Leucine amplifies the effects of metformin on insulin sensitivity and glycemic control in diet-induced obese mice

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Background and objective. The Sirt1/AMPK signaling pathway is a key sensor of energy status and regulates glucose and lipid metabolism. Leucine (Leu) activates Sirt1 by lowering its Km for NAD+ and potentiates other sirtuin/AMPK-activators, resulting in improvement of insulin sensitivity. Since metformin (Met) converges on this pathway, we hypothesized that leucine would amplify its gluco-regulatory effects.

Materials and methods. The effects of Leu (24 g/kg diet) + Met (0.05–0.5 g/kg diet) combinations were compared to standard therapeutic Met (1.5 g/kg diet; ~300 mg/kg BW) on glycemic control in high fat diet induced insulin resistant mice for 6 weeks. The effects of Leu on Met stimulation of Sirt1 and AMPK activities were further evaluated in adipocytes.

Results. Sub-therapeutic levels of Met combined with Leu resulted in increases in Sirt1 activity and in tissue P-AMPK/AMPK ratio and corresponding dose-responsive improvements in fasting and post-prandial glucose, in glucose response to an insulin tolerance test and in the area under the curve in glucose tolerance tests. Changes were evident within 7 days of treatment and sustained throughout the 6-week study duration. The Leu + Met (0.25 g/kg)–combinations produced a comparable effect to a standard therapeutic Met dose, while the Leu + Met (0.5 g/kg diet) resulted in greater improvements. Since resveratrol also synergizes with leucine to augment sirtuin signaling and insulin sensitivity, we tested the addition of resveratrol to Leu–Met and found no additional benefit.

Conclusion. These data demonstrate that adding Leu to Met enables a dose reduction of 66% with improved efficacy and of 83% with comparable efficacy to standard metformin in diet-induced obese mice, and addition of resveratrol does not provide further benefit.

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Abbreviations: ACC, acetyl CoA carboxylase; AMP, adenosine monophosphate; AMPK, 5’-adenosine monophosphate-activated protein kinase; ATP, adenosine triphosphate; BCAA, branched-chain amino acid; BCKD, branched-chain α-ketoacid dehydrogenase; BW, body weight; DIO, diet-induced obese; GTT, glucose tolerance test; HFD, high-fat-diet; HOMAIR, homeostasis model assessment of insulin resistance; HMB, β-hydroxy-β-methyl-butyrate; Leu, leucine; ITT, insulin tolerance test; LFD, low-fat-diet; Met, metformin; NAD, nicotinamide adenine dinucleotide; NAMPT, nicotinamide phosphoribosyltransferase; OCR, oxygen consumption rate; Res, resveratrol; Sirt1, sirtuin 1.

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1. Introduction

Metformin is considered the initial drug of choice for treating type 2 diabetes, as it is highly efficacious, exhibits an excellent safety profile, does not promote weight gain, does not increase the risk for hypoglycemia and has been shown to reduce the risk of diabetes-related comorbidities and death [1]. However, despite these advantages, its therapeutic utility is often limited by the occurrence of dose-related gastrointestinal adverse effects (especially at the full therapeutic dose of 1500 to 2000 mg/day), which often lead to dose reduction and/or compliance issues in 30% of patients and drug discontinuation in up to 10% of patients [2,3]. Moreover, metformin monotherapy often fails to achieve optimal glycemic control due to inter-individual variability in response to drug initiation and maintenance [4].

The blood glucose lowering effect of metformin is caused by suppression of hepatic glucose production as well as increased peripheral glucose disposal [4-7]. Most of these effects are dependent on activation of the 5’ adenosine monophosphate-activated protein kinase/sirtuin1 (AMPK/Sirt1) pathway. Despite some controversy regarding whether metformin activates AMPK as a consequence of a mild inhibition of the mitochondrial respiratory chain complex 1 [8], or due to an inhibition of AMP-deaminase [9], both mechanisms result in increased cellular adenosine monophosphate (AMP) and activation of AMPK, either with or without inhibition of mitochondrial energy production. AMPK activation, in turn, increases the ratio of oxidized to reduced nicotinamide adenine dinucleotide (NAD+/NADH ratio) resulting in increased Sirt1 activity [10]. There is also evidence supporting an AMPK-independent mechanism of action, as hepatic gluconeogenesis has been shown to be reduced in mice lacking hepatic AMPK, suggesting adenosine triphosphate (ATP) depletion rather than direct inhibition of gluconeogenic gene expression as a contributing mechanism for inhibited hepatic glucose production [11].

Insulin resistance characterizes type 2 diabetes, resulting in impaired glucose and lipid metabolism in muscle, adipose tissue and liver. AMPK and Sirt1 are key regulators of energy metabolism and, due to their bidirectional interaction and cross-activation, they are targets of common activators and produce similar metabolic outcomes [12,13]. There is large body of evidence showing an association between decreased activity of the AMPK/Sirt1 axis in obesity, insulin resistance and diabetes, while activation of either one prevents and improves hyperglycemia and insulin resistance [14-18].

Our previous work on the branched-chain amino acid (BCAA) leucine has demonstrated that leucine, as well as its metabolite β-hydroxy-β-methyl-butyrate (HMB), activates Sirt1 by lowering the activation energy for NAD+ [19], enabling Sirt1 activation at a lower NAD+/NADH ratio characteristic of energy replete states. Additionally, leucine and HMB coactivate and amplify the effects of other compounds that converge on the AMPK/Sirt1 axis, thus enabling significant dosage reduction [19]. This synergistic effect has been demonstrated for resveratrol and other polyphenols as well as for metformin [19-21]. Thus, a combination of leucine with a low dose of metformin could substantially reduce the development of metformin’s adverse effects without interfering with its efficacy on improving insulin sensitivity. Consistent with this notion, the short-term efficacy of a combination of HMB and resveratrol with low dose metformin in improving insulin tolerance in db/db mice was recently demonstrated [20]. The present study was designed to more comprehensively evaluate the long-term efficacy of leucine in augmenting the effects of metformin on insulin sensitivity in a mouse model of diet-induced obesity (DIO) and insulin resistance and to determine whether resveratrol can further enhance the response.

2. Research design and methods

2.1. Animals and diets

Six to eight week old male C57BL/6 mice were purchased from Jackson Laboratories. Obesity and insulin resistance were induced via a high-fat diet (HFD) for 6 weeks. The animals were then randomized into one of the following groups with 10 animals/group and kept on their diet for 6 weeks.

For study 1 (treatment groups without resveratrol): 1) Control (low-fat diet (LFD), standard diet (LabDiet 5001)) 2) High-fat diet (HFD, Research Diets, 60% fat), 3) HFD + Leucine (24 g/kg diet) + metformin (0.15 g/kg diet) (Leu + Met 0.15), 4) HFD + Leucine + metformin (0.25 g/kg diet) (Leu + Met 0.25), 5) HFD + Leucine + metformin (0.5 g/kg diet) (Leu + Met 0.5), 6) HFD + metformin control (1.5 g metformin/kg diet) (Met 1.5). The 1.5 g metformin/kg diet concentration was designed as a standard therapeutic dose to achieve a final dosing of ~300 mg/kg BW, and the lower doses were selected as sub-therapeutic doses that we previously found to exert no effect [20].

For study 2 (treatment groups with resveratrol): 1) LFD, 2) HFD Control, 3) HFD + Leucine (24 g/kg diet) (Leu), 4) HFD + Leucine + resveratrol (12.5 mg/kg/diet) (Leu + Res), 5) HFD + Leucine + resveratrol + metformin (0.25 g/kg diet) (Leu + Res + Met 0.25), 6) HFD + Leucine + resveratrol + metformin (0.15 g/kg diet) (Leu + Res + Met 0.15), 7) HFD + Leucine + resveratrol + metformin (0.05 g/kg diet) (Leu + Res + Met 0.05), 8) HFD + metformin control (1.5 g/kg diet) (Met 1.5).

For study 3 (treatment groups with HMB): 1) LFD control, 2) HFD control, 3) HFD + HMB (Ca-HMB, 2 g/kg diet) + metformin (0.15 g/kg diet) (HMB + Met 0.15), 4) HFD + HMB + metformin (0.25 g/kg diet) (HMB + Met 0.25), 5) HFD + HMB + metformin (0.5 g/kg diet) (HMB + Met 0.5), 6) HFD + metformin control (1.5 g metformin/kg diet) (Met 1.5).

Animals were housed in polypropylene cages at a room temperature of 22 °C with a 12 h light/dark cycle. The animals had free access to water and their experimental food throughout the experiment. Body weight was measured every week. Blood glucose was measured in the fed or fasted state using an OneTouch Ultra Glucose meter (Lifescan, Milpitas, CA). At the end of the treatment period (6 weeks) all animals were humanely euthanized with CO2 inhalation. Blood and tissues were collected for further experiments as described below.

This study and all animal procedures were performed under the auspices of an Institutional Animal Care and Use Committee-approved protocol of the Georgia State University.

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and in accordance with PHS policy and recommendations of the Guide.

2.2. Insulin tolerance test (ITT)

Prior to each ITT, food was removed from the mice for 4 to 6 h and basal blood glucose level was measured. Then the mice were injected intraperitoneally with insulin (1.0 U/kg body weight (BW)) in ~ 0.1 ml 0.9% NaCl. Blood glucose was then measured 15, 30, 60, 90 and 120 min after insulin injection. Change in blood glucose over the linear portion of the response curve was then calculated.

2.3. Glucose tolerance test (GTT)

Prior to each GTT, mice were fasted overnight (~16 h) and basal blood glucose level was measured. Then the mice were injected with glucose (1.2 g/kg BW) intraperitoneally and blood glucose was then measured 15, 30, 60, 90 and 120 min after glucose injection. The area under the curve of the response curve was then calculated.

2.4. HOMAIR

The homeostasis model assessment of insulin resistance (HOMAIR) was used as an index of changes in insulin sensitivity. HOMAIR is calculated via standard formula from fasting plasma insulin and glucose as follows: HOMAIR = [insulin (µU/mL) × glucose (mmol/L)]/22.5. The plasma glucose and insulin concentrations were measured using the Glucose Assay Kit from Cayman (Ann Arbor, MI) and the Insulin kit from Millipore (Billerica, MA), respectively.

2.5. Cell culture

Murine 3T3-L1 pre-adipocytes were grown in the absence of insulin in Dulbecco’s modified Eagle’s medium (DMEM, 25 mmol/L glucose) containing 10% fetal bovine serum (FBS) and antibiotics (1% penicillin-streptomycin) (adipocyte medium) at 37 °C in 5% CO2 in air. Confluent pre-adipocytes were induced to differentiate with a standard differentiation medium (DM2-L1, Zen-Bio, NC). Pre-adipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium for further differentiation. Medium was changed every 2-3 days; differentiation was determined microscopically via inclusion of fat droplets.

2.6. Sirt1 activity (Fleur-de-Lys)

Sirt1 activity was measured by using the Sirt1 Fluorimetric Drug Discovery Kit (BML-AK555, ENZO Life Sciences, Farmingdale, NY). The sensitivity and specificity of this assay kit were tested by Nin et al. [22]. Sirt1 activity is assessed by the degree of deacetylation of a standardized substrate containing an acetylated lysine side chain. The substrate utilized is a peptide containing amino acids 379–382 of human p53 [Arg-His-Lys-Lys[Ac]], an established target of Sirt1 activity; Sirt1 activity is directly proportional to the degree of deacetylation of Lys-382. Samples were incubated with peptide substrate (25 µmol/L), and NAD+ (500 µmol/L) in a phosphate-buffered saline solution at 37 °C on a horizontal shaker for 45 min. The reaction was stopped with the addition of 2 mmol/L nicotinamide and a developing solution that binds to the deacetylated lysine to form a fluorophore. Following 10 min incubation at 37 °C, fluorescence was read in a plate-reading fluorimeter with excitation and emission wavelengths of 360 nm and 450 nm, respectively. Resveratrol (100 mM) served as a Sirt1 activator (positive control) and suramin sodium (25 mM) as a Sirt1 inhibitor (negative control). Sirt1 activity was measured in a modified assay using 5 µL of cell lysate. The lysates were prepared by homogenizing cells in ice-cold RIPA buffer plus protease inhibitor mix (Sigma Aldrich, St. Louis, MO). After 10 min incubation on ice, the homogenate was centrifuged at 14,000×g and the supernatant was used for further experiments. Data for endogenous Sirt1 activation were normalized to cellular protein concentration measured via BCA-assay.

2.7. Western blot

The Phospho-AMPK, AMPK, p70S6 kinase, Phospho-p70S6 kinase, 4E-BP1 and Phospho-4E-BP1 antibodies were obtained.

A

B

Fig. 1 – Glucose Tolerance Test (GTT) and body weight after 6 weeks of HFD feeding. Mice were fed an LFD or HFD for 6 weeks. (A) Before randomization to treatment groups, GTT was performed in the LFD group and a representative group of HFD fed animals. * indicates significant different from LFD. (B) Body weight before start of indicated treatments. Data are presented as means ± SEM (n =8-10) (ns = no significant difference between groups).
from Cell Signaling (Danvers, MA). Phospho-acetyl-CoA carboxylase (ACC) and total ACC antibody were obtained from Millipore/Upstate Biotechnology (Lake Placid, NY). β-Actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Protein levels of cell extracts were measured by BCA kit (Thermo Scientific). For Western blot, 10–50 μg protein was resolved on 4%–15% gradient polyacrylamide gels (Criterion precast gel, Bio-Rad Laboratories, Hercules, CA), transferred to either PVDF or nitrocellulose membranes, incubated in blocking buffer (3% BSA or 5% non-fat dry milk in TBS) and then incubated with primary antibody (1:1000 dilution), washed and incubated with horseradish peroxidase- or fluorescence-conjugated secondary antibody (1:10,000 dilution). As previously described[23], total ACC was detected using Streptavidin-conjugated horseradish peroxidase (Amersham Biosciences, Piscataway, NJ). Visualization was conducted using BioRad ChemiDoc instrumentation and software (Bio-Rad Laboratories, Hercules, CA) or Li-COR Odyssey Fc Imaging system (Li-COR Biosciences) and band intensity was assessed using Image Lab 4.0 or Quantity One (Bio-Rad Laboratories, Hercules, CA), with correction for background and loading controls.

2.8. Fatty acid oxidation

Cellular oxygen consumption rate (OCR) was measured using a Seahorse Bioscience XF24 analyzer (Seahorse Bioscience, Billerica, MA) in 24-well plates at 37 °C. 3T3L1 adipocytes were seeded at 40,000 cells per well, differentiated as described above, treated for 24 h with the indicated treatments, washed twice with non-buffered carbonate-free pH 7.4 low glucose (2.5 mmol/L) DMEM containing carnitine (0.5 mmol/L), equilibrated with 550 μL of the same media in a non-CO2 incubator for 30 min, and then inserted into the instrument for 15 min of further equilibration. O2 consumption was measured in three successive baseline measures at eight-minute intervals prior to injection of palmitate (200 μmol/L final concentration). Post-palmitate-injection measurements were taken over a 3-h period with cycles consisting of 10 min break and three successive measurements of O2 consumption.

Fig. 2 – Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT). DIO-mice were fed an LFD or HFD with indicated treatments. After 5 weeks GTT and ITT were performed. Glucose levels were measured at 15, 30, 60, 90 and 120 min after glucose injection (A) or insulin injection (B). The area under the curve from GTT and the change in glucose response from baseline at 30 min after insulin injection were calculated. Data are presented as means ± SEM (n = 10) (ns = no significant difference between groups). Color coding: LFD: black, HFD: orange, Leu + Met 0.15: red, Leu + Met 0.25: green, Leu + Met 0.5: purple, Met 1.5: blue.
2.9. Statistical analysis

All data are expressed as mean ± SEM. Data were analyzed by one-way ANOVA, and significantly different group means (p < 0.05) were separated by the least significant difference test using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, www.graphpad.com).

3. Results

As expected, the six-weeks of obesity induction with HFD caused significant fasting and postprandial hyperglycemia in the GTT and a significant weight gain (Fig. 1). The HFD induced fasting and post-prandial hyperglycemia was not significantly affected by the addition of leucine (Fasting glucose: HFD, 198.3 ± 7.6 mg/dL; HFD + Leu, 174.6 ± 19.2 mg/dL; GTT Area Under the Curve: HFD, 29,163 ± 1532; HFD + Leu, 27,329 ± 1662). However, adding a sub-therapeutic level of 0.15 or 0.25 g/kg diet of metformin to the leucine reduced the HFD-induced hyperglycemia comparable to full-dose metformin (Met 1.5); adding 0.50 g metformin/kg to the leucine diet resulted in a significantly greater reduction in the area under the curve (Fig. 2A). Further, the blood glucose response to insulin (ITT) (Fig. 2B) was improved in the Leu + Met 0.25 and Leu + Met 0.50 groups, comparable to the effect of full-dose metformin, while the Leu + Met 0.15 group did not significantly affect this parameter.

In addition, the leucine/metformin groups significantly reduced fasting blood glucose and insulin levels comparably to full-dose metformin (Fig. 3A and B), and the Leu + Met 0.5 group exerted a significantly greater effect on HOMAIR, resulting in a HOMAIR value not different from the LFD group (Fig. 3C).

A replicate of this study was also conducted with the addition of resveratrol to the leucine/metformin groups. Comparable improvements to those found in the absence of resveratrol were found in GTT (Fig. 4A) and ITT (Fig. 4B), indicating that resveratrol does not enhance the synergistic effects of leucine and metformin on insulin sensitivity.

This study was also repeated with HMB substituted for leucine, using the HMB dose optimized in our previous study [20]. Fig. 5 summarizes the data for the HMB study. Although both, leucine and HMB combinations with metformin exhibited qualitatively comparable outcomes for most parameters, the leucine/metformin combinations exerted quantitatively superior effects on glycemic control in DIO-mice (Fig. 5). The HMB + Met 0.5 combination resulted in a reduction in postprandial glucose level comparable to full-dose metformin; the Leu + Met 0.5 effects were superior to full dose metformin (Fig. 5A and C). Similarly, there was a significant greater reduction in the GTT in

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the Leu + Met 0.5 group compared to Met 1.5 than by HMB + Met 0.5 (Figs. 2A and 5B).

Based on our previous work, we hypothesized that the synergistic effects of leucine or HMB with metformin will involve activation of the AMPK/Sirt1 pathway. Indeed, the Leu + Met combination induced a significant 46% increase in Sirt1 activity in adipocytes (Fig. 6A). Similarly, the HMB + Met combination induced a significant 30% increase in the P-AMPK/AMPK ratio (Fig. 6C). To test whether the effects are dependent on AMPK activation, we measured fatty acid oxidation, an outcome measure of AMPK stimulation, in the presence and absence of an AMPK inhibitor. The palmitate-induced fatty acid oxidation was increased by 24-h Leu + Met treatment compared to control; however, the addition of the AMPK inhibitor Compound C completely blocked this increase (Fig. 6B), indicating AMPK dependence.

Consistent with the in vitro data, the P-AMPK/AMPK ratio and the P-ACC/ACC ratio, an AMPK downstream target, were up to three-fold up-regulated in muscle of the DIO-mice by all leucine/metformin combinations comparable to full-dose metformin (Fig. 7). Since high concentrations of leucine are known to stimulate the mTOR pathway, we measured the downstream targets p70S6Kinase and 4E-BP1 (phosphorylated and total) of this pathway in muscle tissue. Phospho-p70S6K was undetected in all groups and there was no difference among groups for p70S6K and p-4E-BP1/4E-BP1 ratio (Supplementary Fig. 1).

4. Discussion

These data demonstrate that metformin synergizes with leucine to improve hyperglycemia and insulin resistance in a mouse model of obesity and insulin resistance. This synergy results in dose reduction of metformin up to 83% with no loss of efficacy in this model.

The metformin concentrations in this study were based on literature values of full therapeutic dose (300 mg/kg BW) and very low dose (50 mg/kg BW) metformin studies in mice [24,25]. The very low dose was shown to have no independent

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The leucine level used in the treatment groups was based on previous data demonstrating that this is sufficient to increase normal fasting leucine (~0.1 mmol/L) to plasma levels of ~ 0.4–0.5 mmol/L and to achieve tissue Sirt1 activation [21,26,27], consistent with the in vitro dose necessary for Sirt1 activation [28,29]. However, as shown in Fig. 4, this concentration had no independent effect on GTT or ITT.

We recently found both leucine and HMB to allosterically activate Sirt1 directly in a cell-free system, reducing the Km for NAD⁺ and thereby mimicking the effects of caloric restriction [19,30]. This also allows other activators (including metformin) to stimulate Sirt1 at lower concentrations [19,20], as summarized in Fig. 8. Accordingly, a low dose of resveratrol (12.5 mg/kg diet) combined with either leucine or HMB produced significant improvement in adiposity, insulin sensitivity and inflammatory markers in diabetic mice. These effects were superior to an almost 20 times higher dose of resveratrol alone [21].

Metformin’s actions are versatile and still not fully understood. Nevertheless, most of its glucose-lowering effects appear to be mediated through the activation of AMPK, either directly or indirectly by increasing the AMP/ATP ratio in liver and peripheral tissues [1,6,31–33]. Moreover, metformin action also appears to be mediated by Sirt1, as gluconeogenic gene expression was inhibited by metformin stimulation of Sirt1 and GCN5 via both AMPK-independent and dependent mechanisms in the liver of diabetic mice and HepG2 cells [10]. The data presented in this study also indicate that AMPK and Sirt1 modulate the observed effects of the combination of metformin with leucine in muscle, adipose tissue and liver.

The leucine/metformin effects on palmitate-induced fat oxidation were completely blocked by the addition of Compound C (Fig. 6B), and both P-AMPK/AMPK ratio and Sirt1 activity were up-regulated in adipocytes (Fig. 6A and C). Moreover, P-AMPK/AMPK and the downstream target P-ACC/ACC were increased in muscle of the DIO-mice by the combinations as well as by full-dose metformin (Fig. 7). In addition, AMPK-regulated lipogenic genes such as ACC, SCD1 and FAS were inhibited in liver, and there was an associated reduction of the HFD-induced hepatic lipid accumulation and of total liver weight [34].

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In contrast to the salutary effects of leucine described here, others have proposed that elevated blood branched-chain amino acids, including leucine, may contribute to the development of insulin resistance and diabetes [35,36]. However, this observed rise appears to be secondary to aberrant amino acid metabolism, specifically a down-regulation of the branched-chain α-ketoacid dehydrogenase (BCKD), the rate-limiting enzyme of BCAA catabolism, in liver and adipose tissue [37–39]. Thus, it is likely that increased plasma BCAAs is a consequence rather than a cause of insulin resistance [37,39]. In support of this concept, data from a number of studies show that diets high in BCAA restore aberrant BCKD activity and improve glucose and insulin sensitivity [26,27,40,41].

Leucine is also known to stimulate the mTOR pathway, which may promote insulin resistance. However, concentrations above 1.5 mmol/L of leucine appear to be necessary for this effect, which is about three-times higher than the concentration we used in our in vitro data [42,43]. In addition, metformin inhibits mTORC1 signaling both through AMPK-dependent (phosphorylation of raptor) and AMPK-independent (inhibition of Rag GTPases) mechanisms [44]. However, since the Rag GTPases are necessary for the activation of mTORC1 by amino acids including leucine [45], it is very likely that metformin also may blunt the effects of leucine on mTORC1 activation. In line with this, our data demonstrate that there was no stimulation of the mTOR pathway by the leucine/metformin combinations (Supplementary Fig. 1).

We previously demonstrated the short-term efficacy (2 weeks) of a resveratrol/HMB/metformin combination on insulin sensitivity in db/db mice [20]. Therefore, the present study was designed to examine more comprehensively the long-term efficacy (6 weeks) of a leucine/metformin combination and to assess the need for resveratrol in this combination. Since leucine and its metabolite, HMB, exerted qualitatively comparable outcomes for most parameters, the leucine/metformin combinations exerted quantitatively superior effects on glycemic control. The reason for this is not clear, as we previously found these doses of HMB and leucine to exert comparable effects on energy metabolism and adiposity when combined with resveratrol in the DIO mouse model [21]. However, leucine–resveratrol exerted a significantly greater
effect on Sirt1 activation than HMB–resveratrol in adipose and skeletal muscle [21], suggesting that lower Sirt1 activation may contribute to this reduced efficacy.

Our early observations of an interaction between leucine and resveratrol in activating Sirt1 [19–21] suggested that resveratrol may be a useful component in a leucine/metformin based combination for glycemic control. However, comparison of data from Study 1 and Study 2 demonstrates that resveratrol does not provide further benefit, as comparable effects were found in the presence (study 1) and absence (study 2) of resveratrol (Figs. 2 and 4).

The leucine/metformin combination used in this study enabled a dose reduction of metformin up to 83% with no loss of efficacy (Leu–Met 0.25) and up to 66% (Leu + Met 0.5) with improved efficacy in some of the parameters; these are calculated to be equivalent to human doses of 250–500 mg/day. Most adverse effects of metformin are dose-dependent and are most frequently observed only if therapeutic doses (≥1500 mg/day) are achieved. The most prominent symptoms are gastrointestinal such as nausea, vomiting, diarrhea and abdominal pain, which occur in up to 30% of patients and may lead to compliance issues and/or drug discontinuation [3,46,47]. In addition, the presence of co-morbidities, particularly renal impairment, may limit or contraindicate the use of metformin at standard doses [48,49]. Therefore, a combination therapy that enables substantial metformin dose reduction by 83% to achieve comparable efficacy and by 66% to achieve greater efficacy than full-dose metformin. These dose reductions translate into levels of metformin that are not associated with gastrointestinal distress and other adverse effects otherwise associated with metformin, indicating that a leucine–low dose metformin combination may prove useful as a treatment alternative in patients with metformin intolerance or contraindications.

5. Conclusion

Leucine amplifies the effect of metformin on glucose control. These effects are mediated, at least in part, by the activation of the AMPK/Sirt1 pathway (Fig. 8). The addition of resveratrol does not improve these effects and is therefore unnecessary in the formulation for treatment of diabetes. The amplifying effects of the metformin/leucine combination enable a substantial dose reduction of metformin by 83% to achieve comparable efficacy and by 66% to achieve greater efficacy than full-dose metformin. These dose reductions translate into levels of metformin that are not associated with gastrointestinal distress and other adverse effects otherwise associated with metformin, indicating that a leucine–low dose metformin combination may prove useful as a treatment alternative in patients with metformin intolerance or contraindications.

Submission declaration and verification

Data were presented as a Poster Presentation at the ADA 2014 conference, San Francisco (Diabetes 2014; 63 (Suppl 1): A288).

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Authors’ contribution

AB was responsible for design and execution of the cell experiments. LZ was responsible for execution of the animal experiments.
experiments. FL, QC, XC and RW were responsible in the assistance of the completion of animal experiments. BZ, HS and MBZ were responsible for experimental design of the animal studies, data analysis and interpretation. BZ and HS supervised the animal studies and sample analysis. AB, BZ, HS and MBZ were responsible for writing and editing the manuscript.

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**Disclosures**

The authors Antje Bruckbauer and Michael B. Zemel are employees and stockholders of NuSirt Biopharma. All other authors declare that they have no conflict of interest.

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