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# BSN272 Prevents Western Diet-Induced Atherosclerosis and Excess Weight Gain in ApoE<sup>-/-</sup> Mice

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None

# BSN272 Prevents Western Diet-Induced Atherosclerosis and Excess Weight Gain in ApoE<sup>-/-</sup> Mice

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## Abstract

**Objective:** The purpose of this study was to determine whether BSN272 could prevent the development of hyperlipidemia and atherosclerosis in ApoE<sup>-/-</sup> knockout mice fed a Western (high fat and cholesterol, high sugar) diet.

**Background:** BSN272 is a combination drug therapy consisting of D-tagatose and polydatin. D-tagatose has been studied for the treatment of diabetes for several years and has been shown to lower glycated hemoglobin (HbA1c), to reduce cholesterol and triglycerides, as well as prevent weight gain in animals and humans. Polydatin is an antioxidant that appears to promote lipid catabolism and evidence suggests it can reduce diet-induced dyslipidemia in animals.

**Methods:** ApoE-deficient mice were randomized to produce three groups with the same mean body weight. The mice were given the following diets for 16 weeks: Group 1 - standard control diet; Group 2 - Western diet (high fat, high cholesterol diet, high sucrose); Group 3 - Western diet formulated with BSN272. Mice were measured for weight gain, tissue and organ weights, total serum cholesterol and triglycerides and formation of aortic atherosclerosis.

**Results:** Treatment of ApoE<sup>-/-</sup> mice prevented weight gain and lowered total cholesterol compared to mice on a Western diet alone. The effectiveness of BSN272 in lowering cholesterol increased over the time course of the experiment, with cholesterol steadily decreasing over the 16 weeks of treatment. Mice treated with BSN272 also showed significantly lower triglycerides than mice on the standard diet or the Western diet. The addition of BSN272 to the Western diet prevented the formation of atherosclerotic plaques compared to Western diet alone.

**Conclusion:** BSN272 prevents the development of atherosclerosis, excess weight gain, and rise in total cholesterol and triglycerides in ApoE<sup>-/-</sup> mice induced by a Western diet.

## Introduction

Evidence supports a link between obesity and a spectrum of diseases including type 2 diabetes, hypertension, abnormal blood lipids (usually in the form of hyperlipidemia), and increased risk for cardiovascular disease. Hyperlipidemia is typically characterized by elevated levels of triglycerides and low-density lipoprotein (LDL) cholesterol and by low levels of high-density lipoprotein (HDL) cholesterol. This disease commonly manifests in those who are obese and those with type 2 diabetes, and is thought to be a major contributor to the increased incidence of cardiovascular disease seen in these two populations (1,2). Reduction of elevated LDL is a major drug treatment goal and has produced significant reduction in cardiovascular events in patients with cardiovascular disease and diabetes (3). In addition to lowering LDL, research has also shown that raising HDL in persons with low HDL can reduce the number of coronary events, a finding that has led to an interest in the development of drugs that can accomplish this HDL improvement (4).

Elevated serum cholesterol levels have been noted in rodents (5), dogs (6), nonhuman primates (7), and humans (8) consuming a high-carbohydrate diet, particularly one including fructose and sucrose. Recent studies have provided evidence that fructose causes hyperlipidemia postprandially, both directly through the synthesis of fatty acids, and indirectly by increasing liver re-esterification of fatty acids (9). Low-density lipoprotein receptor deficient (LDLR<sup>-/-</sup>) mice fed a high sucrose diet exhibited elevated serum LDL cholesterol concentrations and increased atherosclerosis compared to mice fed an energy-matched diet enriched in saturated fatty acids (10).

D-tagatose, a naturally occurring epimer of fructose, was originally developed as a low-calorie sweetener (1.5 kcal/g compared to 4 kcal/g for sucrose) but was found to have an antihyperglycemic effect in animal and in human studies and showed promise as a treatment for type 2 diabetes and obesity (11, 12, 13). After over 10 years of animal and human studies for use as a food sweetener, D-tagatose was classified as being "generally recognized as safe (GRAS)" by the

FDA and has been used since in food and beverage products with no adverse events reported.

The mechanism by which D-tagatose produces its antihyperglycemic effect in response to a meal is not clear. However, based on studies with fructose and D-tagatose, it has been proposed that D-tagatose is metabolized following a pathway that is essentially the same as that of fructose (13). After absorption from the intestine and transport to the liver, fructokinase phosphorylates D-tagatose to produce D-tagatose-1-phosphate. D-tagatose-1-phosphate can stimulate glucokinase activity (14, 15) leading to increased phosphorylation of glucose to glucose-6-phosphate leading to further activation of glycogen synthase (16). There has been speculation that D-tagatose-1-phosphate can inhibit glycogen phosphorylase in the same manner that fructose-1-phosphate does (13), but this has not been directly shown. By activating glycogen synthase and possibly inhibiting glycogen phosphorylase, D-tagatose-1-phosphate increases glycogen synthesis and inhibits glycogen utilization, explaining, at least in part, the antihyperglycemic effect of the sugar. In addition to the effect on glycogen regulation, D-tagatose inhibits sucrase (17), leading to the suppression of sucrose digestion in the small intestine and inhibits the activity of maltase, at least in vitro, and could slow the digestion of starch. The net effect of the regulation of these enzymes is an increase in glycogen synthesis and storage and a decrease in glycogen utilization. In addition, D-tagatose reduces the absorption and digestion of sucrose and other carbohydrates in the small intestine. D-tagatose has been shown in both animal and human studies to have multiple effects including increase in satiety and weight control, a beneficial effect on abnormal blood lipids, a reduction in atherosclerotic plaque formation, and a reduction in blood glucose and HbA1c levels in patients with type 2 diabetes mellitus (11, 13, 18 - 26).

In addition to its antihyperglycemic effects, D-tagatose has been found to have an effect on blood lipid levels in animals and in humans. In one study, LDL<sup>-/-</sup> mice fed a diet in which high sucrose was replaced with an equivalent amount of D-tagatose had reduced cholesterol, triglycerides and atherosclerosis compared to mice on the diet containing sucrose (24). In a human clinical trial, patients with type 2 diabetes taking D-tagatose were found to have improved HDL levels, increasing from 30 to 41.7 mg/dL over the course of the 14 month study (20). This is interesting in light of evidence suggesting that increasing HDL levels decrease the risk of coronary events. The mechanism by which D-tagatose raises HDL is not

clear, but it should be noted that these patients did lose weight during the study and this may have contributed to the improvement in HDL. In other studies, type II diabetics taking D-tagatose showed a decrease in HbA1c and serum triglycerides (11, 12).

There is considerable interest in the use of trans-resveratrol and its derivatives, including polydatin, for the treatment of many human diseases (27). Extracts derived from *Polygonum cuspidatum* have long been a part of traditional Chinese herbal medicine being used to treat pain, fever, coughs, inflammation and a variety of other ailments (28). Polydatin, a glucoside derivative of resveratrol, is the major component of these extracts. In addition to *Polygonum*, polydatin has been found in wines and grapes (29-32), cocoa (33), peanuts and peanut butter (34), pistachios (35) and almonds (36). As a derivative of resveratrol, polydatin is believed to have many of the same beneficial effects but has some properties that may make it more effective from a pharmacological standpoint than resveratrol. Polydatin is structurally the same as resveratrol except that it has a glucoside group attached to the C-3 position in place of a hydroxyl group. This substitution makes polydatin more water soluble and more resistant to enzymatic breakdown than resveratrol. It is also actively taken up by cells via glucose carriers in the cell membrane instead of being passively transported like resveratrol (37, 38). These properties would suggest that polydatin would have greater bioavailability than resveratrol.

Claims for the many health benefits of polydatin abound. A multitude of studies have presented evidence that polydatin has many positive health effects including anti-inflammatory (39, 40), hepatoprotective (41-44), anti-cancer (45-48), neuroprotective (39, 49-51), and cardioprotective activities (28, 52-55). Pharmacological studies and clinical practice have demonstrated that polydatin also has protective effects against shock (56, 57), ischemia/reperfusion injury (58, 59), congestive heart failure (60), endometriosis (61), and prevention of fatty liver disease and insulin resistance (62), and that it can regulate glucose and lipid metabolism (63). Polydatin has found its way into clinical trials for the treatment of hemorrhagic shock and irritable bowel syndrome (40, 64).

How polydatin is able to have all of these activities is still being studied but multiple mechanisms of action are evident, including; an antioxidant, free radical-elimination mechanism (65-66), activation of protein kinase C (67, 68), inhibition of the activation of renin-angiotensin-aldosterone system and decreasing

the excretion of endothelin 1, TNF-alpha, and angiotensin II (54), reduction of lipid peroxidation levels (37, 69), up regulation of the expression of hippocampal brain-derived neurotrophic factor (70), enhanced insulin sensitivity in the liver as shown by improved insulin receptor substrate 2 expression levels and Akt phosphorylation (63), decreasing the content of malonyldialdehyde (MDA) (50), promoting the activities of total superoxide dismutase (T-SOD), catalase and glutathione peroxidase (GSH-Px) in plasma, and increasing the content of glutathione (GSH) in myocardial tissue (66), restoring decreased deacetylase sirtuin1 activity and protein expression in liver tissue following severe shock (71) and activation of sirtuin (72, 73), suppressing oxidative stress-induced lysosomal instability and mitochondrial injury by increasing the protein expression of SOD2 (71).

The use of polydatin as a potential therapy for dyslipidemia has been suggested primarily by three studies using animal models (52, 53, 74). Arichi et al (74) discovered that orally administered polydatin (100 mg/kg body weight) significantly lowered low-density lipoprotein (LDL)-derived cholesterol by approximately 18% and serum triglycerides by 40% in rats consuming standard chow containing a mixture of corn oil, 10% cholesterol, and 1% cholic acid. Although lower doses of trans-polydatin (50 mg/kg body weight) were ineffective at preventing hyperlipidemia, they were able to prevent the accumulation of cholesterol and triglycerides in the liver, suggesting that lower doses may also be effective but to a much lesser extent. In a study using Syrian golden hamsters, polydatin was found to decrease total cholesterol levels and total triglyceride levels by 47% and 63%, respectively, compared to standard diet (52). In another study using rabbits, the administration of polydatin decreased the serum levels of total cholesterol, triglycerides and LDL ((53). The ratio of total cholesterol to HDL was also reduced.

Insulin, through activation of the Akt pathway and other metabolic pathways, is a major component of metabolic regulation (75). Hao et al. recently found that polydatin activated the Akt signaling pathway in diabetic rats, possibly by phosphorylation of the insulin receptor substrate (IRS), thus reducing blood glucose levels (63). Polydatin may also decrease the expression of intercellular adhesion molecule 1 (ICAM-1) and may reduce white blood cell adhesion, as well as the effects of other cell adhesion molecules and inflammatory cytokines, thought to be active in early atherosclerotic development (76). Additionally, polydatin is also thought to provide protection from

oxidative peroxidation which can result in cell damage (37, 77) and inhibition of oxidation of LDLs which may also play a role in atherosclerosis (28).

In the present study, we have examined the effect of BSN272, a combination of D-tagatose and polydatin, on blood lipids and atherosclerosis in ApoE<sup>-/-</sup> knockout mice. The apoE lipoprotein resides on very low, intermediate, and high density lipoproteins (VLDL, LDL, HDL, respectively) and mediates the removal of atherogenic particles from plasma by acting as a ligand for low density lipoprotein (LDL) receptors. Mice are normally resistant to the development of atherosclerosis. However, inactivation of the apoE gene in mice results in severe hypercholesterolemia and the rapid development of atherosclerosis, particularly when fed a Western type diet consisting of high fat, high cholesterol and high sugar (78-82). The progression and histopathology of lesions in this animal model show features similar to those observed in humans and other species, making these mice good models for evaluating diet composition and potential drugs for their effect on atherosclerotic development (79). We tested the hypothesis that BSN272 prevents Western diet induced elevations in serum triglycerides and cholesterol, and reduces the formation of atherosclerosis in ApoE<sup>-/-</sup> mice.

## Methods

### Materials

D-tagatose and polydatin were provided by Biospherics.net.

### Mice and Diets

Male Jax ApoE<sup>-/-</sup> mice 14 to 18 weeks of age that were 12X backcrossed to C57BL/6J, were obtained from an in-house breeding colony at the University of Kentucky. The mice were given water *ad libitum* and kept on a 12 hour light/dark cycle. The animal-use protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee.

Mice were randomized to produce three groups with the same mean body weight. All mice were started on ground TD.2014 (Harlan Teklad), a 14% protein, low fat, complex carbohydrate diet. Mice in the group receiving BSN272 were acclimated to D-tagatose, due to the potential for gastrointestinal distress that may result from the poor absorption, by adding increasing amounts of D-tagatose to their drinking water daily during a two week run-in phase before beginning their respective diets (Illustration 1).

After the 14 day lead-in phase, the mice in each group

were fed as follows: Group 1 - standard diet (TD.2014); Group 2 - Western diet (high fat, cholesterol, and sucrose containing 21% milk fat, 0.15% cholesterol, 34% sucrose (TD.88137)); Group 3 - Western diet formulated with BSN272 (341 g D-tagatose/kg feed replacing 341 g of sucrose, plus 1 g polydatin/kg feed, TD.110527, custom formulation by Harlan Teklad). Mice were maintained on these diets for 16 weeks. Compositions of the three diets are shown in Illustration 2.

The ratios of kilocalories provided by fat, protein, and carbohydrates (fat / protein / carbohydrates) are as follows: TD.2014 (standard diet); 13/20/67; TD.88137 (Western diet) = 42/15/43; TD.110527 (BSN272 diet) = 52/19/29. Most of the carbohydrate content of the Western diet comes from sucrose. In contrast, while having the highest carbohydrate caloric content, the carbohydrate in the standard chow is from complex carbohydrates found in grain components, there is no sucrose added. In the chow containing BSN272, the sucrose in the Western diet has been replaced by D-tagatose. The energy content of the tagatose containing chows was calculated using the 1.5 Kcal per gram accepted in the US by the FDA for labeling purposes. Other countries use different values. From a physics viewpoint, all sugars have the same energy content when burned in a bomb calorimeter. However, the energy that an organism can extract from a sugar depends upon its ability to metabolize the sugar, and this varies in humans as well as animals with gut microflora and other factors. The initial two-week run in phase provides an adaptation period that increases the useful energy content of tagatose from near zero to something higher.

At the study end point, mice were euthanized using CO<sub>2</sub> followed by cervical dislocation and then the left ventricle was punctured to obtain blood. After the right atrium was cut, mice were exsanguinated by perfusion through the left ventricle followed by removal of tissues (liver, kidneys, spleen, epididymal fat and retroperitoneal fat (both sides), subcutaneous fat (one side only) and aortic tissue from the heart to the iliac bifurcation). The livers, kidneys, spleens and fat tissues were weighed. The hearts with aortas attached were fixed overnight in 4% paraformaldehyde made with phosphate buffered solution, and then transferred to phosphate buffered solution for storage.

#### **Blood analysis**

Total cholesterol and total triglyceride levels were determined using enzymatic assay kits (Wako Pure Chemical, Richmond, VA).

#### **Atherosclerosis measurements.**

Aortas were prepared for atherosclerosis measurements via en face presentation, whereby the entire length of the aorta was removed from the animal, the entire intimal surface and greater curvature of the aortic arch exposed, and the resulting tissue pinned to a dark surface. The atherosclerotic plaques were then traced and quantified using Nikon NIS Elements software (24).

#### **Calculations and Statistics**

Data are presented as the mean  $\pm$  s.e.m. 1-way ANOVA was utilized for analyses. Values of  $P < 0.05$  were considered to be statistically significant. Standard diet,  $n = 9$ ; Western diet,  $n = 13$ ; BSN272 diet,  $n = 15$ .

## **Results**

#### **Food Consumption**

Based upon the weight of food eaten by each group of mice and the number of Kcal/g available for each diet, average daily energy consumption per mouse was calculated. Food consumed was recorded beginning the first day each group was placed on their respective diets (day 15) and not during the 14 day D-tagatose lead-in phase.

Mice on the Western diet consumed the highest number of calories per day ( $12.75 \pm 0.63$  Kcal/mouse/day) followed by the standard diet group ( $11.5 \pm 0.87$  Kcal/mouse/day) and then the BSN272 diet mice ( $10.08 \pm 0.43$  Kcal/mouse/day) (Illustration 3). The difference in caloric intake between BSN272 and standard diet groups was significant ( $P < 0.01$ ), as it was between Western and BSN272 diet groups ( $P < 0.01$ ) and the Western and standard diet groups ( $P < 0.05$ ). Mice on the standard diet ate more food by weight than mice on Western or BSN272 diets. There was no significant difference in the weight of food eaten between the Western and BSN272 diet groups ( $P = 0.17$ ). The difficulties in measuring solid food consumption as well as the variable amount of energy that can be extracted from tagatose suggest that these energy results must be interpreted cautiously.

#### **Body, Tissue and Organ Weights**

##### *Body Weights*

Mice in all three groups were on the standard diet at the beginning of the study and during the two week D-tagatose lead-in period. At the end of the lead-in period the body weights of the mice in the three groups were not significantly different (Illustration 4). On day 15 the mice were placed on their respective diets for 16 weeks. Mice in the BSN272 group lost

weight during the first week they were put on the BSN272 diet and then gained weight at essentially the same rate as mice on the standard diet. Mice on the Western diet gained weight at a faster rate during the 16 weeks and weighed considerably more ( $40.78 \pm 1.72$  g) at the end of the study than mice on standard ( $31.51 \pm 1.11$  g,  $P < 0.01$ ) or BSN272 diets ( $30.53 \pm 0.56$  g,  $P < 0.01$ ). There was no significant difference between mice in the standard diet and BSN272 groups ( $P = 0.39$ ).

#### *Adipose tissue and organ weights*

Mice on the Western diet had increased total adipose tissue (epididymal + retroperitoneal + subcutaneous adipose tissue) compared to mice on both the standard and BSN272 diets (Illustration 5). Consistent with the slight decrease in body weights seen in the mice on the BSN272 diet compared to the standard diet, mice on the BSN272 diet were leaner with smaller amounts of epididymal, retroperitoneal and subcutaneous fat compared to mice on the standard diet ( $P < 0.01$ ).

There was a significant increase in liver weights in mice on the Western ( $2.18 \pm 0.19$  g) and BSN272 diets ( $1.74 \pm 0.061$  g) compared to the standard group ( $1.29 \pm 0.054$  g,  $P < 0.01$ ) (Illustration 6A). The BSN272 diet prevented some of the gain in liver size seen in the mice on the Western diet ( $P < 0.05$ ).

Spleens of the mice on the BSN272 diet were enlarged compared to spleens of mice on both the standard ( $0.100 \pm 0.017$  g) and Western diets (Illustration 6B). There was no significant difference in the sizes of the spleen from the mice on the standard and Western diets, or from the BSN272 and Western diet mice. There was a significant difference in the spleens from the mice on the BSN272 and standard diets ( $P = 0.013$ ). These results are consistent with the results of Kruger et al. (21) who found in a toxicology study of D-tagatose that the spleens of both male and female rats consuming D-tagatose as 20% (w/w) of their diets were significantly larger than spleens from the control group that was not consuming D-tagatose. Interestingly, in a toxicology study spleen weights of rats given 3000 mg polydatin/kg/day were less than the control group not getting polydatin, although the difference was not statistically significant ([http://www.webmedcentral.com/article\\_view/5231](http://www.webmedcentral.com/article_view/5231)).

Hearts from mice on the BSN272 diet weighed slightly less than those from standard ( $0.164 \pm 0.0297$  g) or Western diet mice ( $0.166 \pm 0.015$ ) ( $P < 0.01$  for Western vs. BSN272 and  $P < 0.05$  for standard vs. BSN272) (Illustration 6C).

#### **Serum Lipids**

#### *BSN272 blunts increase in total cholesterol produced by Western diet*

During the D-tagatose 14 day lead-in period all of the mice were on the standard diet (day 1 to 14). At the end of the 14 days serum total cholesterol levels were similar in all groups (Illustration 7). On day 15 the mice in the Western and BSN272 diet groups were started on their respective diets. By day 36, cholesterol had increased dramatically in both of these groups but with a significantly greater increase seen in the Western diet mice. Mice on the BSN272 diet exhibited lower cholesterol levels than mice on the Western diet at all time points through the course of the study. The effectiveness of BSN272 for lowering cholesterol increased over the time course of the experiment, with elevated cholesterol peaking at day 57 and then steadily decreasing over the remaining weeks of treatment. At the end of the study, mice on the BSN272 diet had significantly lower cholesterol than mice on the Western diet ( $591 \pm 35.6$  mg/dL vs.  $904 \pm 28.1$  mg/dL,  $P < 0.01$ ).

#### *BSN272 lowers serum triglycerides compared to mice on either the standard or Western diets.*

At the end of the 14 day lead-in period, triglycerides in the mice in each group were essentially the same (Illustration 8). Mice placed on the Western diet showed elevated levels of triglycerides compared to both standard and BSN272 groups. Interestingly, mice treated with BSN272 showed significantly lower triglycerides ( $108 \pm 5.76$  mg/dL) than mice on the standard diet ( $157 \pm 9.51$  mg/dL,  $P < 0.01$ ) or the Western diet ( $175 \pm 14.86$  mg/dL,  $P < 0.01$ ) at the end of the study period. The drop in triglycerides in the BSN272 diet group occurred after the two weeks.

#### *Mice on the BSN272 diet develop less atherosclerosis than mice on the Western diet.*

The elevations in serum cholesterol were associated with a dramatic increase in atherosclerotic lesion surface area in mice on the Western diet (Illustration 9). The addition of BSN272 to the Western diet significantly prevented atherosclerotic plaque formation in the area of the aortic arch. The mean area covered by plaque in mice on the Western diet was  $25.36 \pm 2.51\%$  compared to  $5.30 \pm 1.31\%$  for mice treated with BSN272. Mice on the standard diet developed very little plaque ( $0.497 \pm 0.19\%$ ),  $P < 0.01$  for all comparisons.

## Discussion

This study examined the effect of replacing sucrose in a high fat, high cholesterol, high sugar diet (Western

diet) with a combination of D-tagatose and polydatin. The high sucrose Western diet promoted obesity, increased total serum cholesterol and triglyceride concentrations, and markedly stimulated the development of atherosclerosis in ApoE<sup>-/-</sup> mice compared to mice on standard chow (low fat, low cholesterol, low sugar). In contrast, a diet that replaced gram-for-gram the sucrose in the Western diet with D-tagatose along with polydatin (BSN272 diet) prevented the weight gain seen with animals on the Western diet.

The mice on the BSN272 diet did not exhibit a gain in body weight witnessed in the Western diet group and weighed slightly, but not significantly, less than the standard diet mice. A previous study also showed that replacing sucrose with D-tagatose in a Western diet fed to LDLr<sup>-/-</sup> mice prevented the development of obesity (24). We obtained the same result with ApoE<sup>-/-</sup> mice. Mice on the Western diet exhibited a higher energy uptake per day compared to standard diet and BSN272 mice and this likely contributed to the development of obesity in mice on the Western diet. The average caloric uptake of mice on the BSN272 diet was less than that of standard diet mice which would account for the overall slightly less body weights of mice in this group. But again, the difference between the BSN272 and standard diet mice was not significant.

There was a significant ( $P < 0.01$ ) drop in weight in the BSN272 group between day 15, when the mice were started on the BSN272 chow, and day 21 when they were next weighed. The mice then gained weight at a rate comparable to the standard diet mice and at the end of the study there was no significant difference in the weight of the mice in these two groups. Food consumption by mice in the BSN272 group was decreased compared to the other two groups during the first week the mice were placed on their respective diets. D-tagatose is known to cause gastrointestinal upset when taken in large enough doses without a gradual increase in intake, which was the reason for the 14 day lead-in period. While there were no outward symptoms of gastrointestinal distress in the mice in the BSN272 group, they did eat less during the first week. After the first week, their food consumption increased and was comparable to the other two groups.

Interestingly, while there was no significant difference in the body weights of the mice in the standard and BSN272 diet groups, the amount of epididymal, retroperitoneal and subcutaneous fat in the BSN272 group was significantly lower than in the standard diet group. Similar results were seen in another study

using LDLr<sup>-/-</sup> mice (24). Polydatin may act as a caloric restriction mimetic like resveratrol.

Mice on the Western diet had livers that weighed about 1.6 times more than the livers from standard diet mice. The replacement of sucrose with BSN272 in the Western diet prevented some of the increase in liver weight compared to standard diet. Previous studies found there was an increase in the size of livers in rats fed D-tagatose. This enlargement was found to be characterized by increased glycogen deposition, with an absence of histopathological changes and no evidence of increased deposition of lipids (83, 84).

After D-tagatose is absorbed into the bloodstream, it is metabolized in the liver by the same pathway used in fructose metabolism and can activate glycogen synthase (16, 85). Similar enlargement of the liver is seen in rats fed fructose. Furthermore, this liver enlargement was reversible upon removal of D-tagatose from the rat's diets and was concluded that the observed liver enlargement had no relevance for the assessment of human safety of D-tagatose.

While BSN272 fed mice exhibited slightly increased serum cholesterol and atherosclerosis compared to standard diet mice, the extent of these changes was far less than those observed in the mice on the Western diet. The livers of ApoE<sup>-/-</sup> mice are unable to remove circulating cholesterol efficiently and as a result these mice have elevated serum cholesterol. Atherosclerotic lesion development is very dramatic in ApoE<sup>-/-</sup> mice fed a Western-type diet and the beginning stages of the disease can be found at 6 weeks (78). With the ApoE<sup>-/-</sup> and LDLr<sup>-/-</sup> mouse models, dietary cholesterol, rather than the level of fat, exerts a major influence on the development of atherosclerosis (86-88). In addition to cholesterol, the fatty acid profile and even the carbohydrate form (i.e. fructose, sucrose) can be manipulated to modify the atherosclerosis phenotype (10, 87, 89). Replacement of sucrose with D-tagatose in a Western diet was shown to decrease serum total cholesterol in LDLr<sup>-/-</sup> mice compared to mice on the Western diet with sucrose (24). We obtained similar results in this study with the ApoE<sup>-/-</sup> mice. Total cholesterol increased in the BSN272 mice upon placing them on their diet, but never reached the levels of the mice on the Western diet. After day 56, cholesterol began to steadily decline in the BSN272 group.

Mice on the BSN272 diet had significantly lower triglycerides at the end of the study, while there was no significant difference in serum triglyceride levels between the mice on the Western and standard diets. Unlike with cholesterol, which showed an increase in the BSN272 group upon start of their diet, triglycerides

dropped between the time when the mice were placed on the BSN272 diet (day 15) and the next time that triglycerides were assayed on day 36 and remained reduced during the course of the study. At the end of the study, triglycerides in the BSN272 mice were significantly lower than in the standard diet group ( $P < 0.01$ ).

Elevations in serum cholesterol concentrations were associated with a striking increase in atherosclerotic lesion surface area in mice fed the Western diet compared to BSN272 and standard diet mice. The addition of BSN272 to the Western diet dramatically reduced the formation of atherosclerotic plaques. Although BSN272 fed mice exhibited increased atherosclerosis compared to standard diet mice, the extent of these changes were far less than those observed in Western diet fed mice.

The results obtained in this study are consistent with a previous 8-week study in which ApoE<sup>-/-</sup> mice were placed on the same BSN272 diet used here (90). That study also showed lower total cholesterol, triglycerides and occurrence of atherosclerotic plaque formation seen in the present study. Results between the studies were very consistent. For instance, plaque area in the eight week study for Western diet group was 4% versus 1% for the BSN272 group, and in the 16 week study 25% for the Western diet group versus 5% in the BSN272 treated mice, a 4-5 fold difference in both studies. Additionally, the difference in cholesterol between the Western diet groups and BSN272 groups was approximately 300 mg/dL (8 week study) and 313 mg/dL (16 week study), demonstrating increasing efficacy with a longer study period. The 16-week BSN272 group triglycerides levels ( $108 \pm 5.76$  mg/dL) were significantly less than mice on the standard diet ( $157 \pm 9.51$  mg/dL,  $P < 0.01$ ) or the Western diet ( $175 \pm 14.86$  mg/dL,  $P < 0.01$ ). These triglyceride results mirror the earlier eight week study (90) which, although not statistically significant, did result in reduced triglycerides in the BSN272 group ( $110.8 \pm 62.5$  mg/dL) compared to the Western diet group ( $141.7 \pm 43.1$  mg/dL), mitigating concerns regarding any potential rebound effect in terms of loss of effectiveness of BSN272 over time. This study extends the length of time of treatment from 8 weeks to 16 weeks and demonstrates the benefits of D-tagatose and polydatin extend beyond the 8 weeks of the original study.

In conclusion, replacing the sucrose with D-tagatose and polydatin in a Western diet prevented obesity, elevations in serum cholesterol, and formation of atherosclerosis compared to mice on the Western diet and resulted in triglyceride levels below that seen in

mice on both the Western and standard diets. The combination of D-tagatose and polydatin could be useful in the management of obesity and hyperlipidemia.

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## Abbreviations

- ANOVA - Analysis of variance
- ApoE<sup>-/-</sup> - apolipoprotein A deficient
- BSN723 – combination drug consisting of D-tagatose and polydatin
- CAT - catalase
- GRAS - generally recognized as safe
- GSH - glutathione
- GSH-Px - glutathione peroxidase
- HbA1c - glycated hemoglobin
- HDL - high-density lipoprotein cholesterol
- ICAM-1 - intercellular adhesion molecule 1
- IDL – intermediate density lipoprotein
- IRS - insulin receptor substrate
- LDL - low-density lipoprotein cholesterol
- LDL<sup>r/-</sup> - low-density lipoprotein receptor deficient
- MDA - malonyldialdehyde
- TNF-alpha – tumor necrosis factor alpha
- SOD2 - Superoxide Dismutase 2
- T-SOD - total superoxide dismutase
- VLDL – very low density lipoprotein

## Illustrations

### Illustration 1

Addition of D-tagatose to drinking water during the two week run-in phase. Increasing amounts of D-tagatose were added to drinking water to acclimate mice to D-tagatose. Mice in Groups 3 and 5 had D-tagatose added to their drinking water.

<b>Day</b>	<b>D-tagatose (g) / 100 ml water</b>
Day 1	2.4
Day 2	4.9
Day 3	7.3
Day 4	9.7
Day 5	12.1
Day 6	14.5
Day 7	17.0
Day 8	19.4
Day 9	21.8
Day 10	24.2
Day 11	26.6
Day 12	29.0
Day 13	31.5
Day 14	34.2

## Illustration 2

Comparison of the three diets feed to the mice.

Diet	Sucrose % by weight	Cholesterol	D-tagatose % by weight	Polydatin	Fat % by weight	Kcal/g
Standard TD.2014	0	0	0	0	4%	2.9
Western TD.88137	34%	1.5 g/kg	0	0	21%	4.5
BSN272 TD.110527	0	1.5 g/kg	34%	1 g/kg	21%	3.7

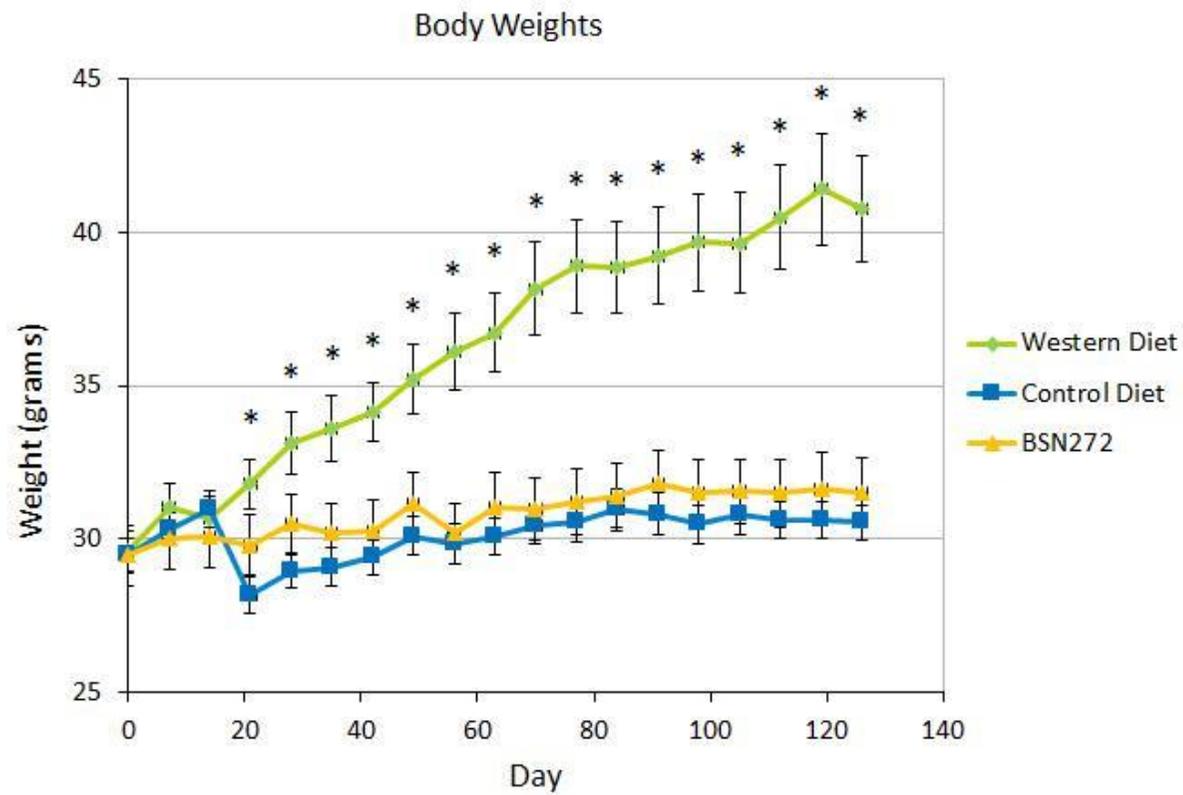
### Illustration 3

Comparison of food consumption and average caloric intake of mice according to diet. Food consumption was determined by weighing food given to the animals and food remaining after every 3 to 4 days. Results are shown as mean  $\pm$  s.e.m. Standard diet, n = 9; Western diet, n = 13; BSN272 diet, n = 15.

Diet	Grams of food/mouse/day	Kcal/gram	Kcal/mouse/day
Standard	3.97 $\pm$ 0.30	2.9	11.51 $\pm$ 0.87
Western	2.83 $\pm$ 0.14	4.5	12.75 $\pm$ 0.63
BSN272	2.72 $\pm$ 0.12	3.7	10.08 $\pm$ 0.43

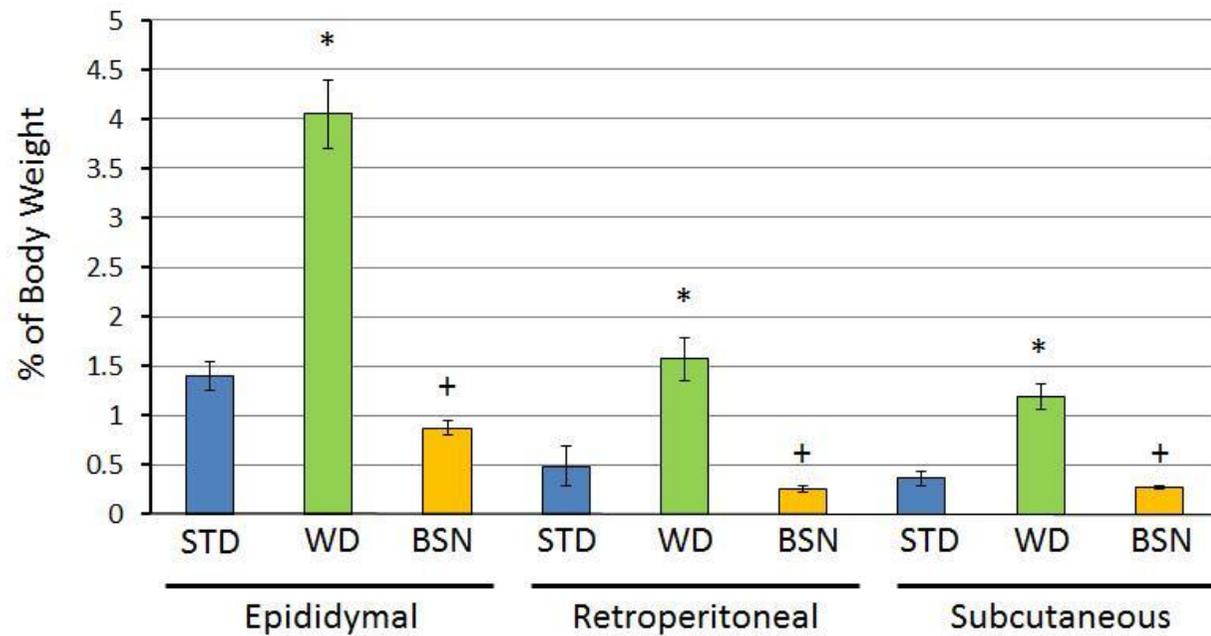
## Illustration 4

BSN272 prevented weight gain in mice associated with the Western diet. Results are shown as mean  $\pm$  s.e.m. Standard diet, n=9; Western diet, n=13; BSN272 diet, n=15. \*P less than 0.01 compared to both Standard and Western diets.



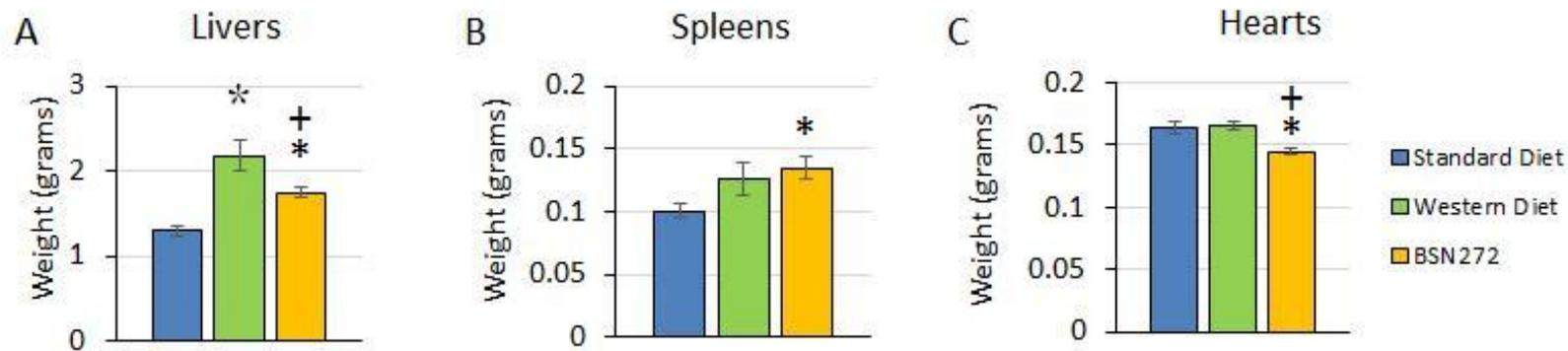
## Illustration 5

Mice fed BSN272 were leaner compared to mice on the Western diet (\* + P less than 0.01, comparisons between all groups). Results are shown as mean  $\pm$  s.e.m. Standard diet, n=9; Western diet, n=13; BSN272 diet, n=15.



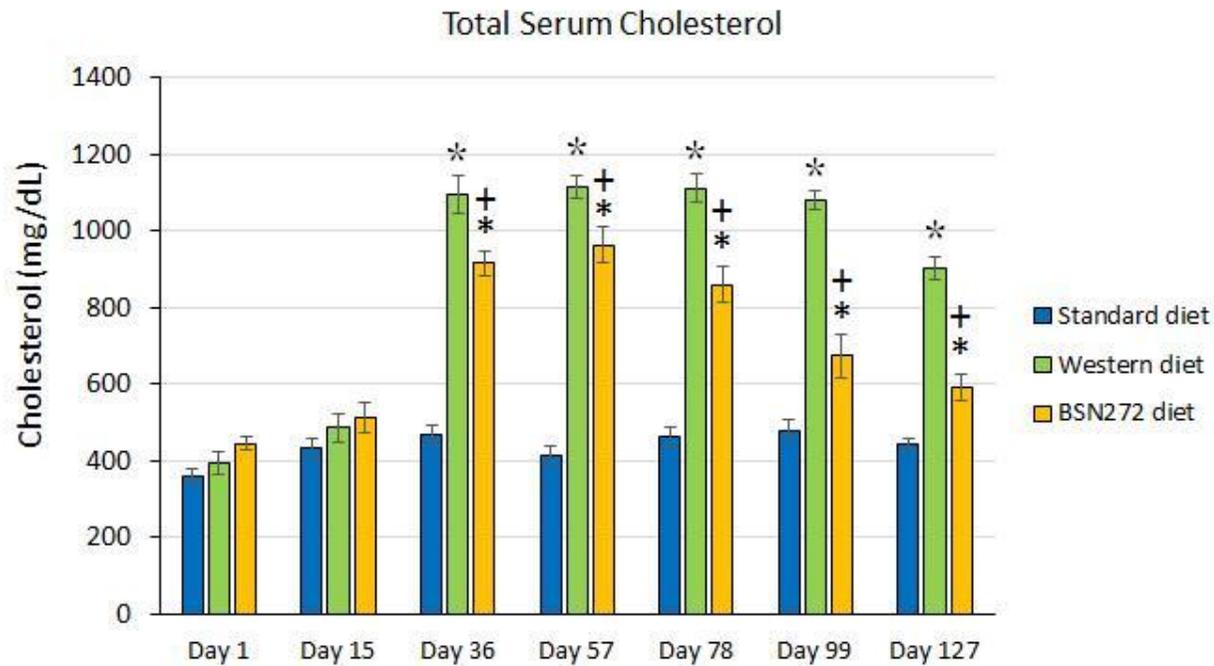
## Illustration 6

Organ weights. (A) Livers.  $P=0.01$ , Western vs Standard;  $P$  less than 0.001, BSN vs Standard;  $P=0.027$ , BSN vs Western. (B) Spleens.  $P=0.013$ , BSN vs Standard. (C) Hearts.  $P$  less than 0.001, BSN vs Western,  $P=0.047$ , BSN vs Standard, Results are shown as mean  $\pm$  s.e.m. Standard diet,  $n=9$ ; Western diet,  $n=13$ ; BSN272 diet,  $n=15$ .



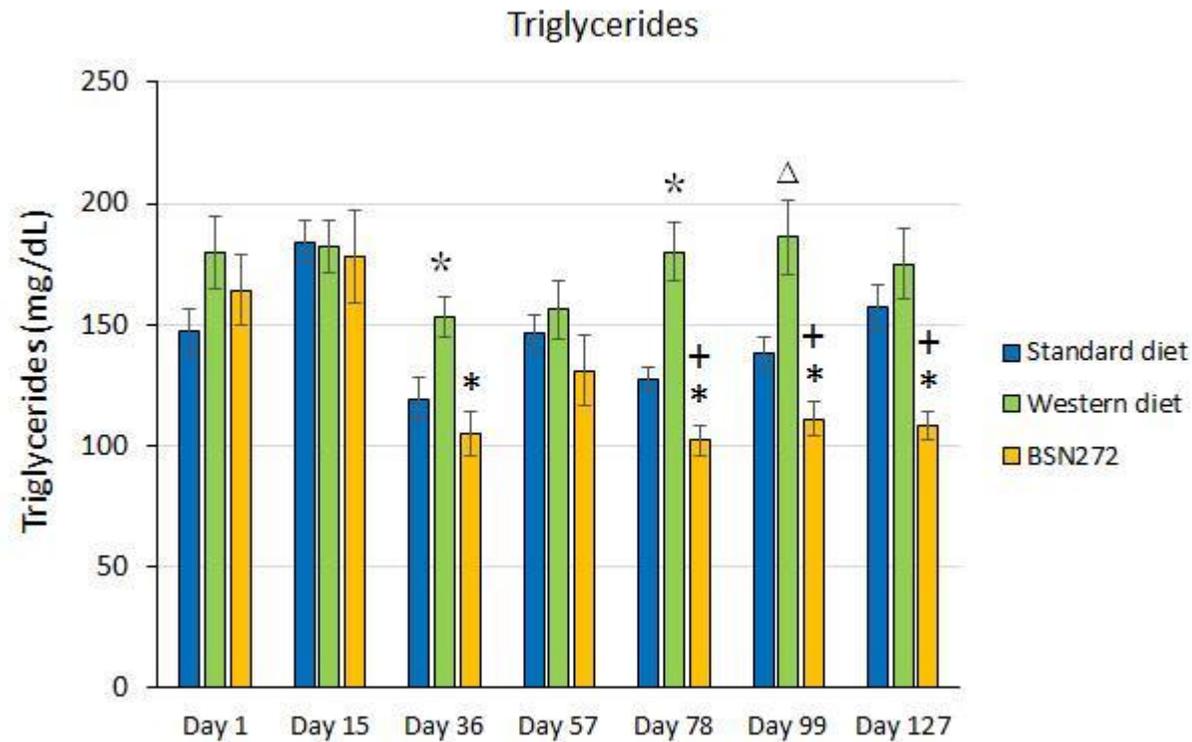
## Illustration 7

BSN272 blunts the rise in serum total cholesterol of mice fed a Western diet. Results are shown as mean  $\pm$  s.e.m. Standard diet, n=9; Western diet, n=13; BSN272 diet, n=15. P less than 0.02, BSN vs Western; P less than 0.001, compared to both Standard and BSN272 diets; P less than 0.02, BSN272 vs Standard.



## Illustration 8

Serum triglycerides. Results are shown as mean  $\pm$  s.e.m. Standard diet, n=9; Western diet, n=13; BSN272 diet, n=15. P less than 0.02, BSN vs Western; P less than 0.02 Western vs Standard; P less than 0.02, BSN vs Standard; P less than 0.03, Western vs Standard.



## Illustration 9

The addition of BSN272 to the Western diet prevented the accumulation of plaque in the area of the aortic arch. Aortas were prepared for atherosclerosis measurements via en face presentation. Results are shown as mean  $\pm$  s.e.m. Standard diet, n=9; Western diet, n=13; BSN272 diet, n=15. \*P less than 0.001 between all groups.

