population, which was evident at the first treatment measurement time (4 weeks) and was sustained throughout the 16 weeks of treatment; therefore, this effect preceded significant changes in body weight and was not correlated with weight change. Similarly, the ITT population exhibited a decline of 4.3 ± 1.6 mm Hg in diastolic pressure ($P=0.05$). A further subgroup analysis was conducted on subjects with hypertension at baseline ($n=35$ [10-13 per treatment group]). Hypertensive subjects exhibited a greater blood pressure response to high-dose NS-0200 (-5.2 mm Hg systolic, -8 mm Hg diastolic pressure; $P < 0.0019$) despite the fact that the 60% of the hypertensive subjects were on a

stable antihypertensive regimen throughout the study. The remaining untreated hypertensive subjects constitute too small a group for reliable inference ($n=7$ for group A, 3 for group B, and 4 for group C), although data from these individuals are consistent with greater anti-hypertensive effects in untreated individuals (placebo-adjusted values of -21.5 mm Hg systolic and -15.7 mm Hg diastolic pressure). There was no effect of treatment on heart rate in either the full cohort or the hypertensive subcohort.

Lipids

There was a reduction in triglycerides ~ 40 mg/dL in the two active treatment groups of the full PP cohort, with no significant difference between the two groups, while the placebo group exhibited an increase ($+55$ mg/dL). The ITT population exhibited qualitatively similar changes in triglycerides (-25 ± 15 mg/mL), although this decrease was not significant ($P=0.068$). NS-0200 treatment resulted in a markedly greater decrease in triglycerides in the subcohort (Figure 4) with hypertriglyceridemia at baseline (~ 140 mg/dL;

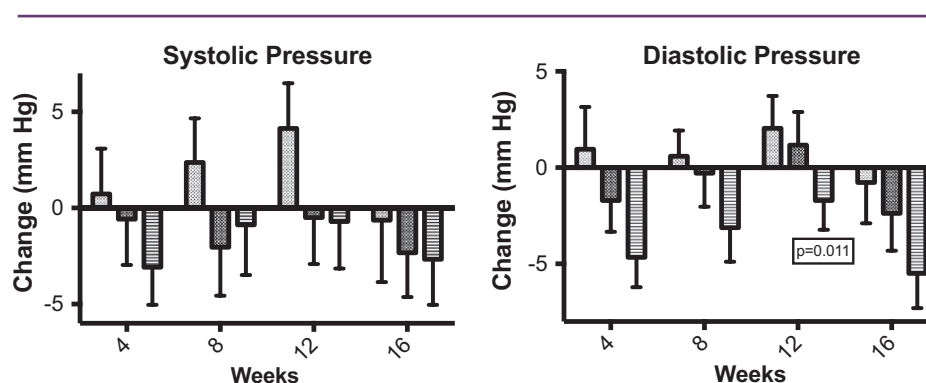


Figure 3 Effects of treatments on blood pressure change in the full per-protocol cohort. Light gray=placebo; stippled=lower-dose NS-0200; horizontal lines=higher-dose NS-0200. Data presented as means \pm SEM.

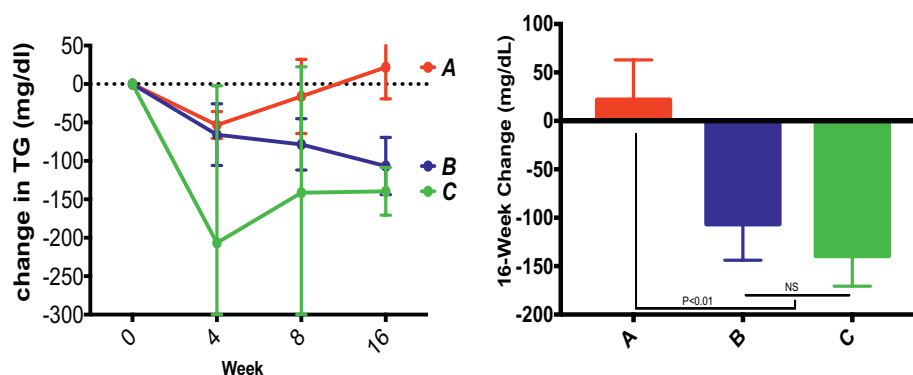


Figure 4 Effects of treatments on triglyceride (TG) change in hypertriglyceridemic subjects. Left panel shows time course, and right panel shows the 16-week change. A=placebo; B=1,100 mg leucine/500 mg metformin/0.5 mg sildenafil twice daily; C=1,100 mg leucine/500 mg metformin/1.0 mg sildenafil twice daily. Data presented as means \pm SEM.

$P < 0.01$). However, there was no significant correlation between weight change and triglyceride change in either the full cohort or the hypertriglyceridemic subcohort.

There was no significant change in cholesterol, low-density lipoprotein, or HDL in any treatment group, although the cholesterol:HDL ratio decreased significantly in both NS-0200 treatment groups ($P < 0.01$), while the placebo group exhibited a slight increase. Cholesterol:HDL change was not correlated with weight change.

Glycemic control

This study was conducted in a predominantly nondiabetic population, with 6 of the 91 ITT subjects qualifying as having type 2 diabetes (2 in each group). Nonetheless, NS-0200 did result in a modest decrease in HbA1c in the PP population that was statistically significant (-0.15% ; $P = 0.002$), with no significant difference between the two doses; the placebo group exhibited a nonsignificant increase of 0.07% . The ITT group exhibited comparable changes ($-0.13 \pm 0.061\%$ and $-0.12 \pm 0.058\%$ for low- and high-dose groups, respectively) that were significant only for the low-dose group ($P = 0.012$). The change in HbA1c exhibited a weak correlation ($r = 0.23$) with weight change that approached statistical significance ($P = 0.051$). There were no significant effects of any treatment on fasting glucose, insulin, or calculated homeostatic model assessment of insulin resistance.

Safety

Treatment-emergent adverse events (TEAEs) in this trial were recently reported (33); in brief, TEAE frequency was similar between placebo and both treatment groups. However, consistent with the presence of metformin, subjects in the active treatment groups did report a greater number of mild to moderate gastrointestinal events than the placebo group, including diarrhea (40.9% vs. 8.3%), nausea (16.7% vs. 12.5%), and vomiting (4.2% vs. 0.6%). There were no serious TEAs in any treatment group.

Discussion

Data from this randomized, controlled trial demonstrate that NS-200 dose-responsively reduces body weight in subjects with obesity in the absence of any prescribed diet or physical activity intervention. The trajectory of this weight loss over the 16-week study period was linear throughout the entire 16 weeks without evidence of a plateau, suggesting that a greater effect may be predicted in studies of longer duration; however, studies of at least a 12-month duration will be required to confirm whether or not this combination results in durable weight loss.

Subjects with elevated circulating triglycerides exhibited a 5-kg weight loss over 16 weeks, approximately twice the weight decrease with NS-0200 found in the overall study group; this was accompanied by a corresponding reduction in triglycerides in this subgroup, although there was no correlation between changes in weight and triglycerides. This is consistent with the mechanism of action of NS-0200 to target synergistic interaction among elements of the Sirt1-AMPK-eNOS network (21). In addition to regulating mitochondrial biogenesis and energy utilization in skeletal muscle and adipose tissue (11), this network is also a key regulator of hepatic lipid metabolism (15) and is suppressed by excess lipids (12-14). Therefore, individuals with greater suppression of

this system are likely to exhibit greater responsiveness to correcting that suppression. However, no independent assessment of Sirt1 activity was conducted to confirm this assumption in this clinical trial.

Prior preclinical data have demonstrated that the metabolic effects of NS-0200 require all three components of the fixed-dose combination with no independent effect of leucine, metformin, or sildenafil at these doses (21). Furthermore, the effects of metformin on body weight have been studied in both diabetic and nondiabetic individuals, with small effects and inconsistent results reported (34). Among diabetics, ~50% of the randomized controlled trials have demonstrated body weight reduction of ~2% with metformin relative to placebo or comparator antidiabetic agent (34), although there is evidence for a modest effect of metformin on weight, especially among those with higher levels of insulin resistance (35). The longest of these trials, the UK Prospective Diabetes Study, reported that patients receiving diet-based treatment gained ~2 kg over the 10-year follow-up period, while those on metformin gained a slightly, but not significantly, smaller amount (~1.5 kg) (36). Studies in nondiabetic patients with or without caloric restriction have also yielded inconsistent placebo-adjusted effects (34). A recent systematic review and meta-analysis reported a small but statistically significant effect of metformin on BMI in children and adolescents (37); however, a recent randomized controlled trial of metformin in children and adolescents demonstrated no significant effect on body weight, body composition, or BMI in either pre- or postpubertal children although there was a small, statistically significant decrease in BMI z score in the prepubertal group (38). The recently released Endocrine Society clinical practice guideline on pediatric obesity notes that, given its limited efficacy, metformin is not considered an appropriate agent for treatment of obesity (39). Similarly, sildenafil has been in extensive clinical use for 20 years with no evidence of independent effects on weight (40-43), and administration of leucine at the dose used in NS-0200 to adults with overweight and obesity increased fat oxidation but did not result in weight loss in the absence of caloric restriction (44). These data indicate that the individual components of NS-0200 have little or no independent therapeutic potential in weight management, supporting the appropriateness of a fixed-dose combination.

Treatment with NS-0200 also exerted significant salutary effects on obesity comorbidities. Treatment resulted in dose-dependent decreases in blood pressure in the active treatment arms, with greater effects noted among hypertensive subjects. The magnitude of this effect is comparable to that observed with antihypertensive treatment; moreover, this antihypertensive effect was manifested at the first measurement time (4 weeks), preceding significant weight change, and exhibited no correlation with weight change, consistent with the antihypertensive effect of NS-0200 being independent of weight loss. Sildenafil at higher doses (≥ 10 mg vs. the 0.5-1.0 mg in NS-0200) is well known to exert vasodilatory effects secondary to phosphodiesterase 5 inhibition as well as eNOS activation, resulting in increased NO/cGMP signaling with consecutive activation of the cGMP-dependent protein kinases to induce vasodilatory, anti-inflammatory, and antiproliferative effects (25-28). The synergy between leucine-induced Sirt1 activation and sildenafil-induced eNOS activation amplifies this effect (21,32) and may enable vasodilatory effects at the lower sildenafil doses used in NS-0200. Moreover, Sirt1 activation was recently demonstrated to increase capillary density and reverse age-related declines in blood flow (45), likely contributing further to the antihypertensive effect of NS-0200.

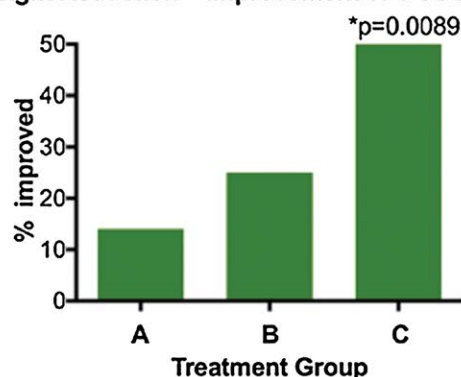
NS-0200 also exerted salutary effects on lipids and modest beneficial effects on glycemic control, consistent with a Sirt1/AMPK-mediated mechanism of action and our previous preclinical data (11,22,23), which suggest pleiotropic effects on cardiometabolic risk factors. Accordingly, we undertook a post hoc assessment to evaluate the apparent pleiotropic effects on cardiometabolic risk by assessing the fraction of subjects in each treatment arm exhibiting significant weight loss (≥ 2 kg) and simultaneous improvement in blood pressure ($\geq 5\%$ improvement), glycemic control (HbA1c reduction of $\geq 0.2\%$), triglycerides ($\geq 10\%$ reduction), cholesterol ($\geq 10\%$ reduction), and/or HDL ($\geq 10\%$ increase). Of subjects in the higher-dose treatment arm, 54% exhibited both weight reduction and improvement in at least two of these comorbidities ($P < 0.0009$ vs. placebo) (Figure 5), and 46% exhibited improvement in at least three comorbidities in addition to weight loss ($P = 0.0014$). This suggests that targeting the Sirt1/AMPK/eNOS energy sensing network with NS-0200 addresses multiple expressions of dysregulated energy metabolism in addition to weight loss. This concept is further supported by our recent report demonstrating that NS-0200 significantly reduced hepatic fat in NAFLD patients with elevated alanine aminotransferase (33), as NAFLD is generally considered

a hepatic manifestation of metabolic syndrome with significant bidirectional relationships with the other cardiometabolic risk factors (46,47); there was no correlation between weight change and hepatic fat change, again suggesting independent effects on weight and hepatic fat. Similarly, the lack of correlation between weight change and change in the other comorbidities reported here provides further evidence for pleiotropic effects of NS-0200 independent of its effect on weight.

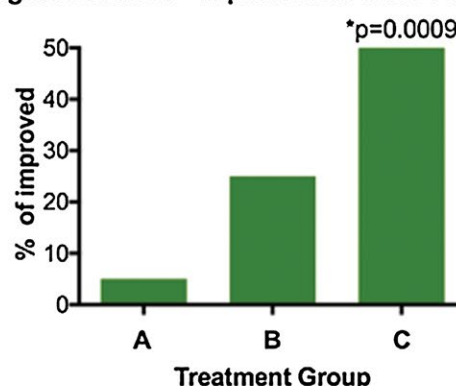
NS-0200 was well tolerated, with no significant effect of treatment versus placebo on overall TEAEs, although the treatment groups did report a greater number of gastrointestinal events, consistent with well-known effects of the metformin component of NS-0200 (48).

This study has several limitations. The study was designed primarily to assess the effects of NS-0200 on hepatic fat in subjects with NAFLD, and the weight and metabolic outcomes described herein were designated as secondary outcomes. Accordingly, potential changes to diet and physical activity were not evaluated. Additionally, because subjects were selected on the basis of elevated liver fat, the reported effects cannot be extrapolated to subjects who do not have NAFLD. Furthermore,

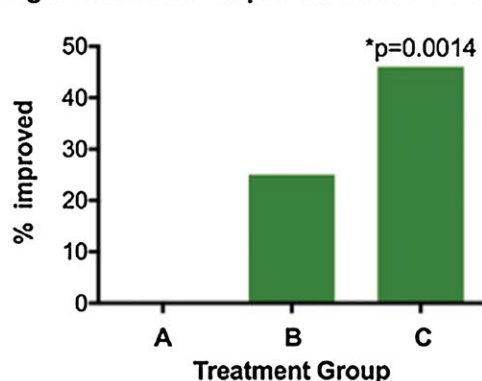
Weight Reduction + Improvement of 1 Co-morbidity



Weight Reduction + Improvement of 2 Co-morbidities



Weight Reduction + Improvement of 3 Co-morbidities



Weight Reduction + Improvement in 4 Co-morbidities

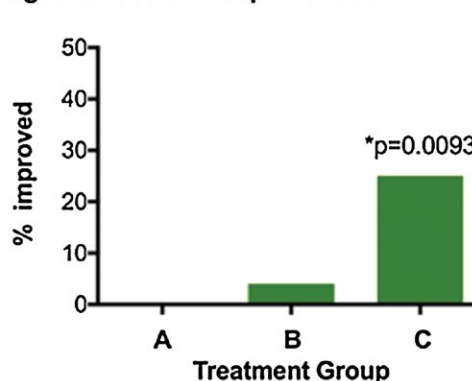


Figure 5 Effects of treatments on improvements in comorbidities in subjects who lost weight (≥ 2 kg). Thresholds for comorbidity improvement were blood pressure ($\geq 5\%$ improvement in systolic and/or diastolic pressure), glycemic control (HbA1c reduction of $\leq 0.2\%$), triglycerides ($\geq 10\%$ reduction), cholesterol ($\geq 10\%$ reduction), and/or HDL ($\geq 10\%$ increase). A=placebo; B=1,100 mg leucine/500 mg metformin/0.5 mg sildenafil twice daily; C=1,100 mg leucine/500 mg metformin/1.0 mg sildenafil twice daily. Significance shown versus placebo.

the 16-week duration of the trial precludes conclusions regarding the durability of the reported effects; however, examination of the weight loss trajectory in the full cohort and the subcohorts described showed no evidence of a weight plateau. These factors are now under study in a follow-up trial.

Overall, data from the present study demonstrate that treatment with NS-0200 resulted in significant, dose-dependent reductions in body weight and concomitant improvements in multiple obesity comorbidities. These data support further development of NS-0200 as a therapy for obesity that exerts potential independent effects on obesity-associated comorbidities. **O**

Acknowledgments

NS-0200-01 Investigator Group: Naga Chalasani, Indiana University, Indianapolis, IN; Raj Vuppalanchi, Indiana University, Indianapolis, IN; Mary Rinella, Northwestern University, Chicago, IL; Mohammad S. Siddiqui, Virginia Commonwealth University, Richmond, VA; A. Sidney Barritt IV, University of North Carolina, Chapel Hill, NC; Giriprasad Rao Korivi, Premier Medical Group, Clarksville, TN; Rohit Loomba, University of California, San Diego, CA; Aasim Sheikh, Gastrointestinal Specialists of Georgia, Marietta, GA; Mia K. Moon, Catalina Research Institute, Chino, CA; Ziad Younes, Gastro One, Germantown, TN; Robert Herring, Quality Medical Research, Nashville, TN; Norman Gitlin, Atlanta Gastroenterology Associates, Atlanta, GA; Matthew Wenker, Sterling Research Group, Cincinnati, OH; John S. Goff, Rocky Mountain Clinical Research, Wheat Ridge, CO.

© 2018 The Obesity Society

References

- Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in obesity among adults in the United States, 2005 to 2014. *JAMA* 2016;315:2284-2291.
- Pi-Sunyer X. The medical risks of obesity. *Postgrad Med* 2009;121:21-33.
- Klein S, Burke LE, Bray GA, et al. Clinical implications of obesity with specific focus on cardiovascular disease: a statement for professionals from the American Heart Association Council on Nutrition, Physical Activity, and Metabolism: endorsed by the American College of Cardiology Foundation. *Circulation* 2004;110:2952-2967.
- Fontaine KR, Redden DT, Wang C, Westfall AO, Allison DB. Years of life lost due to obesity. *JAMA* 2003;289:187-193.
- Prospective Studies Collaboration; Whitlock G, Lewington S, Sherliker P, et al. Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. *Lancet* 2009;373:1083-1096.
- Masters RK, Powers DA, Link BG. Obesity and US mortality risk over the adult life course. *Am J Epidemiol* 2013;177:431-442.
- Ding M, Hu Y, Schwartz J, et al. Delineation of body mass index trajectory predicting lowest risk of mortality in U.S. men using generalized additive mixed model. *Ann Epidemiol* 2016;26:698-703.
- Global BMI Mortality Collaboration; Di Angelantonio E, Bhupathiraju ShN, Wormser D, et al. Body-mass index and all-cause mortality: individual-participant-data meta-analysis of 239 prospective studies in four continents. *Lancet* 2016;388:776-786.
- Cantó C, Gerhart-Hines Z, Feige JN, et al. AMPK regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity. *Nature* 2009;458:1056-1060.
- Banerjee J, Bruckbauer A, Zemel MB. Activation of the AMPK/Sirt1 pathway by a leucine-metformin combination increases insulin sensitivity in skeletal muscle, and stimulates glucose and lipid metabolism and increases life span in *Caenorhabditis elegans*. *Metabolism* 2016;65:1679-1691.
- Ruderman NB, Xu XJ, Nelson L, et al. AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab* 2010;298:E751-E60.
- Pfluger PT, Herranz D, Velasco-Miguel S, Serrano M, Tschöp MH. Sirt1 protects against high-fat diet-induced. *Proc Natl Acad Sci U S A* 2008;105:9793-9798.
- Sparks LM, Xie H, Koza RA, et al. A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. *Diabetes* 2005;54:1926-1933.
- Liu Y, Wan Q, Guan Q, Gao L, Zhao J. High-fat diet feeding impairs both the expression and activity of AMPK in rats' skeletal muscle. *Biochem Biophys Res Commun* 2006;339:701-707.
- Hou X, Xu S, Maitland-Toolan KA, et al. SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem* 2008;283:20015-20016.
- Purushotham A, Schug TT, Xu Q, Surapureddi S, Guo X, Li X. Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. *Cell Metab* 2009;9:327-328.
- Ruderman NB, Carling D, Prentki M, Cacicedo JM. AMPK, insulin resistance, and the metabolic syndrome. *J Clin Invest* 2013;123:2764-2772.
- Bruckbauer A, Zemel MB, Thorpe T, et al. Synergistic effects of leucine and resveratrol on insulin sensitivity and fat metabolism in adipocytes and mice. *Nutr Metab (Lond)* 2012;9:77.
- Bruckbauer A, Zemel MB. Synergistic effects of polyphenols and methylxanthines with Leucine on AMPK/Sirtuin-mediated metabolism in muscle cells and adipocytes. *PLoS One* 2014;9:e89166. <https://doi.org/10.1371/journal.pone.0089166>
- Liang C, Curry BJ, Brown PL, Zemel MB. Leucine modulates mitochondrial biogenesis and SIRT1-AMPK signaling in C2C12 myotubes. *J Nutr Metab* 2014;2014:239750. <https://doi.org/10.1155/2014/239750>
- Bruckbauer A, Banerjee J, Fu L, et al. A combination of leucine, metformin, and sildenafil treats nonalcoholic fatty liver disease and steatohepatitis in mice. *Int J Hepatol* 2016;2016:9185987. <https://doi.org/10.1155/2016/9185987>
- Fu L, Bruckbauer A, Li F, et al. Leucine amplifies the effects of metformin on insulin sensitivity and glycemic control in diet-induced obese mice. *Metabolism* 2015;64:845-856.
- Fu L, Bruckbauer A, Li F, et al. Interaction between metformin and leucine in reducing hyperlipidemia and hepatic lipid accumulation in diet-induced obese mice. *Metabolism* 2015;64:1426-1434.
- Niswender K, Kolterman O, Kosinski M, Zemel MB. Effects of leucine-metformin combinations on glycemic control in type 2 diabetes [abstract 1144-P]. *Diabetes* 2016;65(suppl 1):A300.
- Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J* 2012;33:837a-837d.
- Chrysant SG, Chrysant GS. The pleiotropic effects of phosphodiesterase 5 inhibitors on function and safety in patients with cardiovascular disease and hypertension. *J Clin Hypertens (Greenwich)* 2012;14:644-649.
- Das A, Durrant D, Salloum FN, Xi L, Kukreja RC. PDE5 inhibitors as therapeutics for heart disease, diabetes and cancer. *Pharmacol Ther* 2015;147:12-21.
- Musicki B, Bivalacqua TJ, Champion HC, Burnett AL. Sildenafil promotes eNOS activation and inhibits NADPH oxidase in the transgenic sickle cell mouse penis. *J Sex Med* 2014;11:424-430.
- Mattagajasingh I, Kim CS, Naqvi A, et al. SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 2007;104:14855-14860.
- Koka S, Aluri HS, Xi L, Lesnfsky EJ, Kukreja RC. Chronic inhibition of phosphodiesterase 5 with tadalafil attenuates mitochondrial dysfunction in type 2 diabetic hearts: potential role of NO/SIRT1/PGC-1 α signaling. *Am J Physiol Heart Circ Physiol* 2014;306:H1558-H1568.
- Koka S, Xi L, Kukreja RC. Chronic treatment with long acting phosphodiesterase-5 inhibitor tadalafil alters proteomic changes associated with cytoskeletal rearrangement and redox regulation in Type 2 diabetic hearts. *Basic Res Cardiol* 2012;107:249. <https://doi.org/10.1007/s00395-012-0249-5>
- Fu L, Li F, Bruckbauer A, et al. Interaction between leucine and phosphodiesterase 5 inhibition in modulating insulin sensitivity and lipid metabolism. *Diabetes Metab Syndr Obes* 2015;8:227-239.
- Chalasani N, Vuppalanchi R, Rinella M, et al. Randomised clinical trial: a leucine-metformin-sildenafil combination (NS-0200) vs placebo in patients with non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2018;47:1639-1651.
- Golay A. Metformin and body weight. *Int J Obes (Lond)* 2008;32:61-72.
- Igel LI, Sinha A, Saunders KH, Apovian CM, Vojta D, Aronne LJ. Metformin: an old therapy that deserves a new indication for the treatment of obesity. *Curr Atheroscler Rep* 2016;18:1-8.
- UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet* 1998;352:854-865.
- O'Connor EA, Evans CV, Burda BU, Walsh ES, Eder M, Lozano P. Screening for obesity and intervention for weight management in children and adolescents: evidence report and systematic review for the US Preventive Services Task Force. *JAMA* 2017;317:2427-2444.
- Pastor-Villaescusa B, Cañete MD, Caballero-Villarraso J, et al. Metformin for obesity in prepubertal and pubertal children: a randomized controlled trial. *Pediatrics* 2017;140:e20164285. <https://doi.org/10.1542/peds.2016-4285>
- Styne DM, Arslanian SA, Connor EL, et al. Pediatric obesity-assessment, treatment, and prevention: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 2017;102:709-757.
- Pfizer Labs. US Food and Drug Administration website. Viagra (sildenafil citrate) tablets [product information]. https://www.accessdata.fda.gov/drug-satfda_docs/label/2010/020895s0331bl.pdf. Published January 2010. Accessed May 23, 2018.
- Pfizer Labs. US Food and Drug Administration website. Revatio (sildenafil citrate) tablets [product information]. https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/021845s0041bl.pdf. Published October 2007. Accessed May 23, 2018.
- Ramini GV, Park MH. Update on the clinical utility of sildenafil in the treatment of pulmonary arterial hypertension. *Drug Des Devel Ther* 2010;4:61-70.

43. Giuliano F, Jackson G, Montorsi F, Martin-Morales A, Raillard P. Safety of sildenafil citrate: review of 67 double-blind placebo-controlled trials and the postmarketing safety database. *Int J Clin Pract* 2010;64:240-255.
44. Zemel MB, Bruckbauer A. Effects of a leucine and pyridoxine-containing nutraceutical on body weight and composition in obese subjects. *Diabetes Metab Syndr Obes* 2013;6:309-315.
45. Das A, Huang GX, Bonkowski MS, et al. Impairment of an endothelial NAD⁺-H2S signaling network is a reversible cause of vascular aging. *Cell* 2018;173:74-89.e20.
46. Lonardo A, Nascimbeni F, Mantovani A, Targher G. Hypertension, diabetes, atherosclerosis and NASH: cause or consequence? *J Hepatol* 2018;68:335-352.
47. Patil R, Sood GK. Non-alcoholic fatty liver disease and cardiovascular risk. *World J Gastrointest Pathophysiol* 2017;8:51-58.
48. Bristol-Myers Squibb Company. US Food and Drug Administration website. Glucophage (metformin hydrochloride) tablets [product information]. https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/020357s037s039,021202s021s023lbl.pdf. Revised April 2017. Accessed May 23, 2018.